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Sulforaphane Inhibits Self-renewal of Lung Cancer Stem Cells Through the Modulation of Polyhomeotic Homolog 3 and Sonic Hedgehog Signaling Pathways

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Abstract

Background: Sulforaphane (SFN), an active compound in cruciferous vegetables has been characterized for its antiproliferative capacity. We investigated the role and molecular mechanism through which SFN regulates proliferation and self-renewal of lung cancer stem cells. Methods: Lung cancer stem cells (CD133-positive cells) were isolated by MACs and then measured by flow cytometry. The ability of cell proliferation was assessed by MTT assays and tumorsphere formation assays. The expressions of Sonic Hedgehog (Shh), Smoothened (Smo), Gli1 and Human Polyhomeotic Homolog 3 (PHC3) in cells were measured by quantitative reverse transcription polymerase chain reaction (qPCR) and western blot assays. The expression of transcription factor SOX2 in lung cancer stem cells was also determined by western blot assay. Shh was knocked down by siRNA to further study the role of SFN and Shh signaling pathways in lung cancer. Results: SFN inhibited the proliferation of lung cancer cells and lung cancer stem cells simultaneously. Meanwhile, we observed that Sonic Hedgehog (SHH) signaling pathway, SOX2 and Polyhomeotic Homolog 3 (PHC3) were highly activated in lung cancer stem cells. Knock-down of Shh led to reduced H460 and A549 cells proliferation. Furthermore, we observed that SFN inhibited the activity of PHC3 and SHH signaling pathways in the lung cancer stem cells. In addition, SFN combined with Knock-down of Shh gene showed a greater effect on the proliferation of lung cancer cells. Conclusion: SFN is an effective new drug which can inhibit proliferation of lung cancer stem cells through the modulation of PHC3 and SHH signaling pathways. It provides a novel target for improving therapeutic efficacy for lung cancer stem cells.

Background

Lung cancer is one of the most prevalent cancers which accounts for approximately one-fourth of the cancer incidence and is the second leading cause of death both in China and developed countries around the world[1,2]. Despite initial treatment with conventional therapy has a high remission rate，eventually the disease almost always relapses in the form of resistance to chemotherapy and radiation therapy, therefore the overall 5-year survival rate of patients is less than 5%[3].

Increasing evidence of the existence of cancer stem cells(CSCs) in lung cancer explains why standard chemotherapy or radiotherapy regimens against lung cancer are usually ineffective and result in further tumor recurrence and spread [4].CD133 is a cell membrane glycoprotein and it contains five transmembrane rings. It has been reported that CD133-positive subpopulation of multipotent cells has the biological features of cancer stem cells which possess extremely proliferative and self-renewal characteristics[5,6]. Although CSCs are only a small part of tumors, they have a powerful ability to self-renew and indefinite proliferation. The tumors rich in CSCs are more aggressive and lead to worse clinical outcomes. Therefore, the development of therapeutic strategies and drugs that specifically target CSCs can eradicate tumors effectively and reduce the risk of recurrence and metastasis.

Sulforaphane (SFN) is an isothiocyanate (ITC) found in cruciferous vegetables such as broccoli and cabbage[7,8]. A series of clinical trials have demonstrated that SFN can inhibit the malignant growth of

cancer cells such as pancreatic cancer cells and breast cancer cells and has no obvious toxicity to the normal cells[9-12]. Therefore, it has been suggested safe to use SFN as a potential candidate for cancer treatment. However, the effect of SFN on the development of lung cancer and its mechanism is still unknown.

Sonic Hedgehog (SHH) signaling pathway has an essential role in the control of stem cell growth in embryonic tissues, and it plays a key role in the development of tissues and organs[13].

Recent evidence suggests that the SHH signaling pathway contributes to tumorigenesis when it is mutated or misregulated[14,15]. Since the SHH pathway plays a critical role in the renewal of cancer stem cells, blockade of SHH has evolved as a promising therapy for various types of cancers including lung cancer[16-18]. Sonic hedgehog (Shh), Smoothened (Smo) and Gli1 are important factors in the SHH signaling pathway. Human Polyhomeotic Homolog 3 (PHC3) is a member of the polycomb Group(PcG) protein family [19], and PcG is a family of chromatin-related gene silencing proteins that regulates gene expression program in epigenetics. PcG proteins have essential roles in early embryonic development and have been implicated in embryonic stem cell pluripotency[20]. The PRCs silence tumor-suppressor genes by histone modifications, leading to cancer cell proliferation, metastasis and drug resistance[21]. Thus, PHC3 and SHH signaling pathways have essential roles in controlling stem cell growth in early embryonic development. However, the role of PHC3 and SHH signaling pathways in human lung cancer stem cells still needs to be elucidated.

In this study, we examined the effect of SFN on the proliferation and self-renewal of lung cancer stem cells. Moreover, we analyzed the role of PCH3 and SHH signaling pathways in lung cancer cells and lung cancer stem cells. Furthermore, we analyzed SFN whether it was associated with the proliferation and self-renewal of lung cancer stem cells through the modulation of the PHC3 and SHH signaling pathways.

