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# Tripartite Motif-Containing 44 is Involved in the Tumorigenesis of Laryngeal Squamous Cell Carcinoma, and its Expression is Downregulated by Nuciferine

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Tripartite motif-containing 44 (TRIM44) was reported to be involved in the tumorigenesis of several tumors, but its function in laryngeal squamous cell carcinoma has not been investigated yet. In the present study, we aimed to elucidate the function of TRIM44 in laryngeal squamous cell carcinoma, and identify the compounds which could inhibit TRIM44 expression. Our results showed that TRIM44 was upregulated in tumor tissues and cell lines of laryngeal squamous cell carcinoma. Knockdown of TRIM44 significantly inhibited cell growth of laryngeal squamous cell carcinoma by suppressing TLR4, phosphorylated AKT and phosphorylated NF-κB p65 expression in vitro. Moreover, TRIM44 knockdown inhibited tumor growth in nude mice, which further suggested that TRIM44 exerted oncogenic activity in laryngeal squamous cell carcinoma. Interestingly, it was found that nuciferine significantly inhibited the mRNA levels of TRIM44 after screening a small natural compound library. Our further studies showed nuciferine markedly downregulated the protein levels of TRIM44 and its substrate TLR4 in a concentration-dependent manner in laryngeal squamous cell carcinoma cells. Moreover, the activation of downstream kinases of TLR4 such as AKT signaling pathway was also inhibited by nuciferine. Additionally, nuciferine markedly inhibited cell survival of laryngeal squamous cell carcinoma in a concentration-dependent manner. In contrast, TRIM44 overexpression significantly reduced the cytotoxicity of nuciferine in laryngeal squamous cell carcinoma cells. In conclusion, this study indicated that inhibiting TRIM44 would be a useful strategy for the treatment of laryngeal squamous cell carcinoma, and nuciferine could be a potential chemical applicated in the therapy of laryngeal squamous cell carcinoma.

**Keywords:** laryngeal squamous cell carcinoma; nuciferine; tripartite motif-containing 44; tumorigenesis
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## Introduction

Laryngeal squamous cell carcinoma (LSCC) is a common malignant tumor of head and neck squamous cell carcinoma (HNSC), which accounts for about 1% ~ 5% of all tumors in the body (Mai and Ma 2019). HNSC mainly includes laryngeal carcinoma, nasopharyngeal carcinoma, cervical thyroid carcinoma, etc. At present, surgery, radiotherapy and chemotherapy are the main treatments for early-stage LSCC patients (Baird et al. 2018). However, in late-stage LSCC patients, the 5-year overall survival rate of patients is still very low, and high invasive and acquired chemotherapy resistance are the main causes of LSCC recurrence (Saglam et al. 2007). Therefore, it is very important to reveal the pathogenesis of LSCC and find new target drugs for the treatment of LSCC.

It has been proved that several important inflammatory factors, such as tumor necrosis factor $\alpha$ (TNF$\alpha$) and interleukin-1$\beta$ (IL-1$\beta$), are often released from the injured tissues of LSCC patients (Wang et al. 2016). These inflammatory factors can activate downstream important signaling pathways by acting on Toll-like receptor (TLR4), which can further enhance the malignant process of LSCC (Wang et al. 2016). TLR4 is the first pathogen pattern recognition

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receptor found in Toll-like receptors (TLRs) family, and the C-terminal of TLR4 contains Toll/IL-1 receptor domain, which is mainly used to activate downstream signaling pathways, such as the AKT/mTOR signaling and NF-$\kappa$B signaling pathway (Bahrami et al. 2018). In addition, it has been also reported that TLR4 can be bound to and stabilized by the ubiquitin ligase tripartite motif-containing 44 (TRIM44) (Wei et al. 2019). TRIM44 has been reported to be elevated and verified as a valuable prognostic biomarker in several cancers, such as renal cancer, melanoma, esophageal cancer and prostate cancer (Xiao et al. 2020). Targeting TRIM44 may be a potential target for the treatment of tumors.

