Secretory leukocyte peptidase inhibitor expression and apoptosis effect in oral leukoplakia and oral squamous cell carcinoma

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Abstract. Oral leukoplakia (OL) is one of the most common oral precancerous lesions with the possibility of malignant transformation, ranging from 17 to 24% of patients with a median follow-up of >7 years. Previous research has revealed that compared with normal oral epithelial tissues, the expression of secretory leukocyte peptidase inhibitor (SLPI) protein is significantly reduced in oral squamous cell carcinoma (OSCC). Based on the above-mentioned research, it is known that SLPI is a potential predictive and diagnostic tool for the progression of oral carcinogenesis. Therefore, we investigated the correlation between the abundance of SLPI protein and the different histological grades of OL by immunohistochemistry. The results indicated that the level of SLPI was negatively correlated with the histological grades of the oral premalignant lesions, indicating that it may be a potential predictive tool for the malignant transformation presented in oral precancerous patients. Subsequently, we investigated the biological effects of SLPI using Cell Counting Kit (CCK)-8, Annexin V/PI apoptosis assay and Caspase-Glo® 3/7 assay. The findings revealed that SLPI promoted apoptosis in the Leuk1 and WSU-HN4 cell lines. Mechanistic studies indicated that SLPI, at least in part, regulated cell apoptosis by inhibiting the expression of TNF receptor-associated factor 1 (TRAF1), which has a close relationship with the nuclear factor-κB (NF-κB) pathway.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide and the incidence rate in developing countries is higher than the rate in developed countries (1). The prognosis is poor and the survival rate is barely over 50%. Oral leukoplakia (OL) is one of the most common oral precancerous lesions. It was reported that the percentage of malignant transformation rates of OL range from 17 to 24% of the patients with a median follow-up of >7 years (2). There are no clinical parameters that can predict the potential of malignant transformation in patients with OL. The aim of the present study was to investigate a potential role of SLPI in predicting the development of oral squamous cell carcinoma (OSCC) in patients with OL. Therefore, the development of effective diagnostic methods for at-risk oral premalignant lesions (OPMLs) or early-stage OSCC is essential in order to improve the survival rate of the patients as well as alleviate patient pain.

By using in-depth mass spectrometry-based quantitative shotgun proteomics study of non-invasively collected oral brush biopsies, our previous study (3) indicated that SLPI abundance in OL tissue samples is much lower than the expression of SLPI in human normal oral epithelial tissues, with a more significant decrease in OSCC tissues. Thus, it indicated that SLPI may be a promising, mechanism-based oral cancer biomarker. In the present study, the expression of SLPI in different histological stages of OL was further investigated to determine whether SLPI is closely related to carcinogenesis and may be used as an important reference for grading, screening and treating dysplasia of oral mucosa. SLPI is a 11.7 kDa non-glycosylated serine protease inhibitor of a wide range of serine proteases including neutrophil elastase, catharpin G, chymotrypsin and trypsin. SLPI consists of 107 amino acids possessing two homologous WFDC domains (4-7). It is physiologically produced and secreted in the respiratory, reproductive tract, oral, gastric and colonic mucosa, as well as the lacrimal and salivary glands and other parts (4). According to previous research, the expression of SLPI protein demonstrates diverse patterns in different types of tumors. SLPI is highly upregulated in pancreatic (8), gastric (9), lung (10,11), endometrial (12) and ovarian cancer (13). In contrast, it is

downregulated in human nasopharyngeal (14) and oral squamous cell carcinoma (3,5,15). Furthermore, the roles of SLPI in various types of tumors are not entirely understood. It has been reported that SLPI exerts pro-apoptotic and cell cycle-arrest effects in breast cancer (16). On the contrary (17), SLPI, which provides protection against paclitaxel-mediated cell injury, has pro-growth and pro-survival effects in ovarian cancer. Similarly, it is considered as a proliferation and survival factor for pancreatic cancer cells (8). The role of SLPI in oral cancer has not been entirely researched. However, Wen *et al* (18) reported that SLPI protein exerted an inhibitory effect on cell invasion and migration in oral cancer cell lines. They provided evidence of a potential protective role of SLPI in OSCC and suggested that SLPI may be used for a possible stratification of oral cancer according to the risk of occult metastasis. The aim of the present study was to further investigate the potential predictive value of SLPI for the identification of oral precancerous lesions with high risk of progression to carcinoma and the biological effect in OL and OSCC, especially in OPML. The effective risk prediction of the malignant transformation of oral precancerous lesions is likely to significantly improve the survival rate and to relieve patient pain.

The regulatory mechanisms of SLPI on tumors are not well known. One possible mechanism is through the NF-κB signaling pathway. Several studies have reported that the SLPI protein inhibits the activation of the NF-κB signaling pathway in macrophages (19), alveolar epithelial cells (20) and gingival squamous cell carcinoma-derived Ca9-22 cells (21). It is known that the transcription factor NF-κB is involved in the regulation of inflammatory responses, cell growth, differentiation and apoptosis. Many of the downstream target genes of the transcription factor NF-κB, such as the baculoviral IAP repeat containing 2 (BIRC2), the baculoviral IAP repeat containing 3 (BIRC3), the TRAF1 and TNF receptor-associated factor 2 (TRAF2), are anti-apoptotic genes (22). Furthemore, constitutive activation of the NF-κB signaling pathway in HNSCC (23-25) can promote the survival, growth, migration and secretion of pro-inflammatory and pro-angiogenic cytokines.

