Effect of TTLL12 on tubulin tyrosine nitration as a novel target for screening anticancer drugs *in vitro*

LINGLI XUE, YAN ZENG, CHUAN FANG, WEI CHENG and YADONG LI

Department of Oral and Maxillofacial Surgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, P.R. China

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Abstract. Nitrotyrosine, a structural analogue of tyrosine, is present in cells in pathological conditions and is incorporated into tubulin to form tubulin tyrosine nitration, which disrupts the normal function of microtubules. There is limited research on the functional aspects of tubulin tyrosine nitration in different types of tumor. In the present study, the effect of tubulin tyrosine nitration and tubulin tyrosine ligase like 12 (TTLL12) on the proliferation of SCC-25 cells was investigated. TTLL12-overexpressing cell lines were constructed and used to assess the effect of tubulin tyrosine nitration and TTLL12 on the proliferation of SCC-25 cells via western blotting, immunofluorescent and MTT assays. An TTLL12-stably overexpressing SCC-25 cell line and the enzyme-linked immunosorbent assay were used to establish a novel experiment *in vitro* for screening anticancer drugs targeting tubulin tyrosine nitration by assessing its sensitivity, specificity and repeatability, and using it to find an effective drug. The results demonstrated that the proliferative rate of the control cells was notably inhibited in the presence of nitrotyrosine compared with that of TTLL12-overexpressing cells. The results of the MTT assay revealed that the proliferation of TTLL12-silenced cells was significantly inhibited compared with that of the control group. The sensitivity, specificity and repeatability of the experiment were positive. It was found that nocodazole could have better anticancer effect than paclitaxel. Taken together, the results of the present study suggest that TTLL12 enhances SCC-25 cell survival in the presence of nitrotyrosine by disrupting nitration of the tyrosine residues of tubulin, and tubulin tyrosine nitration may be developed for the basic research of anticancer drugs.

Correspondence to: Dr Yadong Li, Department of Oral and Maxillofacial Surgery, The First Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Chongqing 400016, P.R. China
E-mail: llxxyydd2006@sina.com

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Introduction

During inflammatory conditions, nitric oxide synthases (NOSs), which include endothelial, neuronal and the inducible isoform of NOS, use oxygen and nitrogen to catalyze the production of nitric oxide (NO) (1). NO reacts with another free radical, superoxide (•OO-) to form peroxynitrite (ONOO-) (2) and ONOO- can nitrate tyrosine or protein tyrosine residues to form nitrotyrosine (3). Nitrotyrosine is similar to tyrosine in structure and is incorporated into tubulin by the enzyme tubulin-tyrosine ligase to produce the nitrotyrosination of tubulin, a process which is irreversible (4). Nitrotyrosination of tubulin can induce cell death, which is a way for the human body to remove apoptotic and abnormal cells (5). Long-term chronic inflammation, such as ulcers, gingivitis and periodontitis, may be associated with the malignant transformation of cells, resulting in the occurrence of oral squamous cell carcinoma (OSCC) as the malignant transformation of cells is not effectively eliminated (6,7). Thus, nitrotyrosine is considered a biochemical marker for oral inflammation (8) and oral cancer prognosis (9).

OSCC is one of the most common malignant tumors of the head and neck region (10). The 5-year survival rate of OSCC remains ~50% and has not significantly improved in the past decades (11). The incidence of OSCC was >300,000 cases worldwide in 2017 (12) and is increasing annually (13). Previous studies have focused on OSCC due to its high risk of malignancy, invasion and metastasis (14,15). As it invades adjacent tissues, the tumor has been demonstrated to affect speech, eating, swallowing (16) and breathing (17), which causes the quality of life of patients to decrease substantially (18). Thus, there is an urgent need to identify and develop novel effective treatment for OSCC. The occurrence and development of OSCC is a complex biological process (19), in which the overexpression of oncogenes (for example Notch1, fibroblast growth factor receptor 4 and c-myc plays an important role (20,21). Recently, a novel gene called tubulin tyrosine ligase like 12 (TTLL12) has demonstrated great interest. TTLL12 expression is significantly upregulated in lung adenocarcinoma, colorectal cancer and prostate cancer, and is closely associated with poor prognosis of these patients (22-25). Previous studies have reported that the TTL domain of TTLL12 may be involved in microtubule modification (26,27). The aim of the present study was to investigate the function of TTLL12 and develop an experiment with a novel target (tubulin tyrosine nitration).

Materials and methods

Cell culture. The human OSCC cell line, SCC-25 was purchased from the Center Laboratory of Chongqing Medical University and maintained in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate and 40 μg/ml gentamycin (all purchased from Gibco; Thermo Fisher Scientific, Inc.), at 37˚C in 5% CO_2.

Lentiviral transfection. hTTLL12 complementary DNA was cloned into pTY linkers (Sigma-Aldrich; Merck KGaA), which was transiently transfected into 293T cells (Shanghai Institute of Life Sciences; http://life.fudan.edu.cn/) using calcium phosphate-mediated transient transfection reagent. A lentivirus (Sigma-Aldrich; Merck KGaA) was transfected into SCC-25 cells. The TTLL12-overexpressing cell lines, TTLL12_A and TTLL12_B, and the control clones, control_A and control_B were constructed.

siRNA targeted against TTLL12. A total of two small interfering (si)RNA sequences targeting TTLL12 (siTTLL12-1; 5'-GAGUUCAUCCCCGAGUUUG-3' and siTTLL12-2; 5'-GGAACGAGCUGUGCUACAA-3'), and two negative control sequences [siControl (CONTROL® Non-Targeting siRNA #1) and siLuciferase (GL2 luciferase siRNA)] were purchased from GE Healthcare Dharmacon, Inc. siRNA transfections were performed using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, 100,000 cells were seeded into a 12-well plate and incubated in modified Eagle's medium (Shanghai Institute of Life Sciences; http://life.fudan.edu.cn/) supplemented with 10% fetal calf serum (Shanghai Institute of Life Sciences), 1 mM sodium pyruvate, 40 μg/ml gentamycin (Shanghai Institute of Life Sciences) at a temperature of 37˚C in an environment containing 5% CO_2 for 18 h. Subsequently, cells were further incubated in OPTIMEM (Sigma-Aldrich; Merck KGaA) at a temperature of 37˚C for 3 h, prior to transfection. A total of 10 nM siRNA was transfected per well. After 6 h, the medium was replaced with normal growth medium and cells were incubated until cell lysis. After 24 h, subsequent experimentation was performed.