Nuciferine is a bioactive component of the lotus leaf, which is a kind of aporphine alkaloid (Farrell et al. 2016). It has been proved that the alkaloids in lotus leaf have the effects of lowering blood lipid, anti-free radical activities, inhibiting hypercholesterolemia and arteriosclerosis, and anti-mitosis activities (Farrell et al. 2016). Recently, it has been also reported that nuciferine displays anti-tumor activities in several tumors. Qi et al. (2016) found that nuciferine may be as a novel anti-cancer agent in colorectal cancer and neuroblastoma by using a traditional Chinese medicine network pharmacology method. Zhou et al. (2019a) also found that application of nuciferine could sensitize pancreatic cancer cells to gemcitabine by inhibiting YAP. Nuciferine was also reported to display anti-tumor activities in glioblastoma, non-small cell lung cancer and breast cancer (Liu et al. 2015; Kang et al. 2017; Li et al. 2019). In the present study, we aimed to investigate the function of TRIM44 in LSCC, and evaluate the effects of nuciferine on regulating TRIM44 expression and LSCC cell survival.

## Materials and Methods

*Cells, tissues and chemicals*

Four LSCC cell lines (AMC-HN-8, HEP-2, TU-212 and TU-686) and the normal bronchial epithelial cell line NHBEC were purchased from Bena Culture Collection (Beijing, China). HEK293T cell line was purchased from American Type Culture Collection (ATCC) (Gaithersburg, MD, USA). All cell lines were cultured in DMEM medium (HyClone, Logan, Utah, USA) containing 10% fetal bovine serum (Biological Industries, Beit HaEmek, Israel). Thirty pairs of primary LSCC paracancerous and cancerous tissues were collected from Chengde Central Hospital (Chengde, Hebei, China). All patients were pathologically confirmed, and the fresh tissues were immediately collected and frozen in liquid nitrogen after surgery. The collection of tissues was approved by the Review and Ethics committee of Chengde Central Hospital. Nuciferine (Purity, 99.49%) and other 49 natural products used in this study were all obtained from Selleck Chemicals (Houston, Texas, USA).

*Bioinformatics analysis*

The public tumor database GEPIA matched TCGA normal and GTEx data was used online (http://gepia2.cancer-pku.cn/#analysis) to analyze the expression of TRIM44 in HNSC (subtype filter: mesenchymal (75)).

*Quantitative real-time PCR (qRT-PCR)*

Total RNA was extracted from LSCC cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was reverse-transcribed with PrimeScript reagent kit according to the manufacturer's instruction (Takara, Dalian, China). Then, qRT-PCR was performed with SYBR Green Mix (Takara, Dalian, China). Primers listed as below: TRIM44, forward, 5′-GGCTTGATTTGAGTACCTATT-3′ and reverse, 5′-AGTCCACCTGAGTCTTTGC-3′; GAPDH, forward, 5′-CACCCACTCCTCCACCTTTG-3′ and reverse, 5′-CCACCACCCTGTTGCTGTAG-3′.

*Western blot analysis*

LSCC cells were lysed by sodium dodecyl sulfate (SDS) lysis and prepared for western blot as previously described (Chen et al. 2019). The primary antibodies used in this study were as shown below: anti-TRIM44 antibody (Abcam, Cambridge, UK; Cat. ab236422), anti-GAPDH antibody (Abclonal, Wuhan, China; Cat. AC002), anti-TLR4 antibody (Abcam, Cambridge, UK; Cat. ab13867), anti-phospho-AKT (S473) antibody (CST, Boston, MA, USA; Cat. #4060), anti-AKT antibody (CST, Boston, MA, USA; Cat. #9272), and anti-phospho-p65 (S536) antibody (CST, Boston, MA, USA; Cat. #3033).

*CCK-8 assay*

To evaluate cell growth, LSCC cells were infected with shNC, shTRIM44-1 or shTRIM44-2-derived lentivirus for 0, 1, 3 or 5 days, and then CCK-8 assay was performed according to the manufacturer's instruction (Bimake, Houston, Texas, USA). To evaluate cell survival, LSCC cells were incubated with 0, 25, 50 or 100 $\mu$M nuciferine for 24 hours, and then CCK-8 assay was measured ($\lambda$ = 450).