In the present study, we aimed to investigate whether the loss of SLPI protein was associated with oral cancer progression using IHC. In addition, we explored the biological effects of SLPI in OPML and OSCC cells and the possible regulatory mechanism, especially focusing on changes in the NF-κB signaling pathway.

Materials and methods

Patients and specimens. Sixty-seven formalin-fixed paraffin-embedded (FFPE) specimens were obtained from the Department of Oral Histology and Pathology, of the Shanghai Ninth People's Hospital Affiliated to the Shanghai Jiao Tong University School of Medicine (Shanghai, China) between May 2016 and December 2016. All patients provided written informed consent. The specimens consisted of: 6 OL tissues without dysplasia, 14 OL tissues with mild dysplasia, 10 OL tissues with mild to moderate dysplasia, 7 OL tissues with moderate dysplasia, 13 OL tissues with moderate to severe dysplasia and 17 OSCC specimens. The diagnosis of the samples was confirmed by oral pathologists of the Shanghai

Ninth People's Hospital according to the World Health Organization (WHO) classification system in 2016. The study was approved by the Institutional Ethics Committee of the Shanghai Ninth People's Hospital, Affiliated to the Shanghai Jiao Tong University School of Medicine.

Hematoxylin and eosin (H&E) staining. H&E staining was conducted according to routine protocol (26). After deparaffinization and rehydration, 5-μm tissue sections were stained with hematoxylin solution for 5 min, followed by 5 immersions in 1% acid ethanol (1% HCl in 70% ethanol), and then rinsed in distilled water. Subsequently the sections were stained with eosin solution for 3 min and then dehydrated with graded alcohol and cleared in xylene.

IHC staining. The FFPE specimens were cut in 5-μm tissue sections and then used for IHC as previously described (27,28). The slides were incubated overnight at 4°C with primary antibody diluted in primary antibody diluent (Beyotime Institute of Biotechnology, Beijing, China). The primary antibody used was mouse monoclonal anti-SLPI antibody (dilution 1:100; cat. no. ab17157; Abcam, Cambridge, MA, USA). The negative staining control was phosphate-buffered saline (PBS). For the antigen retrieval, sections were pre-treated by boiling in 0.01 M sodium citrate (Solarbio Science & Technology, Beijing, China) for 20 min in a steaming pressure cooker. Endogenous peroxidase activity was then blocked by 0.3% (v/v) H_2O_2 for 20 min. The sections were incubated for 30 min at room temperature with anti-mouse secondary antibody (cat. no. GK500705; Gene Tech Biotechnology, Shanghai, China), stained with 3,39-diaminobenzidine (DAB) (Gene Tech Biotechnology), and then counterstained with haematoxylin for nuclear staining. The slides were observed under a microscope with x200 and x400 magnification.

IHC scoring. IHC scoring was evaluated independently by two authors as previously described (27). The results were assessed based on staining intensity and staining area. The intensity score was the average of different field scores as follows: 0, negative; 1, weak staining; 2, distinctly enhanced staining; and 3, strong staining. The staining area score was determined by the percentage of positive cells (stained with DAB in the cytomembrane, cytoplasm and cell nucleus) in the slide. Five random fields of one sample (counting >500 cells/field) were analyzed and categorized as follows: 0, 0% positive; 1, 1-25% positive; 2, 26-50% positive; 3, 51-75% positive and 4, 76-100% positive. For the statistical analysis, the expression index (EI) for each specimen was calculated by the following equation: staining intensity score x staining area score; the values given ranged from 0 to 12.

Cell lines. The human HNSCC cell lines WSU-HN4, HN6 and HN30 (kindly provided by the University of Maryland Dental School, Baltimore, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Basalmedia, Shanghai, China) supplemented with 10% heat-inactivated FBS (Gibco; Thermo Fisher Scientific, Carlsbad, CA, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 atmosphere. SCC-9 and SCC-25 cells (kindly provided by the Shanghai Key Laboratory of Stomatology,

Table I. The primer sequences used in the present study.

Name	Forward primer	Reverse primer
SLPI	5'-GCATCAAATGCCTGGATCCT-3'	5'-GCATCAAACATTGGCCATAAGTC-3'
XIAP	5'-AATAGTGCCACGCAGTCTACA-3'	5'-CAGATGGCCTGTCTAAGGCAA-3'
BIRC2	5'-AGCACGATCTTGTCAGATTGG-3'	5'-GGCGGGGAAAGTTGAATATGTA-3'
BIRC3	5'-AAGCTACCTCTCAGCCTACTTT-3'	5'-CCACTGTTTTCTGTACCCGGA-3'
TRAF1	5'-TCCTGTGGAAGATCACCAATGT-3'	5'-GCAGGCACAACTTGTAGCC-3'
TRAF2	5'-GCTCATGCTGACCGAATGTC-3'	5'-GCCGTCACAAGTTAAGGGGAA-3'
BCL2L1	5'-GACTGAATCGGAGATGGAGACC-3'	5'-GCAGTTCAAACTCGTCGCCT-3'
BCL2	5'-GAGGATTGTGGCCTTCTTTG-3'	5'-GCCGGTTCAGGTACTCAGTC-3'
GAPDH	5'-CGGGAAACTGTGGCGTGAT-3'	5'-GTCGCTGTTGAAGTCAGAGGAG-3'

XIAP, X-linked inhibitor of apoptosis; BCL2L1, BCL2-like 1; BCL2, BCL2, apoptosis regulator; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Shanghai, China) were cultured in DMEM/F12 medium (Basalmedia) supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Leuk1 cells (kindly provided by Professor Li Mao of the University of Maryland Dental School) were cultured in keratinocyte serum-free medium (KSF; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 0.2 ng/ml recombinant epidermal growth factor (rEGF). Leuk-1 cell line is spontaneously derived from an OL lesion that was adjacent to OSCC. It exhibits an immortalized but non-tumorigenic phenotype.