Methods

Cell culture

Human non-small cell carcinoma of the lung cancer A549 and H460 cell lines were obtained from the Cancer Institute of Southern Medical University (Guangzhou, China). The cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C in DMEM culture medium (Gibco, Carlsbad, CA, USA) with 10% FBS (fetal bovine serum).

The lung cancer stem cells which express CD133 were isolated by the BD MACs and identified by flow cytometry (FACS). CD133-positive cells were cultured in MEBM basal medium (CBM, New Jersey, USA) to maintain the characteristics of stem cells. CD133-negative cells were cultured in the RPMI-1640 culture medium.

Cell viability assay

The A549 and H460 cells in logarithmic phase were seeded into 96-well plates at a cell density of 1 × 10^4/well, and then different concentrations of SFN were added into each well and incubated together for 48h. The final concentrations of SFN were 0, 2, 4, 6, 8, 10, 12μmol/L, respectively. Then, 10 μL MTT reagent of concentration 5 μg/L was added into the cell medium of each well and incubated for 4 h at 37 °C. Following the removal of the supernatant, 150μL dimethyl sulfoxide(DMSO) was added to dissolve formazan. The absorbance at 490 nm was measured with a microplate reader(Beckman Coulter, Brea, CA, USA). Each reaction was performed in triplicate. At the same time, changes in cell density and cell cycle were observed by optical microscope and flow cytometry.

Isolation and identification of lung cancer stem cells

Lung cancer stem cells were obtained from A549 and H460 cells using CD133 Microbeads by MiniMACS separator(MiltenyiBiotec, Bergish Gladbach, Germany). A549 and H460 cells were collected separately by centrifugation. Different groups of cells (1×10^8 cells/sample) were resuspended in 500μl of the degassed buffer, respectively. Then the cell suspension was added onto the prepared column. The unlabeled cells were collected as they passed through the columns. MS columns were washed with degassed buffer. Then the column was removed from the separator and placed on a new suitable collection tube. Buffer was pipetted onto the column, and a fraction was immediately flushed out with the magnetically labeled cells CD133-positive by firmly applying the plunger supplied with the column.

Flow cytometry analysis

A549 and H460 cells were collected separately by centrifugation, then the cells were washed twice with PBS solution, and up to 1×10^6 cells were resuspended in 500μl of PBS, respectively. The cells were then incubated with PE mouse anti-human CD133 for 30 min at room temperature in the dark. The positive cells were detected using a flow cytometer (BD Biosciences, San Jose, CA, USA) and were analyzed by the CellQuest Analysis Software.

TumorSphere formation assay

Cells were placed in 6-well ultralow attachment plates (Corning Inc.) at a density of 1,000 cells/mL in tumorsphere culture medium(Invitrogen, Carlsbad, CA, USA) supplemented with DMEM with 1% N2 supplement, 2% B27 supplement, and 100ng/mL epidermal growth factor at 37°C in a humidified atmosphere of 95% air and 5% CO2. These cells were then treated with different concentrations of SFN at the same time. Primary spheroids were collected following 14 days of culture, and tumorspheres were measured using an inverted microscope system (magnification, Eclipse Ti-s, Nikon, Tokyo, Japan).

Reverse transcription and qPCR analysis

The total RNA was extracted from the cancer cells and cancer stem cells with Trizol reagents. cDNA was synthesized from 1μg of mRNA with a high capacity cDNA reverse transcription kit according to the manufacturer's instructions. Subsequently, cDNA was amplified by qPCR with the SYBR Premix Ex Taq kit according to the manufacturer's instructions using the ABI7300 Sequence Detection System. The following gene-specific primers were used: Shh(forward)5'-CGC ACC TGC TCT TTG TGG-3',(reverse)5'-GGA GCG GTT AGG GCT ACT CT-3'; Smo(forward) 5'-TCG CTA CCC TGC TGT TAT TC-3', (reverse)5'-GAC GCA GGA CAG AGT CTC AT-3';Gli1(forward) 5'-CTG GAT CGG ATA GGT GGT CT-3', (reverse)5'-CAG AGG TTG GGA GGT AAG GA-3'; PHC3(forward)5'-AGT GGG GAG AGG AGA AGA-3',(reverse) 5'-GGT GGT GGA ACA GAA ACA-3'. The housekeeping gene Beta-actin was used as a loading control. PCR conditions were as follows: one cycle at 95^{o}C for 3 min, followed by 40 cycles at 95^{o}C for 30s, 55^{o}C for 30s, and 72^{o}C for 1 min. All assays were performed in triplicate and were calculated on the basis of $\Delta\Delta$Ct method. The n-fold change in mRNAs expression was determined according to the method of $2^{-\Delta\Delta CT}$.

Western blotting

Protein sample was extracted from 5 ×10^{6} cells with an ice-cold SDS protein lysis buffer. Protein concentration was measured by a Micro BCA Protein Assay Reagent kit. Then protein sample was separated by 10% SDS-PAGE electrophoresis and transferred onto 0.45mm PVDF membranes. The membranes were blocked with 5% non-fat milk in TBST buffer for 1 h, incubated overnight with primary antibody at 4°C and then incubated with peroxidase-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature. The antigen-antibody complexes were visualized using the ECL detection system. The analysis of the bands was conducted by the Image J software. The following antibodies were purchased from commercial sources including anti-Shh (Polyclonal Antibody ,ab53281), anti-Smo (Polyclonal Antibody, ab32575), anti-Gli1(Polyclonal Antibody, ab134906), anti-SOX2(Polyclonal Antibody, ab92494), anti-PHC3 (Polyclonal Antibody, GTX32785) and anti-GAPDH (polyclonal, CWBIO).

