Toosendanin induces apoptosis in Oral Squamous Cell Carcinoma cells through inhibition of p-STAT3 and inhibits tumor growth in a OSCC-PDX model

Ye Wu  
Fujian Medical University

Shaohai He  
Fujian Medical University

Tao Wang  
Fujian Medical University

Lingling Chen  
Fujian Medical University

Dali Zheng  
Fujian Medical University

Lisong Lin (✉ dr_lls@fjmu.edu.cn)  
The First Affiliated Hospital of Fujian Medical University

Research Article

Keywords:

Posted Date: December 1st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2297154/v1

License: ☐ ☐ This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

BACKGROUND

Toosendanin (TSN) has been found to inhibit the proliferation of different types of tumor cell lines. However, there is no data on the role of TSN in oral squamous cell carcinoma (OSCC). The purpose of this study was to evaluate the effects of TSN on OSCC cells in vitro, and to verify its effects on oral squamous cell carcinoma in vivo in a patient-derived xenograft (PDX) model.

METHODS

The effect of TSN on OSCC cells was investigated by cytotoxicity assays and flow cytometry. The expression of proteins was detected by Western blotting. An OSCC PDX model was constructed to further study the role of TSN in regulating the function of oral squamous cell carcinoma.

RESULTS

The cell viability of CAL-27 and HN-6 cells decreased gradually when the concentration of TSN increased from 0.025µM and 0.05µM to 0.1µM, and the apoptosis rate increased. Compared with the control group, the cytotoxic effect of TSN on CAL-27 and HN-6 cells was enhanced in a dose-dependent manner, and it could inhibit proliferation and induce apoptosis at lower doses. TSN can also induce apoptosis by inducing cell cycle arrest and regulating the expression of proteins such as STAT3. After successfully constructing an OSCC-PDX model with high pathological homology to the primary tumor and treated with intraperitoneal injection of TSN, The results showed that TSN could significantly reduce the tumor size of PDX model mice without obvious toxicity.

CONCLUSIONS

The in vivo experiments showed that TSN has a significant inhibitory effect on tumor growth, suggesting that it may be a promising drug for the treatment of oral squamous cell carcinoma. TSN may be an effective potential anticancer drug for the treatment of oral squamous cell carcinoma.

1. Introduction

Oral squamous cell carcinoma (OSCC) is a malignant tumor with a low survival rate, early metastasis and easily recurrence. It is the most common pathological type of oral neoplasm. Its incidence ranks 18th among all tumors, with 378,000 new cases and 178,000 deaths of lip and oral cancer worldwide in 2020 alone. The main treatment methods and strategies are surgery, radiotherapy, chemotherapy, etc. but the 5-year survival rate of patients is still about 65%, and that of advanced patients is less than 30%. With
the progress of modern medicine, the mortality rate of oral squamous cell carcinoma has not decreased significantly. Throughout history, chemotherapy has been used to treat malignant tumors. It can be used as a palliative treatment when the disease has progressed or returned, and can also be used as a comprehensive therapy combined with surgery or radiotherapy to improve the cure rate. Shinichiro Kina et al. found that compared with surgery, neoadjuvant chemotherapy significantly improved the tumor-free survival rate of tongue squamous cell carcinoma patients. However, most of the non-selective cytotoxic drugs used in clinic not only kill tumor cells, but also produce irreversible apoptosis on normal cells, leading to adverse reactions of chemotherapeutic drugs, which limits the therapeutic effect of chemotherapy. Because of its special anatomical structure and function, OSCC is very easy to metastasize, so its surgical treatment always cannot be completely cured, and there are many special antigen sites that affect the efficacy of conventional chemotherapy drugs, such as S Hartmann et al. found that MAGE-A tumor antigen can affect the sensitivity of head and neck cancer cells to cisplatin, 5-fluorouracil, paclitaxel and other chemotherapy drugs. Therefore, we need to find chemotherapy drugs with new therapeutic approaches.

Patients with advanced OSCC with lymph node metastasis are resistant to various chemotherapeutic drugs, and the use of chemotherapeutic drugs in clinical trials is not satisfactory. The exploration of natural plants has never stopped. 57% of the 175 anticancer drugs used in the world come directly or indirectly from natural products. But only 6% have been screened for biological activity, 15% have been evaluated by phytochemistry, and less than 10% of medicinal plants have been studied in detail. Natural plants and their bioactive components have the characteristics of being easy to obtain and low biological toxicity, and play an active role in promoting the inhibition of tumor cells.