Real-time polymerase chain reaction (real-time PCR) and analysis. Total RNA was extracted with TRIzol reagent (Takara Bio, Inc., Shiga, Japan) and 1 μg total RNA was reverse transcribed into complementary DNA (cDNA) in a total volume of 20 μl using the PrimerScript RT reagent kit (Takara Bio). Subsequently, 100 ng of each cDNA was subjected to PCR amplification using the SYBR Premix Ex Taq reagent kit (Takara Bio) by ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The primer sequences were synthesized as displayed in Table I. An initial denaturation step was performed for 5 min at 96°C and 35 cycles were performed with the following PCR program: denaturing at 96°C for 15 sec, annealing at 56°C for 30 sec and elongating at 72°C for 50 sec. The program was completed with a final extension step at 72°C for 5 min. The results were calculated by the $2^{-\Delta\Delta Ct}$ equation.

Western blotting experiments. Sixty micrograms of total protein from the cell lysates of the cell lines were separated by 12% SDS-PAGE. The proteins were then transferred to a 0.22-μm PVDF membrane (EMD Millipore, Billerica, MA, USA) and incubated with a goat polyclonal anti-SLPI antibody (dilution 0.1 μg/ml; cat. no. AF1274; R&D Systems, Minneapolis, MN, USA). The loading control was the mouse monoclonal GAPDH antibody (dilution 1:5,000; cat. no. D190090; Sangon Biotech Co., Ltd., Shanghai, China). The membrane was labeled with HRP-labeled rabbit anti-goat monoclonal IgG (dilution 1:10,000; cat. no. WB0178; Biotechwell, Shanghai, China) or HRP-labeled goat anti-mouse monoclonal IgG (dilution

1:10,000; cat. no. WB0176; Biotechwell) and visualized with an ECL detection system. The quantity of the total protein was assessed using the BCA assay (Sangon Biotech).

Intake of exogenous SLPI. To ensure that exogenous SLPI (Sino Biological, Beijing, China) was absorbed in human oral epithelial cells, the Leica SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) was used to observe the localization of SLPI in WSU-HN4 cells by immunofluorescent assay. SLPI protein (40 μg/ml) or equal-volume ddH$_2$O was added to the cell culture for 1 h. Images were captured using Zen-Software (Carl Zeiss AG, Oberckochen, Germany).

Cell proliferation assay. As previously described (29), Leuk1 and WSU-HN4 cells were plated in 96-well plates at concentrations of 5x10^3 and 8x10^3 cells/well, respectively. After the Leuk1 cells had achieved adherence they were incubated with 20, 40 or 60 μg/ml exogenous SLPI or ddH$_2$O. In addition, the WSU-HN4 cells were mixed with 10, 20 or 40 μg/ml exogenous SLPI or ddH$_2$O. In the beginning of the experiment, Leuk1 cells were incubated with 20, 40 or 60 μg/ml exogenous SLPI or equal-volume ddH$_2$O. After 3 repetitions, the Leuk1 cells were almost dead at the concentration of 60 μg/ml. Based on these results, we reduced the concentration of SLPI in WSU-HN4 cell line. Cell Counting Kit (CCK)-8 solution (10 μl; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well at 0, 24, 48 and 72 h and incubated for 1 h. The number of viable cells in each well were assessed by reading the optical density (OD) of absorbance at 490 nm.

Apoptosis assay. Leuk1 and WSU-HN4 cells, which were separately incubated with 40 μg/ml exogenous SLPI or equal-volume ddH$_2$O, were harvested at 12 h. At the concentration of 40 μg/ml, the outcomes of the CCK-8 assay were statistically significant in Leuk1 and WSU-HN4 cells. Therefore, the concentration of 40 μg/ml was chosen in the assay. According to the manufacturer's protocols, both the adherent cells and the floating cells were harvested, washed twice with cold PBS and resuspended in 1X binding buffer which was obtained from the

FITC Annexin V Apoptosis Detection kit (BD Biosciences, Bedford, MA, USA) at a concentration of 1×10^6 cells/ml. Then, 5 μl FITC Annexin V and 5 μl propidium iodide (PI) were added to cell suspension. The cells were incubated for 15 min at 25°C in the dark, resuspended in 400 μl of 1X binding buffer and then quantified immediately by BD LSR II flow cytometry (BD Biosciences).

Caspase-3/-7 activity assay. The Caspase-Glo® 3/7 assay (Promega, Madison, WI, USA) is a homogeneous, luminescent assay that assesses the activities of caspase-3 and -7. 4,000 cells/well of Leuk1 or WSU-HN4 were seeded in 384-well plates for 24 h. Then different concentrations (0, 10, 20 and 40 μg/ml) of SLPI protein were added to the corresponding wells and incubated for 2, 4, 6, 8, 12 and 24 h. After incubation, 25 μl Caspase-Glo 3/7 reagent was added to each well. The plate was gently mixed using a plate shaker at 300-500 rpm for 30 sec and the contents were incubated at room temperature for 1 h. The luminescence of each sample was assessed in a plate-reading luminometer (SpectraMax i3; Molecular Devices, Sunnyvale, CA, USA).