*Plasmids and transfection*

The pcDNA3.1 vector (EV) was used to construct TRIM44-overexpressing plasmid (TRIM44-OE). EV and TRIM44-OE plasmids were purchased from Genewiz (Suzhou, China). The lentivirus-delivered shRNAs were purchased from GenePharm (Suzhou, China). The sequences of shTRIM44 used were as listed below: shTRIM44-1, 5′-GCCTTTGAAGAATTAAGAAGC-3′; shTRIM44-2, 5′-GCAGAAGGCCCTTCATCTAGT-3′. Above plasmids were transfected into LSCC cells or HEK293T cells with Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA).

*In vivo assay*

$5 \times 10^6$ TU212 cells infected with shNC or shTRIM44-1 were subcutaneously inoculated into the right flanks of nude mice (n = 5 per group) to evaluate the growth ability in vivo. The six-week-old BALB/c nude mice were

obtained from the Institute of model animals of Nanjing University (Nanjing, China). The tumor sizes of nude mice were monitored every two days by using a vernier caliper ($V = 0.5 \times length \times (width)^2$), and the tumor growth curves were calculated. Tumors were also excised at the end of the experiment for further study. The animal study was approved by the Review and Ethics committee of Chengde Central Hospital.

*Statistical analysis*

Data were expressed as mean ± SD. Graphpad Prism 8 was used to draw pictures during the experiments. Data were analyzed by ANOVA followed by Tukey's post hoc test or Students's t test. A $p$ value less than 0.05 was considered to be statistically significant.

## Results

*TRIM44 is upregulated in LSCC*

The function of TRIM44 has not been investigated yet in LSCC. Preliminarily, the public tumor database GEPIA was used to predict the expression levels of TRIM44 in HNSC. As shown in Fig. 1A, the database showed that TRIM44 was significantly upregulated in HNSC tumor tissues. To further confirm the expression of TRIM44 in LSCC, thirty pairs of paracancerous and cancerous tissues were collected. As shown in Fig. 1B, the qRT-PCR analysis showed that TRIM44 was also significantly upregulated in LSCC tumor tissues compared with the paracancerous normal tissues. The qRT-PCR and western blot also showed that TRIM44 was upregulated in four LSCC cell lines compared with the normal human cell line (Fig. 1C, D). These results indicated that TRIM44 was elevated in LSCC.

*Knockdown of TRIM44 suppresses cell growth and tumor growth of LSCC*

Subsequently, to investigate the function of TRIM44 in LSCC cells, TRIM44 was knocked down by shRNAs in AMC-HN-8 and TU-212 cells. CCK-8 assay showed that knockdown of TRIM44 significantly inhibited LSCC cell growth (Fig. 2A, B). The western bot also revealed that TRIM44 knockdown obviously downregulated the expression of its substrate TLR4, and inhibited the activation of its downstream signals, including AKT and NF-$\kappa$B signalings, which were closely involved in mediating tumor cell survival and growth (Fig. 2C, D).

To further confirm the effects of TRIM44 on regulating LSCC cell growth, xenograft models were established. As shown in Fig. 3A, the tumor growth curves showed that knockdown of TRIM44 significantly suppressed the tumor growth in nude mice. At the end of the animal study, tumors were excised (Fig. 3B), and lysed for western blot analysis. As shown in Fig. 3C to Fig. 3E, the protein levels of TRIM44 and phosphorylated AKT were obviously decreased in shTRIM44-group tumors compared with the control, which further indicated that TRIM44 exerted its oncogenic effect by regulating TLR4 signaling in LSCC.