RNA interference

siRNAs for SHH were purchased from Invitrogen and the following sequences were used. SHH-s:5'-ACAGGCUGAUGACUCAGAGGUGUAA-3',SHH-as:5'-UUACACCUCUGAGUCAUCAGCCUGU-3';SHH-s: 5'-GGUGUACUACGAGUCCAAGGCACAU-3', SHH-as:5'- GACUCGUAGUACACC-3';SHH-s: 5'-CCGACAUCAUAUUUAAGGAUGAAGA-3', SHH-as: 5'-UCUUCAUCCUUAAAUAUGAUGUCGG-3. Cells were seeded in 6-well plates at a cell density of 3×10^{5} cells/well in 10% serum medium without antibiotics. After 24h, cells were transfected in Opti-MEM using Lipofectamine RNAi MAX (Invitrogen) and the 20 nmole siRNA was resuspended according to manufacturers instructions. siRNA transfection efficiency in cells was assessed by using a commercially available kit (Blockitalexafluor red oligo, Invitrogen).

Statistical Analysis

Statistical analysis was performed using SPSS16.0 software. Data presented were mean ± SD from three different experiments. Statistical significance between different groups was determined using Students *t*-test. A value of $P<0.05$ was considered statistically significant.

Results

SFN inhibits the growth of lung cancer cells

To explore the role of SFN in lung cancer cells. We first detected cell viability through the MTT assay. The result showed that SFN could inhibit the growth of lung cancer A549 and H460 cells, which was clearly indicated by a declined cell viability trend in a dose-dependent manner following treatment with different concentrations of SFN (Fig. 1A). Electron microscope also shows a significant decrease in the density of A549 and H460 cells following treatments with 8μmol/L SFN for 48h (Fig. 1B). Moreover, the data shows that the number of cells in S phase and G2M phase decreased significantly after treatment with SFN (Fig. 1C).

SFN inhibits the growth of lung cancer stem cells

To investigate whether SFN sensitized to lung cancer stem cell, we first isolated CD133+ cells from the human lung cancer A549 and H460 cells by MACS. Compared with 2.8% and 2.2% after isolation CD133+ cells accounted for 96.4% and 97.8% in A549 and H460 cells separately (Fig. 2Aand 2B).The CD133+cells can form a colony in the tumorsphere culture medium. Pluripotency maintaining transcription factor SOX2 plays an important role in maintaining the distinct characteristics of cells in tumors. In this study, we observed that the expression of SOX2 in CD133 positive cells was higher than that in CD133 negative cells (Fig. 2C).

In sphere formation assay, the A549/CD133+ and H460/CD133+ cells were treated with 0, 4, 8 and 12μmol/L SFN. At the end of 14 days, the total number of spheres and the volume of tumorspheres were measured, and the volume of tumorsphere trend showed a gradual decrease with increasing dose of SFN (Fig. 2D). These results showed that SFN can effectively inhibit the proliferation of lung cancer stem cells.

High expression of SHH and PHC3 signaling pathways in lung cancer stem cells

It has been reported that the SHH and PHC3 signaling pathways play an important role in the control of stem cell growth[7,14]. However, the role of the SHH and PHC3 signaling pathways in lung CSCs is uncertain. As shown in Fig.3A, the mRNA level of the important components of the SHH signaling pathway such as Shh, Smo and Gli1 was markedly increased in human lung CSCs (CD133+/A549 and CD133+/H460 cells) compared to that in human non-lung stem cells (CD133-/A549 and CD133-/H460

cells). We also measured PHC3 expression level in CD133 positive cells and CD133 negative cells. PHC3 mRNA expression level was also markedly increased in CD133 positive cells.

To further determine whether the SHH and PHC3 signaling pathways have an abnormal expression in human lung CSCs, we examined the protein expression level of Shh, Smo, Gli1 and PHC3 in CD133-positive cells and in CD133-negative cells. Interestingly, Shh, Smo, Gli1 and PHC3 protein expression levels were consistent with mRNA transcription level in human lung CSCs(Fig. 3B).

Sulforaphane inhibits PHC3 and SHH signaling pathway in lung CSCs

To further explore whether SFN regulates SHH signaling pathway and PHC3 in lung CSCs, we examined the effects of SFN on the mRNA expression level of Shh, Smo, Gli1 and PHC3 in CD133-positive and CD133-negative cells. As shown in Fig.4A, SFN could markedly inhibit the mRNA expression of Shh, Smo, Gli1 and PHC3 in CD133-positive cells, but this inhibitory effect was not obvious in CD133-negative cells. The expression of these components was also further confirmed by western blotting assay. SFN could also inhibit protein expression of Shh, Smo, Gli1 and PHC3 in the lung cancer stem cells(Fig.4B).