NF-κB luciferase reporter gene assay. The activation of NF-κB was determined by a reporter gene assay. The cells were plated in the 24-well plate to a confluency of 70-80%. After adherence, 600 ng of NF-κB luciferase reporter gene constructs (Beyotime Institute of Biotechnology) was co-transfected with 200 ng of *Renilla* luciferase (Beyotime Institute of Biotechnology) which was used to normalize data for transfection efficiency. After 24 h of transfection, the cells were treated with recombinant human SLPI at 0, 20 or 30 μg/ml. In order to ensure the green fluorescent protein expression of NF-κB luciferase reporter gene, the cells were then cultivated for 12 h. The cell lysates were analyzed via dual luciferase reporter gene assay kit (Beyotime Institute of Biotechnology) on Modulus™ (Turner Biosystems, Sunnyvale, CA, USA).

mRNA expression levels of various anti-apoptotic proteins. The NF-κB signaling pathway regulates different anti-apoptotic proteins, such as XIAP, BIRC2, BIRC3, TRAF1, TRAF2, BCL2L1 and BCL2. When the cells achieved 80-90% confluency in 24-well plate, recombinant human SLPI was added to the corresponding well at the concentration of 0, 10, 20 or 40 μg/ml for 4 h. Using TRIzol-based RNA extraction method, the mRNA expression levels of various anti-apoptotic proteins which were subjected to the transcription factor NF-κB were assessed by real-time PCR.

Statistical analysis. The relationship between the EI of SLPI and the degree of OL was analyzed using Spearman's rank correlation analysis. The correlation coefficient (r) was calculated to determine the correlation degree between the EI of SLPI and the degree of OL. Comparison of the expression of SLPI with clinical parameters was evaluated using the χ^2/Fisher's exact test. Other outcomes were determined using Student's t test or one-way ANOVA. Calculations were carried out by SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). A two-sided P-value of <0.05 was considered to indicate statistically significant differences.

Table II. Correlation between the expression of SLPI and the clinical parameters in OL and OSCC.

Characteristics P-value	Immunoreactivity score, n (%)		
	Low (≤6), n=40	High (>6), n = 2 7	
Age (years)			
≤60	24 (68.57)	11 (31.43)	0.142
>60	16 (50.00)	16 (50.00)	
Sex			
Male	15 (57.69)	11 (42.31)	0.804
Female	25 (60.98)	16 (39.02)	
Anatomic site			
Gingiva	3 (50.00)	3 (50.00)	0.286
Tongue	27 (67.50)	13 (32.50)	
Buccal mucosa	6 (40.00)	9 (60.00)	
Others	4 (66.67)	2 (33.33)	
Disease			
OL	25 (50.00)	25 (50.00)	0.009
OSCC	15 (88.24)	2 (11.76)	

Comparison of the expression of SLPI with clinical parameters was evaluated using the χ^2/Fisher's exact test. OL, oral leukoplakia; OSCC, oral squamous cell carcinoma.

Results

Negative correlation between the SLPI protein expression and the degree of OL. Firebrowse (http://firebrowse.org/), which is a website with data from The Cancer Genome Atlas (TCGA) and demonstrates the different expression of certain proteins in various cancer types through the form of images, was used to analyze the different expression of SLPI in 28 cancer types in the present study (Fig. 1A). It demonstrated that the expression of SLPI had diverse patterns in different types of tumors. As displayed, compared with matched normal tissues, the expression of SLPI in OSCC decreased. To investigate the expression of SLPI at different histological stages of OL, we used the method of IHC. There was a negative association between the expression level of SLPI and the pathological differentiation of oral tissues from various stages of OL to OSCC (r=-0.4922, P<0.05; Figs. 1B-H and 2A), whereas no significant associations were determined between the expression pattern of SLPI and age (P=0.142), sex (P=0.804) or anatomic site (P=0.286; Table II and Fig. 2C-E). In addition, the expression of SLPI between OL and OSCC was significantly different (P<0.05; Fig. 2B).