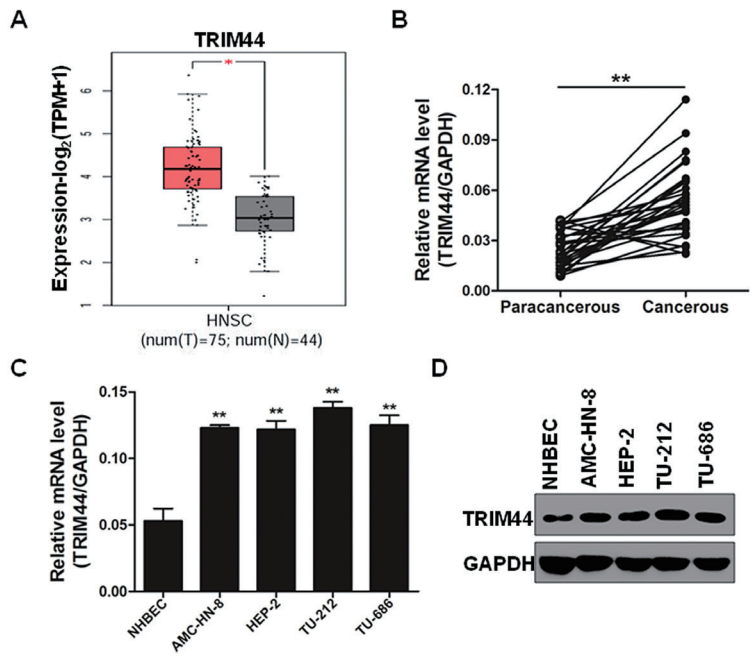


Fig. 1. TRIM44 is upregulated in LSCC.
A. The expression of TRIM44 in HNSC was analyzed by GEPIA online matched with TCGA database. B. Thirty pairs of primary LSCC paracancerous and cancerous tissues were used for qRT-PCR to assess the expression of TRIM44. C and D. LSCC cell lines (AMC-HN-8, Hep-2, TU-212 and TU-686) and the normal cell line NHBEC were used for qRT-PCR (C) and western blot (D) to assess the expression of TRIM44. LSCC, laryngeal squamous cell carcinoma; HNSC, head and neck squamous cell carcinoma; TRIM44, tripartite motif-containing 44.
$^{*}p < 0.05$; $^{**}p < 0.01$.

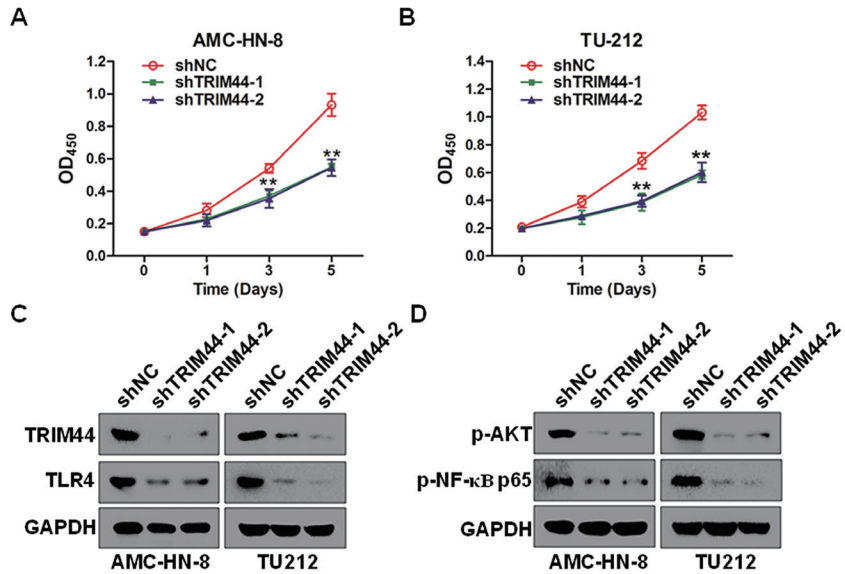


Fig. 2. Knockdown of TRIM44 inhibits LSCC cell growth by suppressing AKT and NF-$\kappa$B activation.
A and B. AMC-HN-8 (A) and TU-212 (B) cells infected with shNC, shTRIM44-1 or shTRIM44-2 were cultured for 0, 1, 3 or 5 days, and CCK-8 assay was measured to assess cell viability. C and D. AMC-HN-8 and TU-212 cells infected with shNC, shTRIM44-1 or shTRIM44-2 were cultured for 3 days, and western blot was measured to assess the expression of TRIM44, TLR4 (C), p-AKT and p-NF-$\kappa$B p65 (D).
$^{**}p < 0.01$.