Cell proliferation assay. In total, 10,000 cells were seeded into 96-well plates. Cell proliferation was assessed via the MTT-based colorimetric assay (Chemicon International), according to the manufacturer's instructions. Dimethyl sulfoxide was used to dissolve the purple formazan crystals. The optical density (OD) of the MTT reaction was measured at 450 nm.

Western blotting. Cells were lysed using 50 μl lysis buffer (Shanghai Institute of Life Sciences) (1 mM DTT, 0.125 mM EDTA, 5% glycerol, 1 mM PMSF, 1 lg/ml leupeptin, 1 lg/ml pepstatin, 1 lg/ml aprotinin and 1% Triton X-100 in 12.5 mM Tris-HCl buffer, pH 7.0) (Shanghai Institute of Life Sciences). Protein concentration was quantified using a BCA Protein Assay kit. The protein samples (20 μg/lane) were separated via SDS-PAGE on a 10% gel and subsequently transferred onto nitrocellulose membranes. The membranes were blocked with 5% dry milk at a temperature of 37˚C for 45 min prior to incubation with primary antibodies against: TTLL12 (1:2,000; cat. no. ab154086; Abcam), tributyl phosphate (TBP) (1:800; cat. no. ab220788; Abcam) and nitrotyrosine (1:1,000; cat. no. ab110282; Abcam) at a temperature of 37˚C for 3 h. Following the primary incubation, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. ab6271; Abcam) at a temperature of 37˚C for 1 h. Protein bands were visualized using the enhanced chemiluminescence kit (cat. no. 15159; Thermo Fisher Scientific, Inc.). The target bands were quantified using Image-Pro Plus J software (version 6.0; National Institutes of Health) with TBP as internal control.

Immunofluorescence. In total, 100,000 cells were cultured on glass coverslips with gelatin (Shanghai Institute of Life Sciences) at 37˚C for 12 h, fixed with 4% cold paraformaldehyde at room temperature for 20 min and incubated with 2.5% bovine serum albumin in phosphate buffered saline (PBS) for 1 h. After washing with PBS twice, cells were incubated with primary antibodies against TTLL12 (1:2,000; cat. no. ab154086; Abcam), overnight at 4˚C. Following the primary incubation, cells were incubated with a peroxidase-conjugated IgG secondary antibody (1:1,000; cat. no. A0545; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. Nuclei were counterstained with 1 μM DAPI at 37˚C for 5 min and cells were observed under a Leica Sp8 confocal scanning laser microscope (magnification, x400; Leica Microsystems, Inc.).

Determining negative and positive controls. Paclitaxel is known to exert a therapeutic effect on OSCC and increases tubulin tyrosine nitration (28,29); thus, it was used as a positive control in the present study. Thiirene was used as a negative control as it does not affect tubulin tyrosine nitration (30), and no evidence associating thiirene and tumor progression has been documented in the following: PubMed (https://pubmed.ncbi.nlm.nih.gov), Web of Science (http://login.webofknowledge.com), Ovid (http://ovidsp.ovid.com), Science Direct (https://www.sciencedirect.com), Wan Fang (http://www.cquc.net) or the China National Knowledge Infrastructure databases (https://www.cnki.net).

Stable TTLL12-overexpressing SCC-25 cells (experimental group) were treated with 400 μM nitrotyrosine and 100 μM paclitaxel or thiirene (all Shanghai Institute of Life Sciences) at 37˚C for 24 h, while cells in the control group were treated with 400 μM nitrotyrosine at 37˚C for 24 h. Cells were harvested in lysis buffer (Shanghai Institute of Life Sciences) (1 mM DTT, 0.125 mM EDTA, 5% glycerol, 1 mM PMSF, 1 lg/ml leupeptin, 1 lg/ml pepstatin, 1 lg/ml aprotinin and 1% Triton X-100 in 12.5 mM Tris-HCl buffer, pH 7.0) (Shanghai Institute of Life Sciences)and subjected to western blotting. Tubulin tyrosine nitration levels were quantified using Quantity One version 4.62 software (Bio-Rad Laboratories, Inc.) and normalized to TBP. Error bars represent the mean ± standard error of the mean (SEM) of three independent experiments.

Determining the optimum concentration of nitrotyrosine and drug. TTLL12-overexpressing cells were seeded into 96-well plates at a density of $1x10^4$ cells/well and cultured in modified Eagle's medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 40 μg/ml gentamycin (Shanghai

Institute of Life Sciences) in a 5% CO_2 incubator at 37˚C for 6 h. Different concentrations of nitrotyrosine (800, 400, 200, 100 and 50 μM) and paclitaxel or thiirene (40, 20, 10 and 5 μM) were added into the wells to determine the optimal concentration of nitrotyrosine and drug by square matrix titrimetry (31). After cells were cultured at 37˚C for 24 h, the MTT assay was subsequently performed to assess cell proliferation.