Different expression of SLPI in Leuk1 and HNSCC cell lines. To confirm our previous results (3) and the IHC data, the levels of SLPI protein in Leuk1 and 5 HNSCC cell lines were researched using western blot analysis. The protein levels of SLPI in HNSCC cell lines were significantly lower than the
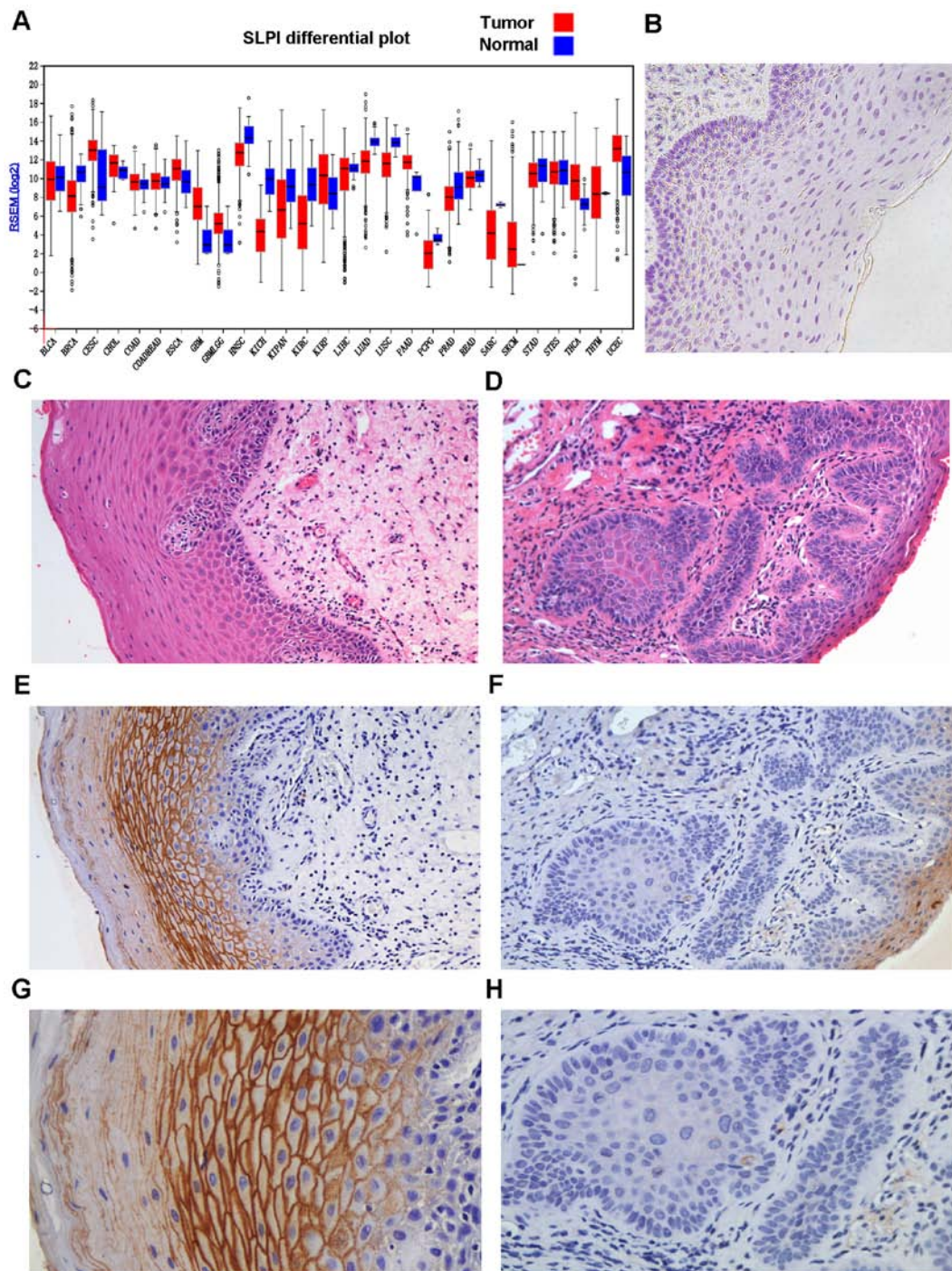
Figure 1. (A) The mRNA expression of SLPI in different tumors and protein expression of SLPI in OSCC and OPML by IHC staining. The different mRNA expression of SLPI in tumor tissues and matched normal tissues. Data from The Cancer Genome Atlas (TCGA). Red, tumor tissues; Blue, matched normal tissues. (B) Negative control (original magnification, x100). Negative staining control was phosphate-buffered saline (PBS). (C) The histological image of H&E staining is from the same tissue sample displayed in E and G (original magnification, x200). (D) The histological image of H&E staining is from the same tissue sample shown in F and H (original magnification, x200). (E) Strong SLPI immunoreactivity is displayed in OL tissue sample with mild dysplasia (original magnification, x200). (F) Negative SLPI immunoreactivity is detected in OSCC (original magnification, x200). (G) Strong SLPI immunoreactivity in OL tissue sample with mild dysplasia, which is from the same tissue sample shown in E (original magnification, x400). (H) Negative SLPI immunoreactivity in OSCC, the same tissue sample displayed in Fig. 1F (original magnification, x400). BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical and endocervical cancers; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; COADREAD, colon and rectal adenocarcinoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; GBMLGG, glioma; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIPAN, pan-kidney cohort (KICH+KIRC+KIRP); KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; STES, stomach and esophageal carcinoma; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma.