Toosendanin (TSN, C₃₀H₅₈O₁₁) is a triterpenoid found in toosendan plants. It is a triterpenoid compound extracted from neem bark by Chinese scientists in the 1950s. It has been used to dispel ascariasis in the digestive tract. In the 1970s, while purifying and analyzing the chemical structure of TSN, some studies on its pharmacology were also carried out. Its pharmacological effects are extremely extensive, including but not limited to selectively blocking the release of acetylcholine from nerve endings, significant anti-botulism, and so on. Furthermore, TSN inhibits significantly the proliferation of hepatoma cell line (BEL7404), glioblastoma cell line (U251), neuroblastoma cell line (SH-SY5Y), promyelocytic leukemia cell line (HL-60), prostate cancer cell line (PC3), colorectal cancer cell line (SW480), breast cancer (MDA-MB-468) and lymphoma cell line (U937). Compared with similar chemotherapy drugs, TSN has a lower IC₅₀, and the access is relatively simple. TSN has a multi-target regulatory effect on a variety of tumor cell lines, and inhibits the proliferation of tumor cells through its downstream signal, so it has a good anticancer prospect. Zhang et al. first reported that TSN can inhibit the proliferation of tumor cells at the molecular level, and its mechanism may be related to the induction of necrosis, apoptosis and autophagy of tumor cells. However, the specific mechanism of TSN's anti oral squamous cell carcinoma effect is still unclear.
Patient-derived xenograft tumor model (PDX) has gradually become a hot topic in recent years\textsuperscript{15,16}. It has achieved good results in tumor drug screening and clinical transformation research, and is considered to be a valuable tool for preclinical tumor screening\textsuperscript{17}. PDX model can fully reflect the heterogeneity of original tumors and create tumor microenvironment that cannot be simulated in CDX model\textsuperscript{18}. This is a reproduction of the very similar behavior and performance of tumors in patients, especially in terms of tumor drug response\textsuperscript{19,20}. PDX is a tumor model created by transplanting tumor tissues of cancer patients into immunocompromised mice, which is the first cancer research method used by the National Cancer Institute (NCI)\textsuperscript{21}.

The inhibitory effects of TSN on cancer can be seen in the basic experiments of gastrointestinal and other systemic tumors, but it has not been reported in oral head and neck squamous cell carcinoma. Therefore, we have used BALB/c nude mice and NOD-SCID nude mice to establish OSCC-PDX model, and further explored the effect of TSN on process of development and occurrence of OSCC and its curative effect in vivo and in vitro.

2. Material And Methods

2.1. Cell culture and reagents

Normal oral epithelial cells (HOEC) Normal oral fibroblasts (HPG) OSCC (CAL27 and HN6) cell lines were taken from the Fujian Key Laboratory of Oral Diseases and cultured in high glucose DMEM with Fetal Bovine Serum (FBS) heat-inactivated at 10%. The cells were cultured in the following conditions: 37 °C in a 5% CO\textsubscript{2} humidified incubator.

2.2. Materials

Toosendanin (MF: C\textsubscript{30}H\textsubscript{38}O\textsubscript{11}, MW: 574.62, purity: HPLC ≥ 98%, Cat.No HY-N0263) was purchased from MedChemExpress Co., Ltd, US. Mice were provided by Beijing HuaFuKang Biotechnology Co., Ltd.

2.3. Cell Survival Assay

The cell viability and the killing ability of TSN to cells were measured by CCK8 method. Inoculated cells were cultured overnight at 37°C with 5% CO\textsubscript{2} in 96-well plates with 8\times10\textsuperscript{3} cells per well. In cell culture medium, TSN was dissolved in dimethyl sulfoxide (DMSO) (the maximum concentration of DMSO was 0.05% (v/w), and used DMSO to prepare different concentrations of TSN (0.625-100μM). A 0.05% DMSO solution was used to treat the control group of cells. Cells were incubated at 37 °C in a 5% CO\textsubscript{2} cell incubator for 24 hours and 48 hours, respectively. Then each well was incubated for one hour at 37 °C in 5% CO\textsubscript{2} with 10 μl CCK8 added. The absorbance was detected at 450 nm with a microplate reader. The 50% maximum growth inhibition concentration (IC\textsubscript{50}) was calculated by nonlinear regression analysis according to the concentration of log and cell viability. Three replications of all experiments were
conducted, and the standard deviation was less than 5%. Each treatment's effect was evaluated as a percentage of cell survival, with untreated control cells considered to be 100% alive.