In vitro experiment with novel target (tubulin tyrosine nitration) to screen anticancer drugs. Stable TTLL12-overexpressing SCC-25 cells were seeded into 96-well plates at a density of 10,000 cells/well and incubated in modified Eagle's medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 40 μg/ml gentamycin. (Shanghai Institute of Life Sciences) at 37˚C for 6 h. MEM (Shanghai Institute of Life Sciences) (20 μl) supplemented with nitrotyrosine (final concentration, 400 μM) and anticancer drugs (final concentration, 10 μM) was added to the cells and further incubated at 37˚C for 24 h. Paclitaxel and thiirene were used as the positive and negative controls, respectively. Following incubation, the upper medium was gently removed and 100 μl buffer [90 mM Mes pH 6.7, 1 mM EGTA, 1 mM $MgCl_2$, 10% (v/v) glycerol and 0.5% (v/v) Triton X-100; Shanghai Institute of Life Sciences] was added to the cells and further incubated at 37˚C for 3 min. Once again, the upper medium was gently removed and 100 μl PBS supplemented with 3.7% paraformaldehyde and 0.05% Tween-20 (Shanghai Institute of Life Sciences) was added. Following incubation at 37˚C for 10 min, the upper medium was gently removed and cells were further incubated with 100 μl PBS containing anti-nitrotyrosine antibody (1:1,000; cat. no. 16-207, clone 1A6, HRP conjugate, Sigma-Aldrich; Merck-KGaA) for 1 h at room temperature. Once again, the upper medium was gently removed and well was washed once with 200 μl PBS supplemented with 0.05% Tween-20. Finally, the upper medium was gently removed and 100 μl OPD-H_2O_2 (Shanghai Institute of Life Sciences) was added at 37˚C for 3 min, prior to addition of 50 μl H_2SO_4 (Shanghai Institute of Life Sciences) (2 mol/l) at 37˚C for 3 min. The absorbance was detected at a wavelength of 450 nm, using a microplate reader (Thermo Fisher Scientific, Inc.). The Nitrotyrosine ELISA kit (K4158-100, Hycult Biotech Inc.) was used for detection in strict accordance with the manufacturer's instructions and repeated three times.

Sensitivity, specificity and repeatability of the experimental method. *In vitro* experiments must have good sensitivity, specificity and repeatability, therefore the following experiments are done.

Sensitivity of the experiment. Stable TTLL12-overexpressing SCC-25 cells were seeded into 96-well plates at a density of 10,000 cells/well and incubated in modified Eagle's medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 40 μg/ml gentamycin (Shanghai Institute of Life Sciences) at 37˚C for 6 h. The following concentrations of nitrotyrosine (80, 40, 20, 10, 5 and 2.5 μM) were added to the cells. Paclitaxel and thiirene were used as the positive and negative controls, respectively. Following incubation, the upper medium was gently removed and 100 μl buffer [90 mM Mes pH 6.7, 1 mM EGTA, 1 mM $MgCl_2$, 10% (v/v) glycerol and 0.5% (v/v) Triton X-100; Shanghai Institute of Life Sciences] was added to the cells and further incubated at 37˚C for 3 min. Once again, the upper medium was gently removed and 100 μl PBS supplemented with 3.7% paraformaldehyde and 0.05% Tween-20 (Shanghai Institute of Life Sciences) was added. The Nitrotyrosine ELISA kit (cat. no. K4158-100; Hycult Biotech Inc.) was used for detection in strict accordance with the manufacturer's instructions and repeated three times.

Specificity of the experiment. It is well-known that carboplatin (CDDP), 5-fluorouracil (5-FU), cyclophosphamide (CTX) and methotrexate (MTX) exert notable effects on cancer development but have no effects on microtubules (32-35). Stable TTLL12-overexpressing SCC-25 cells were seeded into 96-well plates at a density of 10,000 cells/well and incubated in modified Eagle's medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 40 μg/ml gentamycin. (Shanghai Institute of Life Sciences) at 37˚C for 6 h. MEM (Shanghai Institute of Life Sciences) (20 μl) supplemented with nitrotyrosine (final concentration, 400 μM) and anticancer drugs (CDDP, 5-FU, CTX and MTX) (Shanghai Institute of Life Sciences) (final concentration, 10 μM) was added to the cells and further incubated at 37˚C for 24 h. The rest of the experiment was performed as aforementioned.

Repeatability of the experiment. Stable TTLL12-overexpressing SCC-25 cells were seeded into 96-well plates at a density of 10,000 cells/well and incubated in modified Eagle's medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 40 μg/ml gentamycin (Shanghai Institute of Life Sciences) at 37˚C for 6 h. MEM (Shanghai Institute of Life Sciences) (20 μl) supplemented with nitrotyrosine (final concentration, 400 μM) and samples (Paclitaxel, thiirene, CDDP, 5-FU and CTX) (final concentration, 10 μM) was added to the cells and further incubated at 37˚C for 24 h. The rest of the experiment was performed as aforementioned. It was the intra-batch assay. The same experiment was subsequently done over 4 different days for the inter-batch assay.

Determining the validity of the experiment. In total, 40 compounds were randomly selected by Shanghai Institute of Life Sciences and assessed in the experiment. Stable TTLL12-overexpressing SCC-25 cells (experimental group) were treated with 400 μM nitrotyrosine and 10 μM nocodazole (Shanghai Institute of Life Sciences) at 37˚C for 24 h, while cells in the control group were treated with 400 μM nitrotyrosine at 37˚C for 24 h. Cells were harvested in buffer and subjected to western blotting. Tubulin tyrosine nitration levels were quantified using Quantity One software and normalized to TBP. Error bars represent the mean ± SEM of three independent experiments.