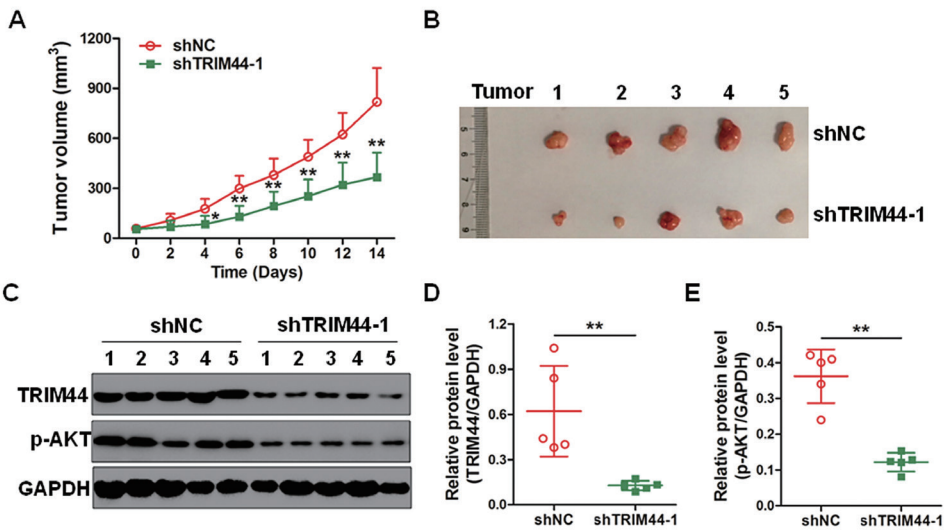


Fig. 3. Knockdown of TRIM44 inhibits tumor growth in nude mice.
A and B. TU212 cells infected with shNC or shTRIM44-1 were subcutaneously inoculated in nude mice to evaluate the growth ability in vivo, and the tumor growth curves were calculated (A). Tumors were also excised at the end of the experiment (B). n = 5 mice per group. C-E. Tumors were prepared for western blot to evaluate the expression of TRIM44 and p-AKT. GAPDH was used as a loading control (C). And the optical density was also measured (D and E).
$^{*}p < 0.05$; $^{**}p < 0.01$.

*Nuciferine is screened out to inhibit the expression of TRIM44 in LSCC cells*

In order to further identify the compounds that could inhibit the expression of TRIM44, we collected 50 natural compounds for a small-scale screening. As shown in Fig. 4A, TU212 cells were treated with DMSO or 50 natural chemicals (25 $\mu$M) for 24 hours, and then cells were prepared for qRT-PCR to assess the expression of TRIM44. Among the results, we found that nuciferine could inhibit the expression of TRIM44 more effectively (Fig. 4A, B). Then, we further verified that nuciferine could inhibit TRIM44 expression in a concentration-dependent manner in both of AMC-HN-8 and TU-212 cells (Fig. 4C).