2.4. Annexin V-FITC/PI Double Staining

Annexin V-FITC/PI double fluorescence detection kit (Uelandy, China, Cat.No F6012L) was used to detect apoptosis. CAL27 and HN6 cells (3.5 × 10⁵ cells per well) were inoculated into 6-well plates, and TSN (0–20µM) concentration gradient was selected to stay overnight for 48 hours. The collected trypsin digested cells and supernatant were centrifuged for 5 minutes at the rate of 200 g, precipitated in 100 µl buffer and re-suspended, and stained for 10–15 min at 15 °C-20 °C. Immediately analyzed by flow cytometry, the excitation wavelength was 488nm and the emission wavelength was 525 nm.

2.5. Western Blot

CAL-27 cells were treated with TSN (0, 0.025, 0.05 and 0.1 µM) for 72 hours, and then put into the lysis buffer. After cracking, the sample is cooked, the protein sample (40 µg) is graded in 10% sodium dodecyl sulfate polyacrylamide gel, and then transferred to the polyvinylidene fluoride membrane. After sealing with blocking solution, the membrane was washed with bovine serum albumin, and then incubated with primary antibody at 4 °C for the night. First antibody: anti-STAT3(Abcam, Cat.No ab-68153,1:1000), anti-p-STAT3(Abcam, Cat.No ab-267373,1:1000), and goat anti-rabbit second antibody. Finally, the developer is used to visualize the protein bands in the imaging system. The internal control was GAPDH. The gray values of proteins were measured by IMAGE J software, and statistical analysis was carried out.

2.6. In vivo study of PDX model

The tumor specimens in this study were provided by the Affiliated Hospital of Fujian Medical University (Approval Number: FJMU-IACUC 2021-0461) and the written informed consent of each participant was obtained. Part of the tumor resected from the patient was immediately stored in DMEM. Then 2–3 mm³ tumor blocks were implanted into the groins of 4-week-old male NOD/SCID mice. When the primary mouse tumor grew to about 100 mm³, it was transplanted into the second-generation NOD/SCID mice. Similarly, when the tumor of the second generation of mice grew into 100 mm³, it was transplanted into Balb/c nude mice. The mice were randomly divided into 3 different treatment groups and 1 group of blank control group with 7 mice in each group. PDX tumor-bearing mice were treated by intraperitoneal injection of 5-Fluorouracil (positive control group 5mg/kg), TSN (5mg/kg) and TSN (10mg/kg) for two weeks. Based on the formula below, we calculated the volume of the tumor:

\[
\text{volume} = \text{length} \times \text{width}^2 \times 0.5
\]

2.7. Histological Examination

Paraformaldehyde-fixed tumor sections were embedded in paraffin, and hematoxylin-stained sections were routinely examined, and hematoxylin was stained routinely. Observing and capturing the slides was performed using a fluorescence inverted microscope.
2.8. Statistical Analysis

In all statistical analyses, \( P < 0.05 \) was used as the significance level. The means ± standard error (SD) was used to report all results. Comparing two groups of independent samples was done using Student's t-test. One-way ANOVA analysis was used to determine the statistical significance of multiple groups.

3. Result

3.1. Cell Survival Assay

In order to evaluate the inhibitory effect of TSN on the proliferation of HN6 and CAL27 cell lines, the cell viability of HN6 and CAL27 cells treated with different concentrations of TSN (0–10 µM) at different time points (24 hours and 48 hours later) was measured, and the cytotoxicity of TSN was evaluated. Observation under a fluorescence inverted microscope (Fig. 1A B) showed that obvious inhibition of cell proliferation was observed at low drug concentration. It is also demonstrated that, with the increase of drug concentration, the number of normal adherent cells decreased significantly, and apoptosis products such as cell vacuoles and fragments appeared, the cell morphology having changed irregularly. Apoptosis induced by TSN is in a dose-dependent manner. IC\(_{50}\) (TSN-HOEC) = 3.436 µM, IC\(_{50}\) (TSN-HPG) = 0.6875 µM, IC\(_{50}\) (TSN-CAL27) = 0.1012 µM and IC\(_{50}\) (TSN-HN6) = 0.2168µM. (Fig. 1C). The IC\(_{50}\) of TSN on normal oral cells is higher than that of tongue squamous cell carcinoma.

3.2. Annexin V-FITC/PI Double Staining

In order to determine whether TSN can directly induce apoptosis in CAL27 and HN6 cells, after 48 hours of treatment with different concentrations of TSN (0-0.1 µM) (Fig. 2A). The flow cytometry (Fig. 2A) showed that the apoptosis rate of CAL27 cells increased after TSN treatment, and the number of apoptosis and the early (\( P < 0.001 \)) and late (\( P < 0.001 \)) apoptosis rates rose in a dose-dependent manner after TSN treatment (Fig. 2B).