Statistical analysis. Each experiment was repeated three times. Statistical analysis was performed using SPSS 10.0 software (SPSS, Inc.). Tubulin tyrosine nitration levels and the ratio of optical density (OD) 450 nm are presented as the mean ± standard deviation. One-way analysis of variance followed by Tukey's post hoc test was used to compare differences between multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

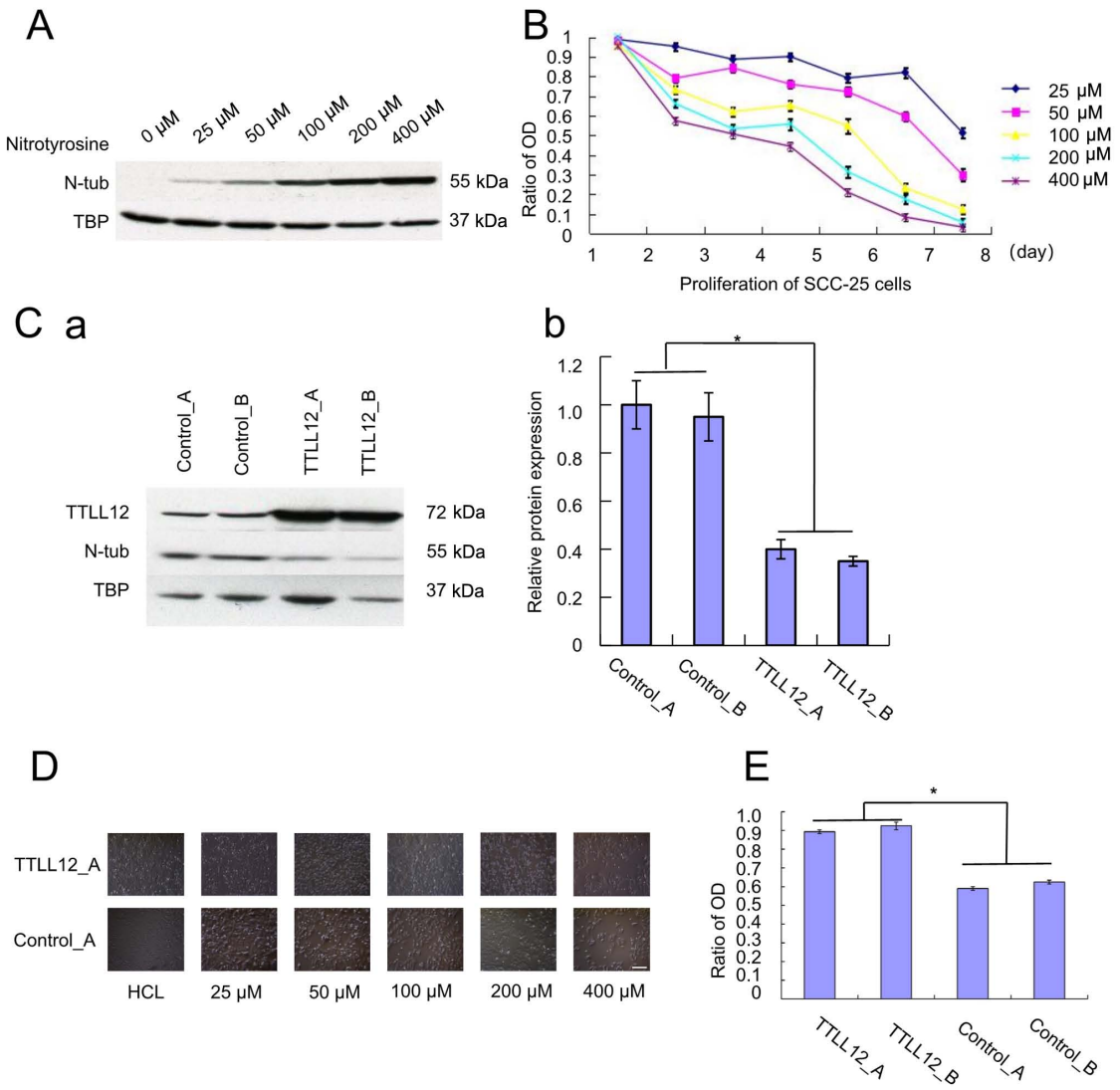

Figure 1. Effect of overexpressing TTLL12 and nitrotyrosine on the proliferation of SCC-25 cells. (A) Cells were subjected to western blot analysis to determine protein expression. (B) Cell proliferation was assessed via the MTT assay every 24 h. The ratio of OD 450 nm was calculated as OD assay/OD control. Error bars represent the mean ± SEM of three independent experiments. (C) TTLL12-overexpressing clones, TTLL12_A and TTLL12_B, as well as control_A and control_B cells were treated with 400 μM nitrotyrosine for 24 h. (Ca) Cells from each group were subjected to western blot analysis to determine protein expression. (Cb) The blots were quantified via densitometry and normalized to TBP. Tubulin tyrosine nitration levels of all groups are presented relative to the average tubulin tyrosine nitration level of control_A. Error bars represent the mean ± SEM of three independent experiments. (D) TTLL12_A and control_A cells were cultured in different concentrations of nitrotyrosine (0-400 μM) for 24 h. Scale bar, 100 μm. (E) TTLL12_A, TTLL12_B, control_A and control_B cells were treated with 20 μl modified Eagle's medium supplemented with 400 μmol/l nitrotyrosine for 24 h, while the untreated group received medium supplemented with HCl. Cells proliferation was assessed via the MTT assay. The ratio of OD 450 nm represents the OD 450 nm of treated cells normalized to that of untreated cells. Error bars are represent the mean ± SEM of three independent experiments. *P<0.05. TTLL12, tubulin tyrosine ligase like 12; OD, optical density; TBP, tributyl phosphate, SEM, standard error of the mean; N-tub, nitrotyrosine tubulin.