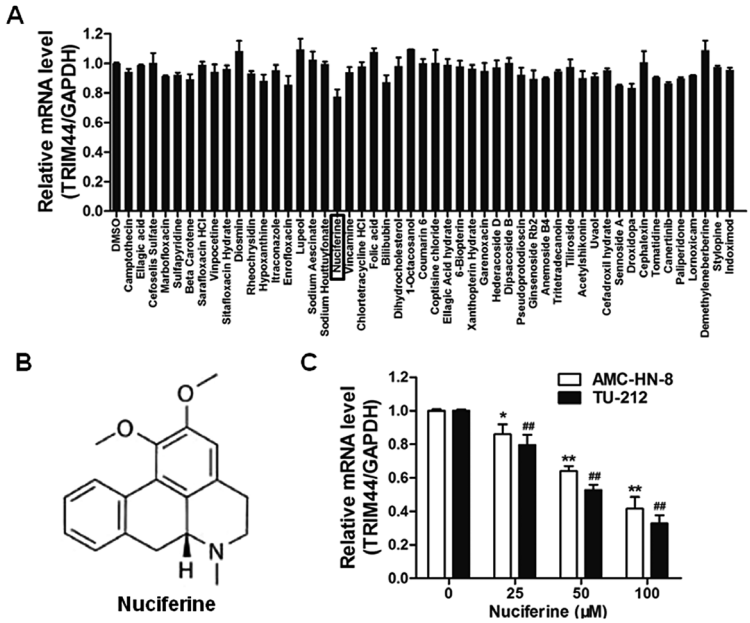


Fig. 4. Nuciferine is screened out to inhibit the expression of TRIM44 in LSCC cells.
A. TU212 cells were treated with DMSO or 50 natural chemicals (25 $\mu$M) for 24 hours, and then cells were prepared for qRT-PCR to assess the expression of TRIM44. B. The chemical structure of nuciferine. C. AMC-HN-8 and TU-212 cells were treated with increasing concentrations of nuciferine for 24 hours, and then cells were prepared for qRT-PCR to assess the expression of TRIM44.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{\#\#}p < 0.01$.

*Nuciferine displays anti-tumor activity in LSCC cells by suppressing TLR4 expression*

Then, we detected the effects of nuciferine on TRIM44 protein expression by western blot. As shown in Fig. 5A, nuciferine markedly downregulated the protein levels of TRIM44 and its substrate TLR4 in a concentration-dependent manner in both of AMC-HN-8 and TU-212 cells. Moreover, the activation of downstream kinases of TLR4 such as AKT signaling pathway was also inhibited (Fig. 5B). Not surprisingly, nuciferine markedly inhibited LSCC cell survival in a concentration-dependent manner in both of AMC-HN-8 and TU-212 cells (Fig. 5C). Then, TRIM44 was overexpressed in LSCC cells, and TRIM44 overexpression could activate TLR4/AKT signaling (Fig. 5D). Interestingly, overexpression of TRIM44 effectively inhibited the anti-LSCC effect of nuciferine (Fig. 5E). These results showed that nuciferine displayed its anti-tumor activity by suppressing TRIM44 in LSCC cells.

## Discussion

TRIM44 is a member of TRIM family, which contains B-box, coiled-coil and zinc finger domains (Urano et al. 2009). As E3 ubiquitin ligases, TRIM family proteins are involved in the process of ubiquitination, and mediate many physiological processes, including cell proliferation, DNA repair and signal transduction (Watanabe and Hatakeyama 2017). It has been demonstrated that TRIMs are involved in the poor prognosis of tumors, and expected to become a new target for tumor therapy (Hatakeyama 2011). For example, the elevation of TRIM44 was significantly associated with the poor prognosis of patients with renal cell carcinoma (RCC), and silence of TRIM44 inhibited RCC cell proliferation and migration (Yamada et al. 2020). Similar effects of TRIM44 acted as an oncogene were found in melanoma (Wei et al. 2019), esophageal cancer (Xiong et al. 2018), intrahepatic cholangiocarcinoma (Peng et al. 2018), glioma (Zhou et al. 2019b), etc. But the function of TRIM44 in LSCC has not been investigated yet. In our present study, the expression levels and function of TRIM44 in LSCC were analyzed, and we found that TRIM44 was upregulated in both of the tumor tissues and cell lines of LSCC. Meanwhile, silence of TRIM44 could significantly inhibit the cell growth and tumor growth of LSCC. These findings indicated that targeting TRIM44 could be a potential strategy for the therapy of LSCC.

To search for the compounds that could inhibit the expression of TRIM44, we then did a small-scale natural compounds' screening. We found that nuciferine, a bioactive component of the lotus leaf, could significantly inhibit the mRNA levels of TRIM44 in LSCC cells. Wei et al. (2019) recently reported that TRIM44 could promote melanoma cell growth by stabilizing TLR4 protein. Szczepanski et al. (2009) also reported that triggering of TLR4 promoted head and neck squamous cell carcinoma development. Given these reports, we next evaluated the protein levels of TRIM44 and TLR4 after nuciferine treatment in LSCC cells, and we found that nuciferine markedly inhibited the protein levels of TRIM44 and TLR4 in LSCC cells.