3.3. Western Blot

STAT3 is related to the proliferation of tumor cells, and the down-regulation of p-STAT3 expression will induce tumor cell apoptosis. With the aim to knowing whether TSN has inhibitory effect on STAT3 pathway, and determining of the above indexes by Western blotting to determine whether TSN inhibited STAT3 phosphorylation and promoted apoptosis of CAL27 cells (Fig. 3A). The results showed that p-STAT3 decreased significantly and caspase3 increased significantly (\( P < 0.001 \)) (Fig. 3B). TSN blocked the activity of STAT3 in a concentration-dependent manner. However, there was no statistical significance between the two concentration groups of Group 0.05µM and Group 0. 1µM.

3.4. TSN treatment in PDX Models
According to our previous studies on tumor cells in vitro, TSN can effectively inhibit the growth of OSCC. After the establishment of OSCC-PDX model, the primary tumor and the PDX tumor were stained with HE, and the four immunohistochemical indexes of CK56, Ki67, p53 and P16 were determined (Fig. 4).

The results showed that the OSCC-PDX model was constructed successfully, it has high pathological homology with patient tumor.

Randomly divided into two groups and after 14 days of intraperitoneal injection treatment (Fig. 5C), three mice in the NC group and one mouse in the 5-FU group died, the related tumor was removed (Fig. 5D). There was no significant difference in body weight between NC group and TSN group, which suggested that TSN had no obvious toxicity to mice(Fig. 5A). Compared with NC group, the tumor volume of TSN group was smaller and the weight was less, either concentration of TSN was statistically significant for tumor size reduction. There was no significant difference between the positive control 5-FU group, while the high concentration (10mg/kg) TSN group showed more therapeutic effect. \(P< 0.001\) (Fig. 5B).

The apoptosis and proliferation of tumor cells was verified by detecting the tumor tissue by Ki67, Caspase 3 and TUNEL(Fig. 6(A) -D)). As shown in Fig. 6E and F, TSN promoted the apoptosis of tumor cells (the number of orange-red spots in TUNEL increased), there was statistical significance among the three groups \((P< 0.001)\), and inhibited the proliferation of tumor cells (the number of brown spots in Ki67 decreased and the number of brown spots in Caspase 3 increased). For caspase 3, which represents apoptosis, there is no statistical difference among the three groups (ns). This result coincides with the results of ours in vitro experiment.

4. Discussion

Many previous studies have shown that TSN plays a significant role in promoting apoptosis of tumor cell lines such as blood system, kidney, lung, prostate, liver and breast, and can enhance the effect of chemotherapeutic drugs on drug-resistant tumor cells\(^{12,13}\). However, the mechanism of OSCC has not been elucidated. For this reason, this study used human HN6, CAL27 cells, and OSCC-PDX model to investigate the mechanism and effects of TSN on oral squamous cell carcinoma.

It is found that TSN effectively inhibited the growth of OSCC cells at very low concentration in vitro, which has cytotoxicity and promotes apoptosis in a time-and dose-dependent manner. However, it has low toxicity to normal oral epithelial cells HOEC and normal oral fibroblasts HPG. These findings were consistent with other types of tumors reported in the literature, which have also identified TSN as an anticancer agent\(^{24,25}\).

The results of flow cytometry showed that TSN could significantly induce early and late apoptosis of OSCC cells. It has been found that TSN may induce apoptosis through STAT3 pathway. Signal transducer and activator of transcription 3 (STAT3) is a special transcription factor. Current studies have shown that there are abnormal signal transduction and abnormal activation of STAT3 in many malignant tumor cells\(^{26,27}\). Up-regulation of p-STAT3 expression can induce tumor cell proliferation, while its down-
regulation can promote tumor cell apoptosis. It is a key point in activating a variety of growth factors or cytokine signal pathways. In addition, it regulates genes that are involved in cellular growth, metastasis, and angiogenesis. It is regarded as a promising target for cancer therapy. We also found that TSN inhibits the expression of p-STAT3 by blocking the STAT3 pathway in CAL27 cells, and the inactivated STAT3 cannot bind to the caspase3 promoter, which further affects the expression of downstream protein caspase 3, leading to direct cytotoxicity and apoptosis\textsuperscript{28}.