Results

Effect of different concentrations of nitrotyrosine on the proliferation of SCC-25 cells. SCC-25 cells were treated with different concentrations of nitrotyrosine (0-400 μM) to determine the concentration of nitrotyrosine that effects tubulin tyrosine nitration. Western blot analysis demonstrated that the concentration of tubulin tyrosine nitration increased in relation to the concentration of nitrotyrosine (Fig. 1A). The proliferation of SCC-25 cells was assessed for 7 days via the MTT assay every 24 h. The results demonstrated that the proliferation of SCC-25 cells was inhibited following treatment with nitrotyrosine, whereby the higher the concentration of nitrotyrosine, the greater the inhibition of proliferation of SCC-25 cells (Fig. 1B). The tyrosine residues of tubulin were nitrated to form tubulin tyrosine nitration, which causes cells to die (36).

Effect of overexpressing TTLL12 on tubulin tyrosine nitration and SCC-25 cell proliferation. The TTLL12-overexpressing cell lines, TTLL12_A and TTLL12_B, and the control clones, control_A and control_B were constructed, and maintained in medium supplemented with 400 μM nitrotyrosine for 24 h. The results demonstrated that tubulin tyrosine nitration was significantly lower in TTLL12-overexpressing clones compared with the control clones (P<0.05; Fig. 1C). Subsequently, in order to assess the effect of overexpressing TTLL12 on cell proliferation, TTLL12_A and control_A cells