expression in the Leuk1 cell line (Fig. 3A). Furthermore, compared with the mRNA level of SLPI in Leuk1 cells, the levels were remarkably downregulated in most of the HNSCC cell lines by real-time PCR (Fig. 3B).
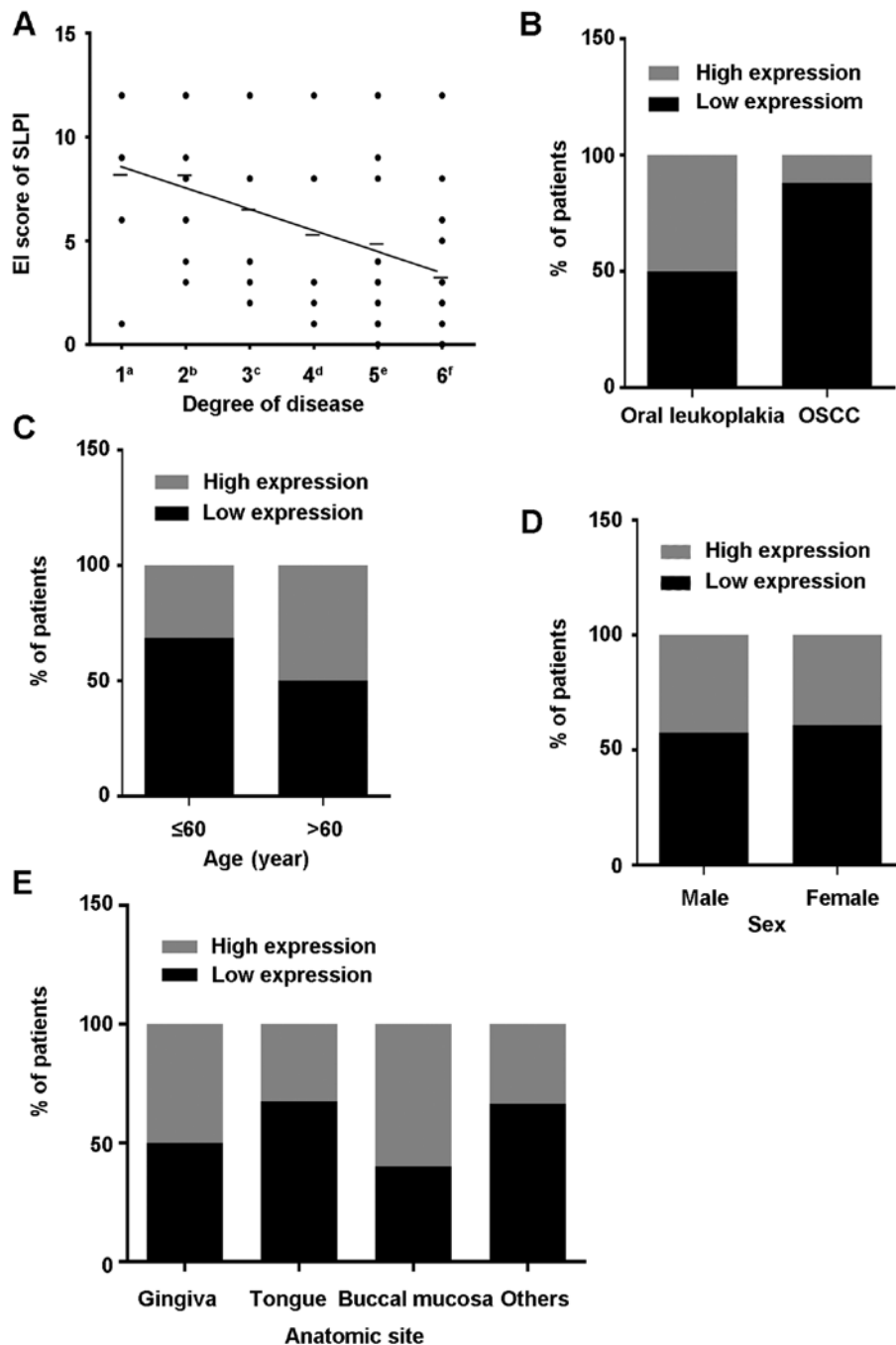
Figure 2. Evaluation of SLPI protein expression in OSCC and OPML. (A) The correlation between SLPI expression level and the pathological differentiation of the oral tissues from various stages of OL to OSCC (r=-0.4922, P<0.05). (B) The percentage of OL and OSCC patients with high or low expression of SLPI protein (Fisher's exact test, P<0.05). (C-E) The relationship between expression index (EI) scores and age, sex (Fisher's exact test, P>0.05) or anatomic site (χ^2 test, P>0.05). [a]OL without dysplasia; [b]OL with mild dysplasia; [c]oral leukoplakia with mild to moderate dysplasia; [d]OL with moderate dysplasia; [e]OL with moderate to severe dysplasia; and [f]oral squamous cell carcinoma (OSCC).

Exogenous SLPI can be absorbed in cells. According to previous research, the invasion effect of SLPI in WSU-HN12 cell lines has been investigated (18). It is well-known (30) that the WSU-HN4 and WSU-HN12 cell lines were derived from the same patient. Furthermore, the WSU-HN4 cell line was derived from the primary squamous cell carcinoma of the tongue and it has an epithelial phenotype and low invasive capacity. The WSU-HN12 cell line exhibits a mesenchymal phenotype and has high invasive capacity. It was derived from a nodal metastasis in the patient from whom the HN-4 cells