SHH silencing is associated with a reduction in lung cancer cell proliferation

In order to further identify the role of the SHH signaling pathway in the proliferation of lung cancer cells, we silenced the Shh gene in A549 and H460 cell lines. A549 and H460 cells were transfected with siRNAs against shh and maintained for 48 h. The efficiency of siRNA transfection was established by Stealth negative control. The transfection efficiency was determined by western blotting. The shh protein expression in shh-siRNA transfected cells was suppressed by approximately 85% in A549 cells and 95% in H460 cells compared with the control(Fig. 5AD). When the Shh gene was silenced successfully, the ability of shh-siRNA transfected cells to form tumorsphere became lower than that of the control group (Fig. 5BE). Further research revealed that the inhibitory effect of SFN on cell proliferation still exists in Shh-siRNA cells. In the cell proliferation assay, the cell viability also showed a gradual decrease with increasing dose of SFN both in the siRNA-SHH group and the NT-siRNA group. SFN combined with Shh gene silencing had a greater effect on the proliferation of lung cancer cells(Fig. 5C and 5F).As no prior report on the relationship between SHH and PHC3 in lung cancer exists, we employed a further study expression of PHC3 in shh-siRNA transfected cells. Interestingly, PHC3 expression level showed a significant decrease in shh-siRNA cells compared with the control(as shown in Fig.5AD).

Discussion

A series of scientific studies have shown that SFN can induce apoptosis in a variety of human cancers [22], leading to cell cycle arrest and inhibition of malignant growth. SFN is an isothiocyanate (ITC) extracted from cruciferous vegetables. In our study, we observed that SFN can inhibit the activity of lung cancer cells in vitro.

CD133 is an important marker of cancer stem cells[23,24]. Therefore, we separated lung cancer stem cells which express CD133-positive from the human lung cancer A549 and A460 cells. A series of research studies [25,26] have reported that pluripotency maintaining transcription factor SOX2 plays an important role in maintaining the stem characteristics of cells in tumors. Here, we demonstrate that SOX2 is highly expressed in CD133-positive lung cancer cells. Therefore, CD133 positive cells possess the characteristics of lung cancer stem cells. To investigate whether SFN affects lung cancer stem cells, we performed the sphere formation assay. We observed that the volume and the number of tumorspheres were gradually decreased with the increasing dose of SFN. This result indicates that SFN has the potential to inhibit the proliferation of lung cancer stem cells. However, the mechanism of the phenomenon still needs to be elucidated.

In recent years, several studies have reported that the SHH signaling pathway plays a critical role in the development and progression of lung cancers [27-29]. It has also been reported that the SHH signaling pathway could regulate self-renewal and proliferation of cancer stem cells and increase tumor invasiveness [30]. In this study, we have demonstrated that the SHH signaling pathway was highly upregulated in lung CSCs, suggesting that the hyperactive SHH signaling may regulate the expression of stemness genes in lung CSCs and play important roles in the cell proliferation and progression of lung CSCs. Furthermore, when the Shh gene was silenced successfully, the ability of shh-siRNA transfected cells to form tumor sphere was decreased as compared to that of the control group. Therefore, we speculate that the SHH signal pathway plays an important role in the proliferation of lung cancer cells.

To explore whether SFN inhibits the proliferation of lung cancer stem cells through the SHH signaling pathway, we further investigated the expression of key components in the SHH signaling pathway in cells which were treated by SFN. The results showed that SFN can obviously reduce the mRNA and protein expression of Shh、Smo and Gli1 in CD133-positive cells as compared to CD133-negative cells. In this study, we also observed that this effect of SFN-induced cell viability inhibition also occurred in A549 andH460 cells in which the Shh gene was silenced. Furthermore, the inhibitory effect of SFN on cell proliferation was stronger in the siRNA-SHH group than in the control group. Therefore, targeted Shh gene therapy is inferred to be a new way to treat lung cancer.

PHC3 is one of the members of PRCs, which have essential roles in early embryonic development and have been implicated in embryonic stem cell pluripotency[31,32]. It has been reported that PHC1 is important in regulating stem cells [33,34]. Other previous studies have shown that PcG complexes control cellular proliferation and favor tumorigenesis[35,36].AM Deshpande[37] reported that PHC3 expression was abnormal in osteosarcoma. In this study, we also investigated whether PHC3 like PHC1 plays the same role in regulating stem cells. Our data indicated that both the mRNA and protein expressions of

PHC3 were also markedly increased in human lung CSCs. Further research also showed that SFN inhibited the mRNA and protein expressions of PHC3 in CD133-positive cells as compared to CD133-negative cells. Moreover, PHC3 presented the same expression pattern as SHH signaling in lung cancer. However, there is no evidence to support the interaction between SHH and PHC3. Therefore, we investigated the protein expression of PHC3 in Shh silenced cells. Here, we demonstrated that knockdown of shh suppressed PHC3 protein expression in A549 and H460 cells.

Conclusion

This study provides evidence that SFN can serve as a potent anticancer agent, inhibit the proliferation of lung cancer cells and kill the lung cancer stem cells. Furthermore, this study, for the first time, demonstrates that the SHH and PHC3 signaling pathways work together in lung cancer stem cells and aberrant activation of these signals promotes tumorigenesis and progression of lung cancer. These findings suggested that SFN could be exploited in a novel therapeutic avenue for lung cancer treatment by regulating the SHH and PHC3 signaling pathways.