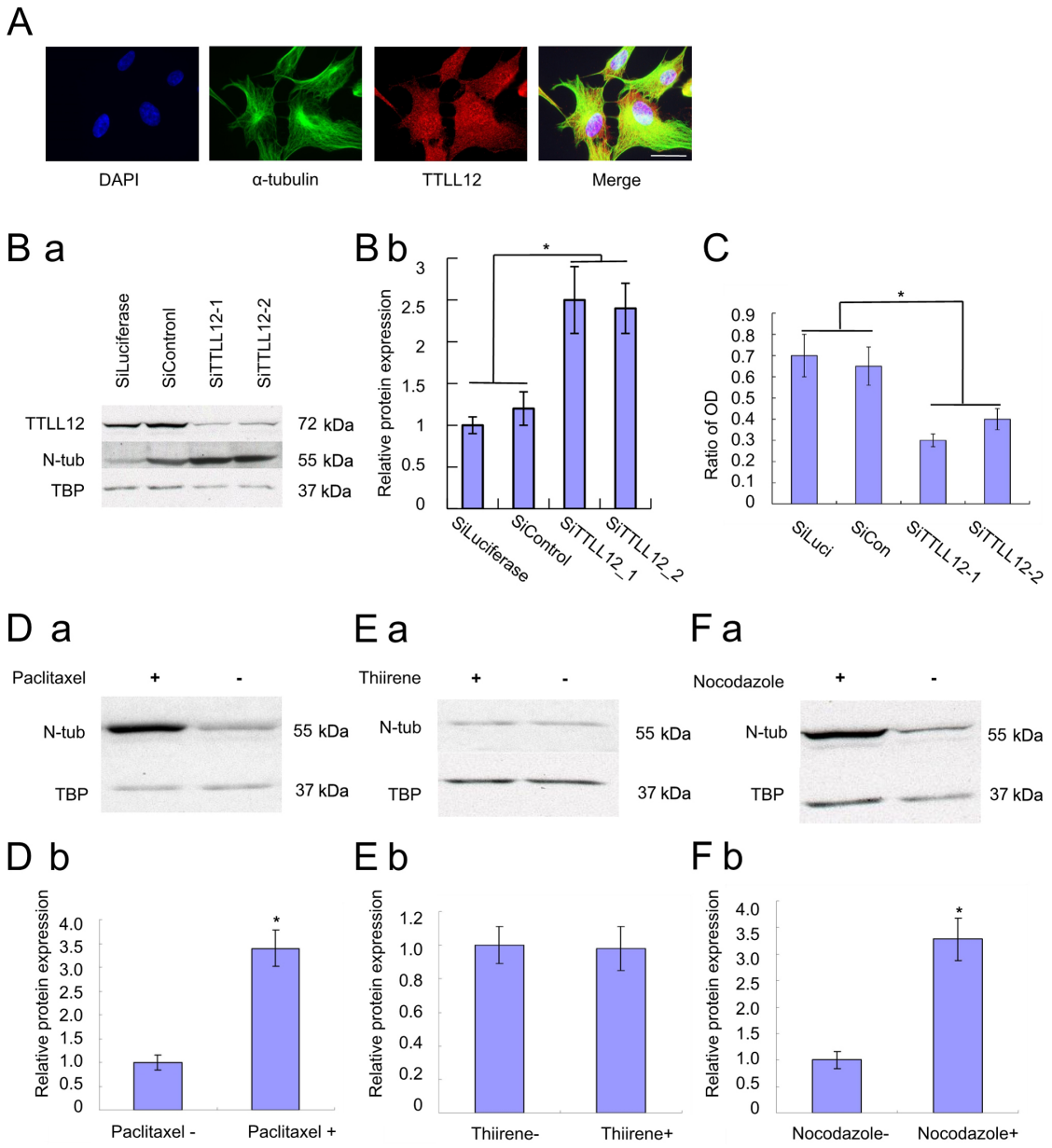

Figure 2. Immunofluorescence of TTLL12, and the effects of TTLL12 silencing and drugs on nitrotyrosine tubulin. (A) Overexpressing cells were used for the immunofluorescence assay to investigate the localization of TTLL12. Scale bar, 25 μm. (B) siTTLL12-1, siTTLL12-2, siLuciferase and siControl cells were treated with 400 μM nitrotyrosine for 24 h. (Ba) Cells from each group were subjected to western blot analysis to determine protein expression. (Bb) The blots were quantified via densitometry and normalized to TBP. Tubulin tyrosine nitration levels of all groups are presented relative to the average tubulin tyrosine nitration level of control siRNAs. Error bars represent the mean ± SEM of three independent experiments. (C) SiTTLL12-1, SiTTLL12-2, SiLuciferase and SiControl cells were treated with 20 μl modified Eagle's medium supplemented with 400 μmol/l nitrotyrosine or 400 μmol/l HCl for 24 h. Cell proliferation was assessed via the MTT assay. The ratio of OD represents the OD 450 nm of treated cells normalized to that of untreated cells. Error bars represent the mean ± SEM of three independent experiments. (D) Stable TTLL12-overexpressing SCC-25 cells were treated with 400 μM nitrotyrosine. Paclitaxel (10 μM) was added to cells in the experimental groups, while nothing was added to the control cells. (a) After 24 h, cells from each group were subjected to western blot analysis to determine protein expression. (b) The blots were quantified via densitometry and normalized to TBP. Nitrotyrosine tubulin levels are presented relative to the average of control. Error bars represent the mean ± SEM of three independent experiments. (E) Cells were treated with 10 μM thiirene. (F) Cells were treated with 10 μM nocodazole. Nitrotyrosine tubulin levels were compared with the treatment and control groups. *P<0.05. TTLL12, tubulin tyrosine ligase like 12; si, small interfering; SEM, standard error of the mean; OD, optical density; TBP, tributyl phosphate; N-tub, nitrotyrosine tubulin.

were treated with different concentrations of nitrotyrosine (0-400 μM) for 24 h and observed under an inverted microscope. The results demonstrated that the proliferation of the control cells was notably inhibited following treatment with nitrotyrosine compared with the TTLL12-overexpressing cells (Fig. 1D). TTLL12-overexpressing and control clones were treated with 400 μM nitrotyrosine for 24 h. The results demonstrated a statistically significant increase in the proliferation of TTLL12-overexpressing clones compared with the control clones (P<0.05; Fig. 1E). TTLL12 localization was assessed via immunofluorescence. Nuclei were stained with DAPI (blue), TTLL12 stained red and α-tubulin stained green (Fig. 2A).

Effect of silencing TTLL12 on tubulin tyrosine nitration and SCC-25 cell proliferation. TTLL12 expression was downregulated following siRNA transfection, which was verified via

Table I. Determining the optimum concentration of nitrotyrosine and drug.

Concentration of drug		Concentration of nitrotyrosine				
		50 μM	100 μM	200 μM	400 μM	800 μM
5 μM	Paclitaxel	0.427±0.031	0.596±0.043	0.714±0.052	0.846±0.061	1.126±0.109
	Thiirene	0.187±0.011	0.195±0.009	0.224±0.019	0.253±0.022	0.225±0.021
10 μM	Paclitaxel	0.647±0.041	0.864±0.061	1.110±0.129	1.097±0.055	1.230±0.097
	Thiirene	0.110±0.009	0.126±0.011	0.130±0.014	0.122±0.011	0.147±0.015
20 μM	Paclitaxel	0.801±0.067	1.104±0.096	1.135±0.109	1.116±0.091	1.235±0.115
	Thiirene	0.108±0.091	0.124±0.011	0.132±0.012	0.126±0.014	0.139±0.012
40 μM	Paclitaxel	0.810±0.064	1.130±0.122	1.180±0.096	1.281±0.108	1.332±0.113
	Thiirene	0.110±0.089	0.129±0.093	0.133±0.101	0.147±0.107	0.149±0.112

Table II. Sensitivity assay results.

Concentration of nitrotyrosine	80 μM	40 μM	20 μM	10 μM	5 μM	2.5 μM
OD, 450 nm	1.330±0.119	1.291±0.107	1.118±0.113	1.086±0.064	0.846±0.076	0.681±0.054

OD, optical density.

Table III. Specificity assay results.

	Paclitaxel	Thiirene	CDDP	5-FU	CTX	MTX
OD, 450 nm	1.102±0.042	0.157±0.012	0.108±0.014	0.179±0.011	0.207±0.014	0.251±0.017

OD, optical density; CDDP, carboplatin; 5-FU, 5-fluorouracil; CTX, cyclophosphamide; MTX, methotrexate.

Table IV. Intra-batch and inter-batch reproducibility assay results.

	Intra-batch assay (OD, 450 nm)			Inter-batch assay (OD, 450 nm)		
	Mean	SD	CV	Mean	SD	CV
Paclitaxel	1.107	0.047	4.25	1.084	0.031	2.86
Thiirene	0.163	0.011	6.75	0.143	0.006	4.20
CDDP	0.114	0.004	3.50	0.121	0.007	5.79
5-FU	0.186	0.008	4.30	0.179	0.011	6.15
CTX	0.216	0.017	7.87	0.229	0.016	6.99

OD, optical density; SD, standard deviation, CV, coefficient of variation; CDDP, carboplatin; 5-FU, 5-fluorouracil; CTX, cyclophosphamide.

western blotting. The TTLL12-silenced cell lines, siTTLL12-1 and siTTLL12-2, and control cells, siLuciferase and siControl, were treated with 400 μM nitrotyrosine for 24 h. Western blot analysis demonstrated that tubulin tyrosine nitration was significantly higher in TTLL12-silenced cells compared with the control cells ($P<0.05$; Fig. 2B). Furthermore, downregulation of TTLL12 led to an increase in tubulin tyrosine nitration. The results of the MTT assay demonstrated that the proliferation of TTLL12-silenced cells was significantly inhibited compared with the control cells ($P<0.05$; Fig. 2C). In addition, downregulation of TTLL12 decreased cell proliferation of TTLL12-silenced cells compared with the control cells (Fig. 2C).