As the ultimate objective of our study, we wish to verify the in vivo efficacy of TSN in inhibiting PDX model. In cancer drug screening, human tumor cell lines are cultured and mouse xenotransplantation of human cells growing under the skin of immunodeficient animals is performed. These models have an obvious flaw, which is their artificial influence on tumor cell lines, which are usually passed along from generation to generation in rich culture media. Genetic and epigenetic heterogeneity of primary tumors may not generally be represented by these models\textsuperscript{29,30}. On the other hand, the PDX model retains the differentiation, morphological, structural and molecular biological characteristics of the primary tumor, restores the tumor microenvironment in vivo more accurately, retains the heterogeneity of the original tumor tissue, and improves the accuracy of predicting the antitumor effect of drugs\textsuperscript{31,32}. According to the immunohistochemical results of mouse tumor mass and patient tumor, our OSCC-PDX model has high pathological homology with patient tumor, and angiogenesis can be seen in mouse tumor, indicating that this model greatly simulates the tumor microenvironment in vivo and retains the heterogeneity of the original tumor tissue. Therefore, the results of our study on OSCC-PDX mouse model have considerable credibility. It can be seen that the effect of TSN on PDX model mice is similar to that of 5-FU, and the higher the concentration, the better the therapeutic effect. According to the results of immunohistochemical experiment in vivo, we can see that TSN can significantly promote tumor apoptosis, which can be reflected in TUNEL experiment, and we measured the downstream protein caspase 3 of STAT3 signal pathway, and its expression is relatively weak, which is similar to that of Western Blot. It can be concluded that TSN has a positive effect on the chemotherapeutic effect of oral squamous cell carcinoma.

To sum up, our experimental results showed that TSN may induce apoptosis by inhibiting STAT3 in a dose-dependent manner. However, the application of TSN is limited by its own properties such as low water solubility, low thermal stability, high photosensitivity and low bioavailability\textsuperscript{33}.

The rate and degree of the drug from the site of administration to the systemic circulation is called the bioavailability of the particular drug, which is the main factor to be considered to measure the good efficacy of the drug. This needs further research and has great potential for further treatment in the clinical environment. Therefore, the results of this study may have direct practical significance, but further experiments are needed to evaluate the efficacy of TSN in patients with OSCC before clinical use.

**Abbreviations**
Declarations

Disclosure

The authors report no conflicts of interest in this study.

Ethical Approval

The tumor specimens in this study were provided by the Affiliated Hospital of Fujian Medical University (Approval Number: FJMU-IACUC 2021-0461).

Competing interests

The authors declare that they have no competing interests in relation to the present work.

Authors' contributions

All authors made substantial contributions to the present study. YW, LSL, DLZ and SHH carried out the concepts, design, definition of intellectual content, literature search, data acquisition, data analysis, manuscript review and manuscript editing. LLC and TW carried out the literature search. SHH drafted the manuscript text and prepared figures 1-6. All authors read and approved the final manuscript.

Funding

This work was supported by Subsidy Foundation for Educational and Scientific Research Projects of units directly under Fujian Provincial Department of Finance [Grant No. 2022-639] and the Open Project of the Stomatology Key Laboratory of Fujian Province [Grant No. 2019kq06]

Availability of data and materials

All the articles included in the present paper are available in the Pubmed.

References


Figures
Figure 1

The result of cell survival assay. (A) HOEC and HPG observed under fluorescence inverted microscope (48 h). (B) CAL27 and HN6 observed under fluorescence inverted microscope (48 h). (C) Fitting curve of HN6 CAL27 HOEC and HPG treated with TSN (48 h).
Figure 2

Induction of apoptosis by TSN in OSCC cells. (A) Flow cytometry images. (B) Early and late apoptosis of CAL27 induced by TSN.

*P < 0.05, **P < 0.01, ***P < 0.001
Figure 3

TSN induce OSCC apoptosis via p-STAT3 inhibition. (A) Western blot analysis of total STAT3 and p-STAT3, caspase 3 and cleaved-caspase 3 in CAL27 whole-cell lysates after treatment with TSN. (B) Western blot analysis of total STAT3 and p-STAT3 in CAL27 whole cell lysates.

*P < 0.05, **P < 0.01, ***P < 0.001
Figure 4

Immunohistochemical results of the primary tumor and the PDX tumor. (A) HE. (B) CK56. (C) Ki67. (D) P53. (E) P16.
Figure 5

Construction of OSCC-PDX model and treatment effect of TSN on tumor growth in a PDX model. (A) Body weight of mice in each group. Data are expressed as the mean ± SDs, n=7. (B) Fig 9: Tumor volume of each group. Data are expressed as the mean ± SDs, n=7. (C) Groups of mice at the end of the experiment.

*P < 0.05, **P < 0.01, ***P < 0.001
Figure 6

Immunohistochemical results of the PDX tumor. (A)-(D) Immunohistochemistry staining (HE, Ki67, Caspase 3 and TUNEL) of tumor tissues. (E) Statistical histogram of Ki67 and Caspase 3. (F) Statistical histogram of TUNEL.

*P < 0.05, **P < 0.01, ***P < 0.001