Abbreviations

SFN: Sulforaphane; ITC: isothiocyanate; CSCs: cancer stem cells; LCSs: Lung cancer stem cells; SHH : sonic hedgehog; Hh: Hedgehog; PHC3: Polyhomeotic Homolog 3; PcG: polycomb Group;

qPCR: quantitative reverse transcription polymerase chain reaction; DMSO:dimethyl sulfoxide;

FACS: flow cytometry.

Declarations

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FW and CY conceived and designed the study. FW and JZ carried out the clinical data analysis and wrote the manuscript. SW and MW analysed the data and wrote part of the manuscript. CQ, XL and LM and QL performed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable, A549 and H460 cell line were obtained from the Cancer Institute of Southern Medical University (Guangzhou, China), and did not require ethics approval for their use in this study.

Consent for publication

Not Applicable.

Competing interests

The authors declare no competing interests

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Figures

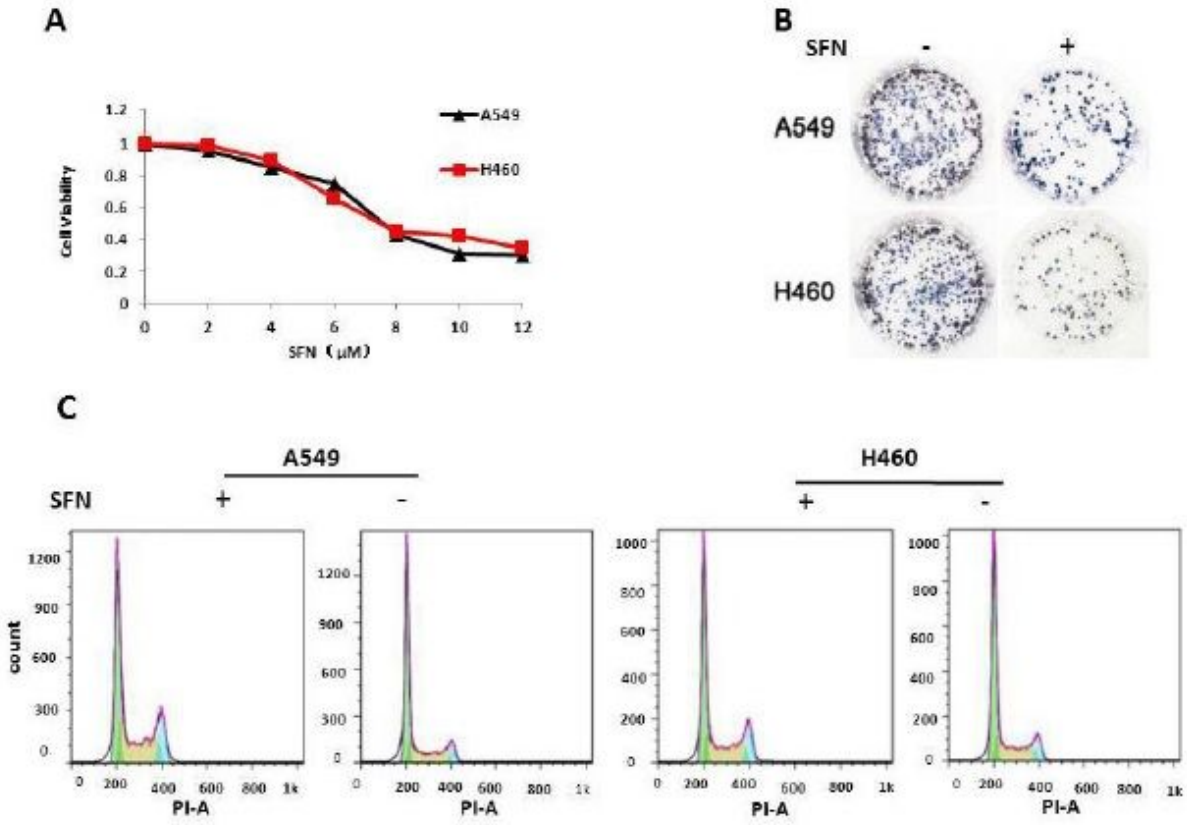

Fig. 1 Inhibitory effect of SFN on cell proliferation

(A) Inhibitory effect of SFN on viability of A549 and H460 cells in a dose -dependent manner following treatments with 0, 2, 4, 6, 8, 10 and 12 μmol/L SFN for 48h by MTT assay.

(B,C) Inhibitory effect of SFN on cell density of A549 and H460 cells following treatments with 8 μmol/L SFN for 48h by optical microscope and flow cytometry.

Figure 1

Inhibitory effect of SFN on cell proliferation (A) Inhibitory effect of SFN on viability of A549 and H460 cells in a dose -dependent manner following treatments with 0, 2, 4, 6, 8, 10 and 12 μmol/L SFN for 48h by MTT assay. (B,C) Inhibitory effect of SFN on cell density of A549 and H460 cells following treatments with 8 μmol/L SFN for 48h by optical microscope and flow cytometry.