Effects of paclitaxel and thiirene on tubulin tyrosine nitration. Western blot analysis demonstrated that tubulin tyrosine nitration increased in cells treated with paclitaxel, suggesting a positive association between the two (Fig. 2D). Thus, paclitaxel

Table V. Novel experiment results.

Sample	OD, 450 nm	Sample	OD, 450 nm	Sample	OD, 450 nm	Sample	OD, 450 nm
Paclitaxel	1.091±0.043	1	0.132±0.010	2	0.147±0.013	3	0.151±0.011
4	0.129±0.012	5	0.136±0.013	6	0.158±0.018	7	0.235±0.019
8	0.321±0.025	9	0.351±0.027	10	0.358±0.029	11	0.247±0.021
12	0.241±0.019	13	0.411±0.037	14	1.043±0.042	15	0.412±0.022
16	0.314±0.019	17	0.452±0.031	18	0.189±0.009	19	0.338±0.026
20	0.371±0.019	21	0.121±0.011	22	0.365±0.024	23	0.146±0.015
24	0.352±0.029	25	0.347±0.017	26	0.122±0.009	27	0.157±0.014
28	0.163±0.013	29	0.221±0.019	30	0.289±0.017	31	0.113±0.008
32	0.167±0.009	33	0.148±0.011	34	0.128±0.012	35	0.168±0.014
36	0.278±0.016	37	0.263±0.019	38	0.410±0.031	39	0.247±0.022
40	0.162±0.011						

OD, optical density.

was used as a positive control. Conversely, the results demonstrated no significant differences in tubulin tyrosine nitration in cells without or with thiirene (Fig. 2E), suggesting that thiirene does not affect the level of tubulin tyrosine nitration. Thus, thiirene was used as a negative control.

Determining the optimum concentration of nitrotyrosine and drug. The results of the square matrix titrimetry assay demonstrated that the OD 450 nm value of 400 μM nitrotyrosine and 10 μM paclitaxel was ~1.0, while the OD 450 nm value of thiirene was lower, and the ratio of the positive to negative control (P/N value) was the highest. Thus, 400 μM was considered the optimal concentration of nitrotyrosine, and 10 μM the optimal concentration of drug (Table I).

Sensitivity, specificity and repeatability of the experiment. The results of the sensitivity assay demonstrated that the OD 450 nm value of paclitaxel with a minimum concentration of 2.5 μM was 0.681, which was higher than the OD 450 nm value of 10 μM thiirene (0.122) (Table II). Thus, the sensitivity of the novel experiment was considered positive.

The results of the specificity assay demonstrated that the OD 450 nm value of paclitaxel (1.102) was considerably higher compared with the other drugs (Thiirene, 0.157; CDDP, 0.108; 5-FU, 0.179; CTX, 0.207; MTX, 0.251; Table III). Thus, the specificity of the novel experiment was considered positive (Table III).

The results of the repeatability assay demonstrated that the coefficient of variation (CV) of the intra-batch assay was 3.50-7.87%, while the CV of the inter-batch assay was 2.86-6.99%. Given that both values were <10%, the repeatability of the experiment was considered positive (Table IV).

Application of the experiment. The results of the last experiment demonstrated that the OD 450 nm value of most samples was <0.452 (Table V), and only the OD 450 nm value of sample no. 14 was >1 (1.043; Table V), showing that shwed nocodazole could have an improved anticancer effect compared with paclitaxel. Western blot analysis demonstrated that tubulin tyrosine nitration increased in cells treated with nocodazole, suggesting a positive association between the two (Fig. 2F).

Discussion

Microtubules are the basic units of the cytoskeleton, which exist in all eukaryotic cells (37). They are polymerized by microtubule protein and can be assembled into cilia, flagella, axon, neural tube, basal granule, centriole and spindle (38). Microtubules participate in the formation of the cytoskeleton, maintenance of cell morphology, cell contraction, pseudopodia movement, material transport and cell division (39,40). Prior to functioning, microtubules need to be coated with tubulin tyrosine ligase (TTL) (41). TTL is involved in nitrotyrosine-mediated post-translational modification of tubulin (4,42). In the presence of nitrotyrosine, TTL induces tubulin tyrosine nitration, which significantly changes the structure of microtubules, decreases the adhesive ability of cells, causes cell deformation and intracellular transport obstacle, and eventually leads to cell apoptosis and organ function loss (43,44).

Previous studies have detected nitrotyrosine in fresh-frozen human tissue samples and have demonstrated that nitrotyrosine is an important marker for oral cancer prognosis and survival (9). However, there is a lack of studies assessing the effect of nitrotyrosine on the proliferation of oral cancer cells. The results of the present study demonstrated that the proliferation inhibition of SCC-25 cells was proportional to the content of nitrotyrosine, which suggest that nitrotyrosine inhibits SCC-25 cell proliferation. TTLL12-overexpressing clones (TTLL12_A and TTLL12_B) and control clones (control_A and control_B) were constructed and treated with nitrotyrosine. The results demonstrated that TTLL12 promoted the proliferation of SCC-25 cells by inhibiting the formation of tubulin tyrosine nitration. Furthermore, TTLL12-silenced clones (siTTLL12-1 and siTTLL12-2) and control clones (siLuciferase and siControl) were constructed and treated with nitrotyrosine. The results demonstrated that tubulin tyrosine nitration formation increased, and the proliferation of SCC-25 cells was inhibited in TTLL12-silenced cell lines compared with the control cells. Following overexpression

of TTLL12, tubulin tyrosine nitration formation decreased, and cells continued to proliferate by escaping the attack of tubulin tyrosine nitration. Conversely, TTLL12 silencing increased tubulin tyrosine nitration and inhibited cell proliferation. Taken together, these results suggest that TTLL12 may function as a potential oncogene. Although the immunofluorescence results demonstrated that TTLL12 appeared to overlap with α-tubulin, the molecular mechanism by which TTLL12 regulates tubulin tyrosine nitration remains unclear and requires further investigation. It can be hypothesized that TTLL12 and nitro have the same binding site on α-tubulin, and thus competes with α-tubulin. It was hypothesized that when TTLL12 is overexpressed, it becomes difficult to nitrate the tyrosine residues of tubulin, thus tubulin tyrosine nitration decreases.