originated. It is considered an appropriate choice to research the other biological effects of SLPI in the low invasive WSU-HN4 cell lines. By comparing a blank group with the exogenous SLPI group in the WSU-HN4 cells, we found that exogenous SLPI protein was absorbed in cells. In some cells, partial proteins were also localized in the nucleus (Fig. 3C-F). A previous research (31), has demonstrated that SLPI can enter the cytoplasmic and nuclear fractions of macrophages and it was hypothesized that it may be the arginine-rich nature of SLPI that enables it to interact with the predominantly
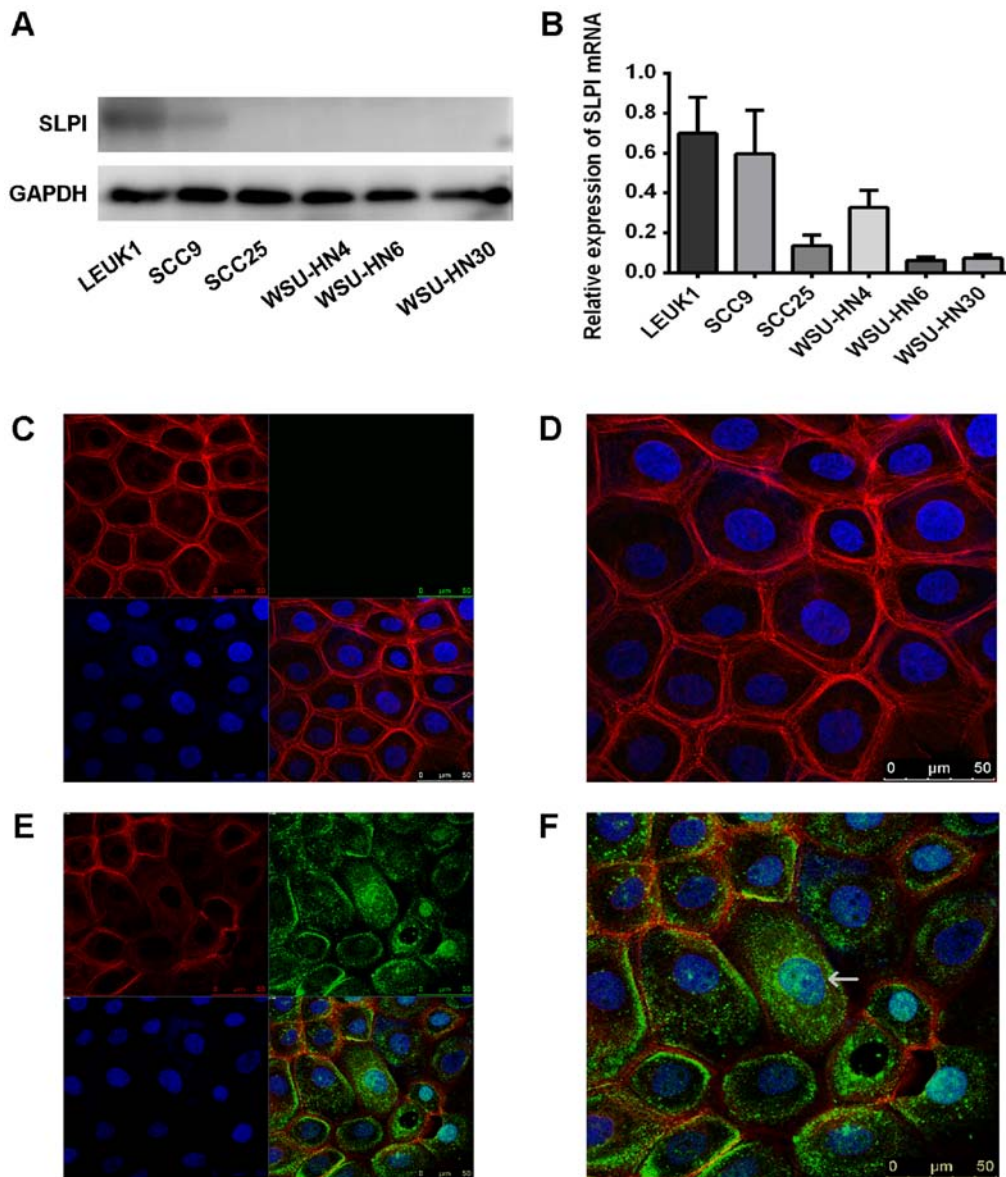
Figure 3. Intake of exogenous SLPI protein and the mRNA and protein expression of SLPI *in vitro*. (A) The protein expression level of SLPI in Leuk1 and OSCC cell lines. (B) The mRNA expression level of SLPI in Leuk1 and OSCC cell lines. (C and D) The images of WSU-HN4 cells incubated without exogenous SLPI protein. (E and F) The images of WSU-HN4 cells incubated with 40 μg/ml exogenous SLPI protein for 1 h. Cell nucleus was stained with DAPI (Blue). Cytoskeleton was stained with phalloidine (Red). SLPI was stained with FITC secondary antibody (Green). The data are shown as the mean ± SEM. The white arrow indicates protein imported into the nucleus.

negatively-charged cell membrane and be internalized; and SLPI is a small molecule (~11 kDa). Researchers have found that it prevents the interaction of p65 with the NF-κB consensus region by binding directly to the NF-κB binding sites in macrophages. Thus, it may exhibit similar regulatory mechanisms in OPML and OSCC. Future studies are needed to clarify this hypothesis.

Reduced cellular proliferation in Leuk1 and WSU-HN4 cell lines with exogenous SLPI. To investigate the biological effects of SLPI protein in OPML and OSCC, Leuk1 and WSU-HN4 cell lines were incubated with exogenous SLPI protein for 0, 24, 48 and 72 h and the proliferation rate was analyzed using CCK-8 assay. The proliferation rates of Leuk1 cells significantly decreased both in the 40-μg/ml exogenous SLPI protein group and in the 60-μg/ml group (P<0.05, Fig. 4A).

Due to the fact that most cells were killed at a concentration of 60 μg/ml SLPI protein, we reduced the concentration to 10, 20 or 40 μg/ml in the WSU-HN4 cells. Similarly, compared with the control group, the proliferation rate of the WSU-HN4 cells was significantly slower in the 40-μg/ml exogenous SLPI protein group (P<0.05, Fig. 4B). These data revealed that SLPI inhibited cell growth in Leuk1 and WSU-HN4 cell lines, especially at the concentration of 40 μg/ml.