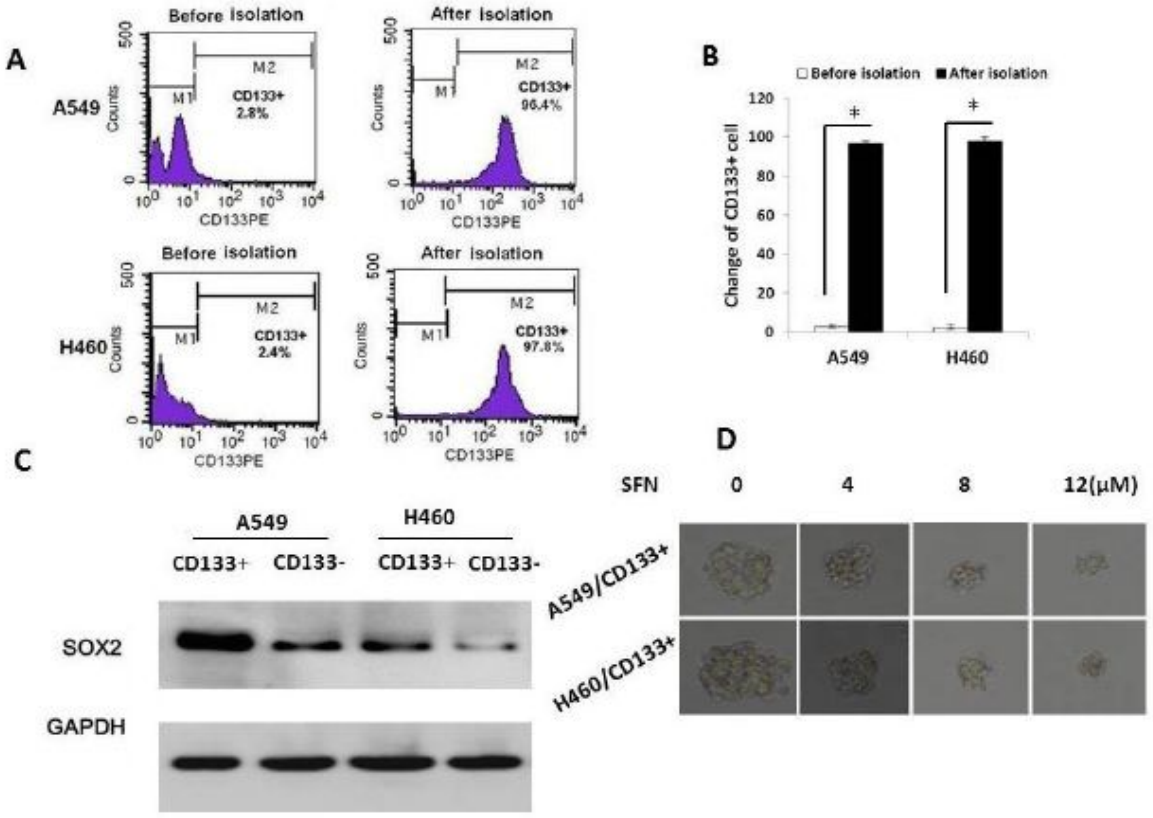

Fig. 2 Sorting of lung cancer stem cells and tumor sphere formation assay

(A,B) Before and after sorting, the percentage of CD133 positive cells are 2.8%, and 96.4% in A549 cells and 2.4% and 97.8% in H460 cells, respectively. The data present as means ± SD from three independent experiments.*P<0.05 compared to pre sorting group.

(C) Western blotting data shows high expression of stem cell gene SOX2 in human lung CSCs.

(D) The volume of tumor spheres of A549/CD133+ and H460/CD133+ cells following treatments with 0, 4, 8 and 12 μmol/L SFN for 7days. The data present as means ± SD from three independent experiments.*P<0.05 compared to the control.

Figure 2

Sorting of lung cancer stem cells and tumor sphere formation assay (A,B) Before and after sorting, the percentage of CD133 positive cells are 2.8%, and 96.4% in A549 cells and 2.4% and 97.8% in H460 cells, respectively. The data present as means ± SD from three independent experiments.*P<0.05 compared to pre sorting group. (C) Western blotting data shows high expression of stem cell gene SOX2 in human lung CSCs. (D) The volume of tumor spheres of A549/CD133+ and H460/CD133+ cells following treatments with 0, 4, 8 and 12 μmol/L SFN for 7days. The data present as means ± SD from three independent experiments.*P<0.05 compared to the control.