Malignant tumors (for example oral, liver and gastric cancer) seriously threaten the well-being and quality of life of affected patients (45). Currently, there are no effective treatments for cancer, and comprehensive treatment is extensively applied in the clinical setting (46). Chemotherapy, as the only effective systemic treatment, began in 1948 (folic acid antagonists were used as antitumor drugs to treat leukemia), which plays an irreplaceable role in the comprehensive treatment of cancer (47). Due to the heterogeneity of tumors (48), different individuals have different reactions to the same drug, which affects the treatment outcome. In response to this problem, some medical studies have proposed to use a drug sensitivity experiment to provide individualized treatment to improve the clinical efficacy (49,50). *In vitro* drug sensitivity experiments have been extensively used in the last century due to their effective and efficient outcomes (51). However, the microenvironment *in vivo* may lead to the emergence of drug resistance (52), which decreases the efficacy of drug sensitivity experiments *in vitro* to guide clinical individualized treatment. However, effective methods (drug sensitivity experiments *in vitro* and *in vivo*) to identify potential effective anticancer reagents remain (53,54).

The majority of drug sensitivity experiments *in vitro* aim to assess the anticancer effect of drugs by detecting the ratio of living cells to dead cells after the tumor cells are cultured with drugs (55). Novel effective drugs that can kill tumors are identified by drug sensitivity experiments; however, the anticancer molecular mechanism of these drugs remains unknown. This notably disrupts the clinical application of novel drugs. Thus, additional experiments are required to identify the anticancer molecular mechanism of novel drugs, which are time consuming and expensive, and delay the treatment of patients.

Based on this concept, the present study established a novel type of drug sensitivity test targeting tubulin tyrosine nitration *in vitro* by combining the research results with the enzyme-linked immunosorbent assay (ELISA). This novel assay can screen out new effective anticancer drugs and determine the anticancer molecular mechanism of these drugs. Paclitaxel exerts a notable therapeutic effect on OSCC and increases tubulin tyrosine nitration (28,29); thus, it was used as a positive control in the present study. Conversely, thiirene is not associated with tumors and does not affect tubulin tyrosine nitration (30); thus, it was used as a negative control in the present study. TTLL12-overexpressing cells were treated with the samples and nitrotyrosine, and tubulin tyrosine nitration was detected via ELISA. If the OD value of the samples was greater than that of the positive control, the samples were considered to have a positive anticancer effect. The results of the experiment demonstrated that nocodazole increased tubulin tyrosine nitration, and its OD 450 nm value was high, thus, it may be considered a promising anticancer drug.

The novel experiment has the following characteristics: It is simple, sensitive, fast, specific, cheap, stable and easy to be popularized. This experiment consists of a few steps and simple technologies, including cell culture and ELISA, which are both easy to perform. The highly efficient biocatalysis of enzymes is used in the novel experiment, so that markedly low contents of tubulin tyrosine nitration can be detected, and the novel experiment has high sensitivity. The experiments lasts for ~32 h and detection occurs using a 96-well plate, thus, the overall time required is relatively short and the experiment is fast. The combination of anti-nitrotyrosine antibody and tubulin tyrosine nitration is that of antibody and antigen, thus, the experiment has high specificity. Special or valuable materials are not involved, thus the materials are easily attained and affordable. The experiment comprises only a few steps, few reagents and few variation factors, which facilitates good repeatability and stability. Considering the aforementioned advantages, the experiment can be easily popularized and used for large-scale screening. The experiment can automatically and mechanically detect multiple 96-well plates simultaneously, whereby one 96-well plate can analyze 16 samples at a time. Thus, if multiple 96-well plates are detected in parallel, hundreds of samples can be detected within 32 h. This high-throughput test has another benefit in that the results of the experiment not only present the anticancer effect of the sample, but also reveal its anticancer molecular mechanism. If the sample participates in the process of microtubule modification, whereby it can promote the formation of tubulin tyrosine nitration, tumor cells will experience apoptosis. Simultaneously, tubulin tyrosine nitration will combine with the antibody, which will eventually increase the OD value (56). If the OD value of the sample is higher than that of the positive control, this suggests that the sample is a promising anticancer drug. Such a drug can kill cancer cells, and its anticancer effect may even be better than that of paclitaxel; its anticancer molecular mechanism involves its participation in the process of microtubule modification, which promotes the formation of tubulin tyrosine nitration, thus causing tumor cell apoptosis. This information can greatly speed up the application of the drug in the clinical setting, and is expected to improve patients survival outcomes. To the best of our knowledge, the present study was the first to use TTLL12-overexpressing cell lines to screen anticancer drugs, paving the way for future research on novel anticancer drugs targeting the microtubule system. However, our results need to be verified *in vivo* in the future.

In conclusion, the results of the present study demonstrated that TTLL12 promoted SCC-25 cell survival in the presence of nitrotyrosine by decreasing the formation of tubulin tyrosine nitration. The novel experiment described in the present study is simple, sensitive, rapid, specific, affordable, stable and easy to be popularized, and has good application for basic research on anticancer drugs.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YL designed the present study. CF and WC participated in statistical analyses. CF interpreted the western blot results. WC performed immunofluorescence assessment of the cells. YL, LX and YZ interpreted the results, and prepared and drafted the initial manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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