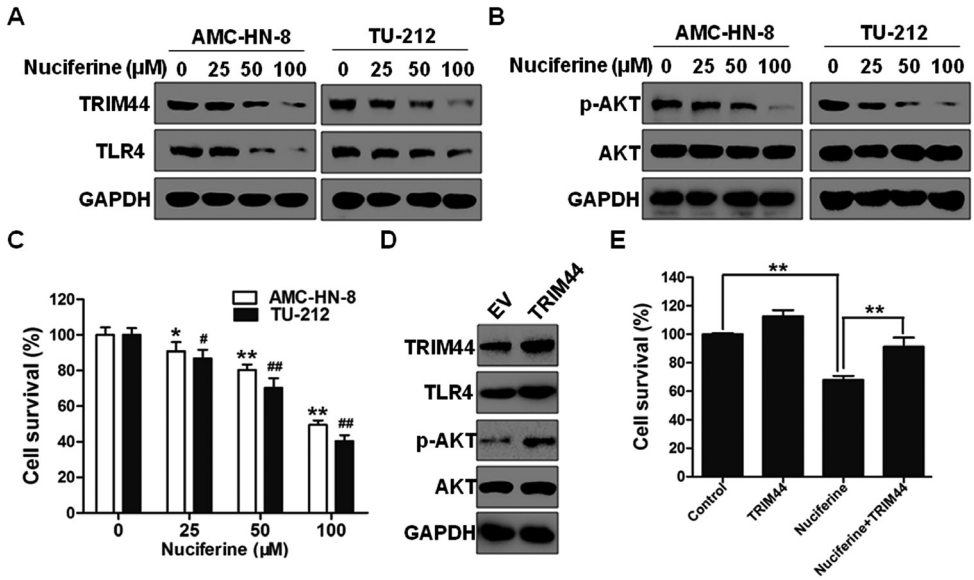


Fig. 5. Nuciferine displays anti-tumor activity in LSCC cells by suppressing TLR4 expression.
A and B. AMC-HN-8 and TU-212 cells were treated with increasing concentrations of nuciferine for 24 hours, and then cells were lysed for western blot to evaluate the expression levels of TRIM44, TLR4 (A), p-AKT and AKT (B). GAPDH was used as a loading control. C. AMC-HN-8 and TU-212 cells were treated with increasing concentrations of nuciferine for 24 hours, and then CCK-8 was performed to evaluate the cell survival. D and E. TU-212 cells were transfected with empty vector (EV) or TRIM44-overexpressing plasmids for 24 hours, and then transfected cells were prepared for western blot analysis (D), or treated with 50 $\mu$M nuciferine followed by CCK-8 analysis (E).
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$.

It has been reported that nuciferine has good anti-tumor effects in several cancers (Qi et al. 2016), but its effect in LSCC has not been known yet. In the present study, we also examined the effects of nuciferine on LSCC cell survival, and we found that nuciferine could markedly inhibit LSCC cell survival. Then, we examined the mechanism of nuciferine in LSCC cells, and overexpression of TRIM44 could significantly abolish the antitumor activity of nuciferine in LSCC cells. However, our current results only report the effects of nuciferine on LSCC from the cellular level, but its effect on animal level has not been elucidated and will be focused on in our future experiments. In addition, we will optimize the structure of nuciferine in our future experiments in order to find more effective compounds.

## Conclusion

In the present study, nuciferine was screened out to downregulate the expression of TRIM44 in LSCC cells, and overexpression of TRIM44 could significantly abolish the anti-LSCC activity of nuciferine. In conclusion, the present study demonstrated a new mechanism of nuciferine in suppressing LSCC progression, and inhibiting TRIM44 could be a useful strategy for the treatment of LSCC.

## Author Contribution

Qing-Hua Li, Li-Ping Sui and You-Xin Guo participated in the conception and design of the study. Yun-Hua Zhao, Bao-Gang Chen, Jian Li, Zhi-Hong Ma, Zhi-Hong Hu and Yan-Li Tang performed the experiments. Qing-Hua Li and You-Xin Guo interpreted the data and produced the draft of the manuscript. All authors read and approved the final manuscript.

## Conflict of Interest

The authors declare no conflict of interest.

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