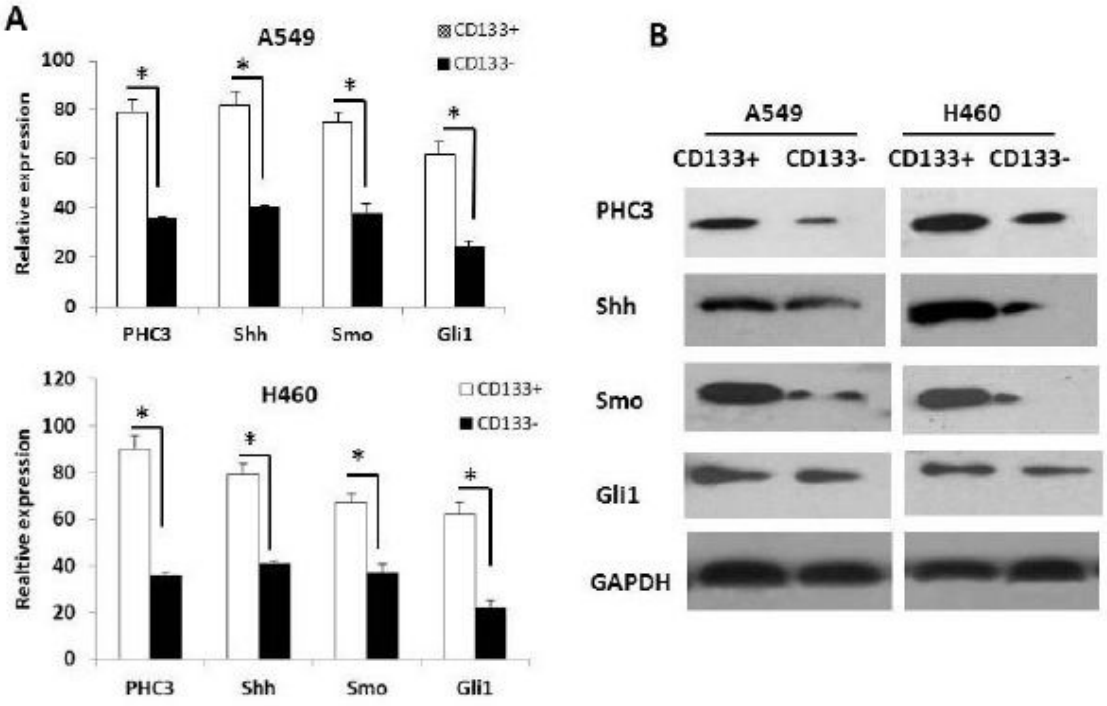

Fig. 3 Expression of PHC3, Shh, Gli1 and Smo in human lung CSCs and human non-lung CSCs.

(A) qPCR data shows PHC3, Shh, Gli1 and Smo expression in human lung CSCs (CD133+/A549 and CD133+/H460 cells) and in human non-lung stem cells (CD133-/A549 and CD133-/H460 cells). Each group was performed in triplicate. *P<0.05 compared to CD133 negative group.

(B) Western blotting data shows PHC3, Shh, Gli1 and Smo expression in human lung CSCs (CD133+/A549 and CD133+/H460 cells) and human non-lung stem cells (CD133-/A549 and CD133-/H460 cells).

Figure 3

Expression of PHC3, Shh, Gli1 and Smo in human lung CSCs and human non-lung CSCs. (A) qPCR data shows PHC3, Shh, Gli1 and Smo expression in human lung CSCs (CD133+/A549 and CD133+/H460 cells) and in human non-lung stem cells (CD133-/A549 and CD133-/H460 cells). Each group was performed in triplicate. *P<0.05 compared to CD133 negative group. (B) Western blotting data shows PHC3, Shh, Gli1 and Smo expression in human lung CSCs (CD133+/A549 and CD133+/H460 cells) and human non-lung stem cells (CD133-/A549 and CD133-/H460 cells).

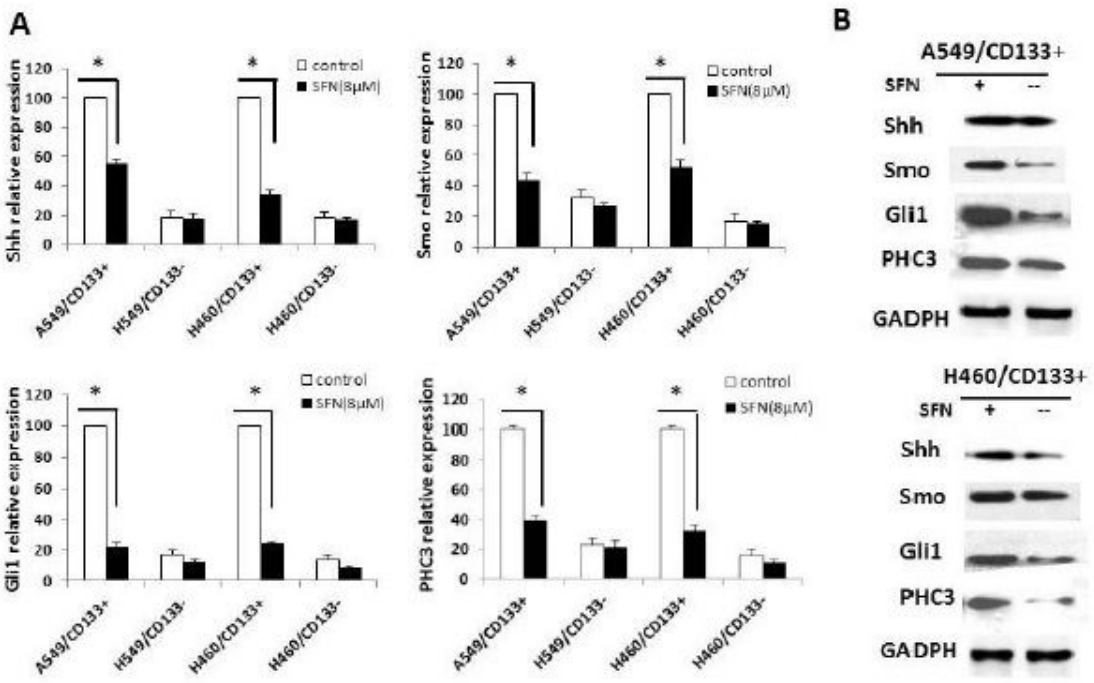

Fig. 4 SFN inhibits expression of PHC3, Shh, Gli1 and Smo in human lung CSCs.

(A) qPCR data show PHC3, Shh, Gli1 and Smo expression in human lung CSCs (CD133+/A549 and CD133+/H460 cells) and human non-lung CSCs (CD133-/A549 and CD133-/H460 cells) were treated with 8μmol/L SFN for 48 h. Each reaction was performed in triplicate. **P*<0.05 compared to the control.

(B) Western blotting data show PHC3, Shh, Gli1 and Smo expression in CD133+/A549 and CD133+/H460 cells were treated with 8μmol/L SFN for 48 h.

Figure 4

SFN inhibits expression of PHC3, Shh, Gli1 and Smo in human lung CSCs. (A) qPCR data show PHC3, Shh, Gli1 and Smo expression in human lung CSCs (CD133+/A549 and CD133+/H460 cells) and human non-lung CSCs (CD133-/A549 and CD133-/H460 cells) were treated with 8μmol/L SFN for 48 h. Each reaction was performed in triplicate. *P<0.05 compared to the control. (B) Western blotting data show PHC3, Shh, Gli1 and Smo expression in CD133+/A549 and CD133+/H460 cells were treated with 8μmol/L SFN for 48 h.

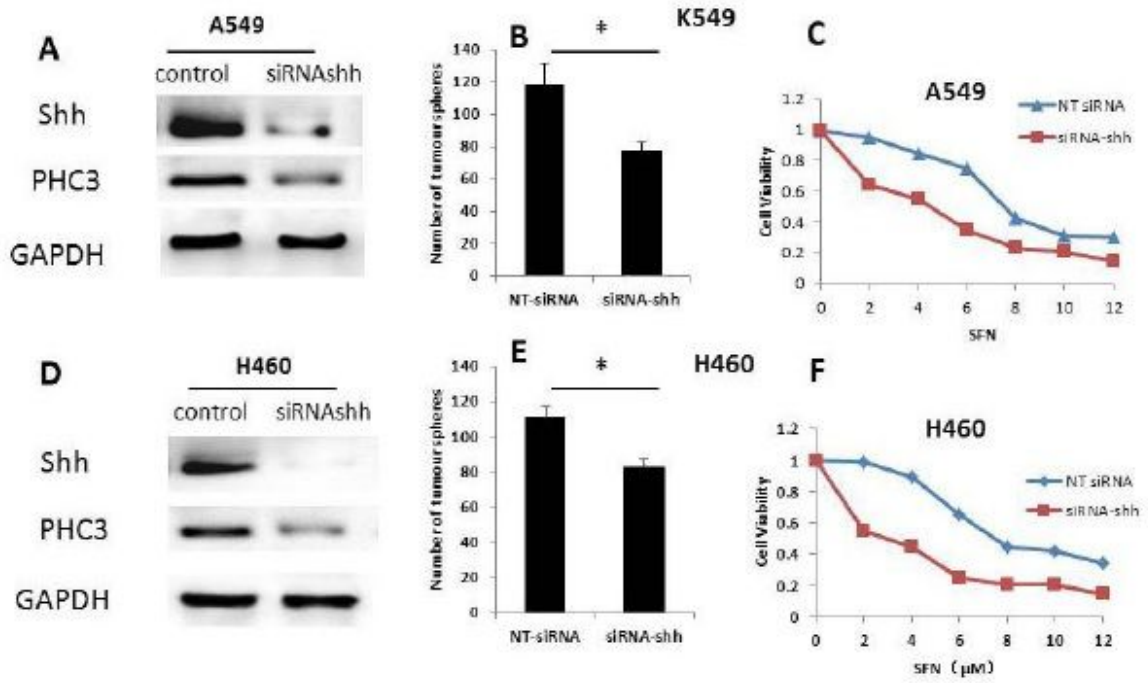

Fig.5 Effect of SHH signal pathway on cell proliferation.

(A, D)The expression of Shh and PHC3 protein was tested by western blotting when cells were transfected with siRNA against shh for 48h.

(B, E) The number of tumor spheres of A549 and H460 cells when cells were transfected with siRNA against shh for 7 days. *P<0.05 compared to the NT-siRNA.

(C, F) Inhibitory effect of SFN on viability of A549 and H460 cells by MTT when cells were transfected with siRNA against shh for 48h.

Figure 5

Effect of SHH signal pathway on cell proliferation. (A, D)The expression of Shh and PHC3 protein was tested by western blotting when cells were transfected with siRNA against shh for 48h. (B, E) The number of tumor spheres of A549 and H460 cells when cells were transfected with siRNA against shh for 7 days. *P<0.05 compared to the NT-siRNA. (C, F) Inhibitory effect of SFN on viability of A549 and H460 cells by MTT when cells were transfected with siRNA against shh for 48h.