Exogenous SLPI promotes apoptosis in the Leuk1 and WSU-HN4 cell lines. We investigated whether SLPI could inhibit cell growth by arresting the cell cycle at some point, however the evidence was not sufficient (data not shown). To analyze whether the suppressive effect of SLPI protein was caused by pro-apoptotic effect, Leuk1 and WSU-HN4 cells with exogenous SLPI or ddH$_2$O were assessed using PI and
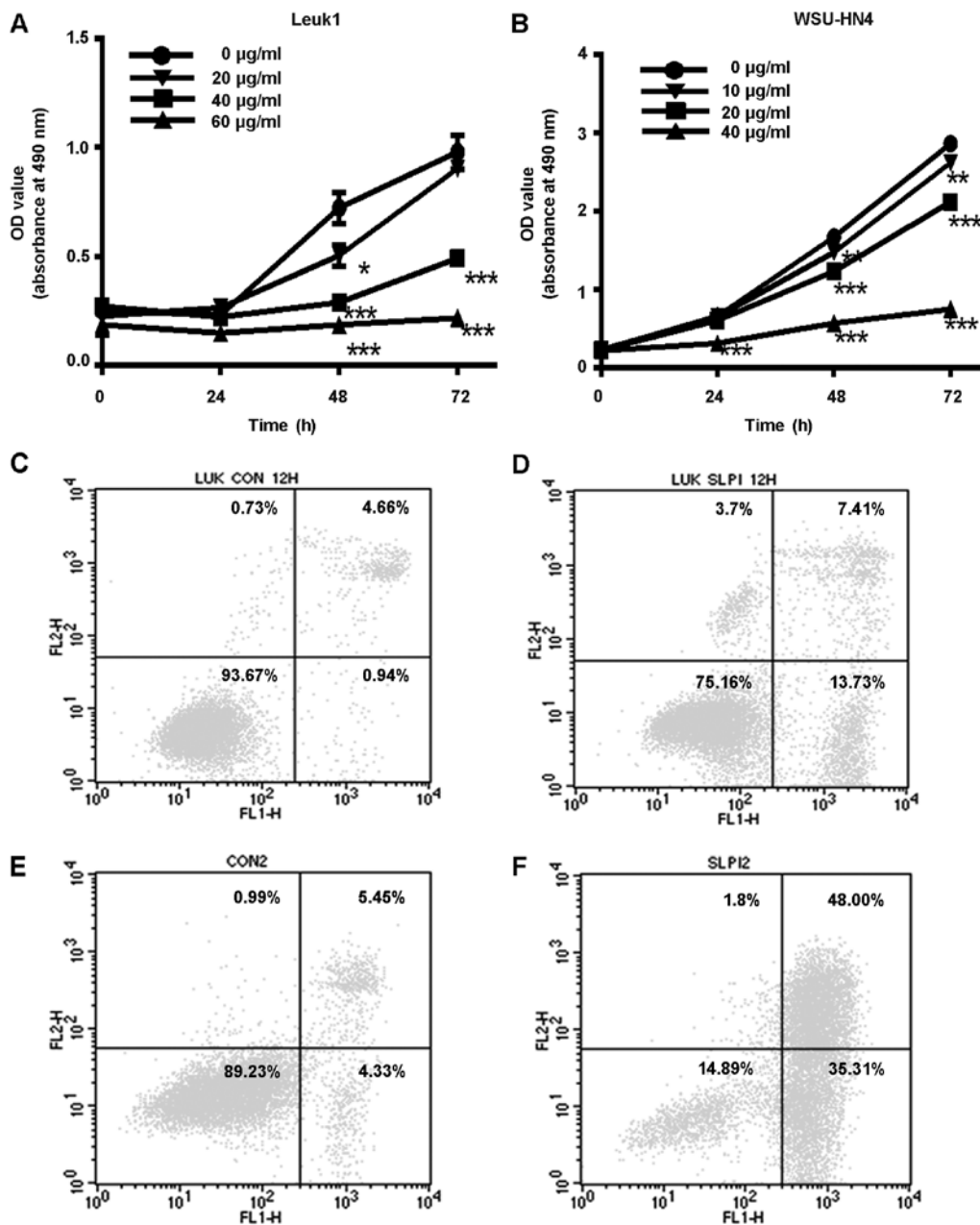
Figure 4. Effects of exogenous SLPI protein on cell proliferation and apoptosis in Leuk1 and WSU-HN4 cell lines. (A and B) SLPI reduced cellular proliferation in Leuk1 and WSU-HN4 cell lines using CCK-8 assay. (C and D) SLPI induced apoptosis in Leuk1 cell line using Annexin V/PI. (E and F) SLPI promoted apoptosis in WSU-HN4 cell line. (C and E) control group, (D and F) 40 μg/ml exogenous SLPI group. The data are expressed as the means ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs. 0 μg/ml SLPI group.

Annexin V. The proportion of early apoptotic cells in Leuk1 and WSU-HN4 cells incubated with exogenous SLPI was 13.73 and 35.31%, respectively, which was notably higher than the ratio of early apoptotic cells in the control cells with ddH$_2$O (0.94 and 4.33%) (Fig. 4C-F). It is a new discovery that SLPI has the pro-apoptotic biological effect both in OPML and in OSCC.

SLPI protein activates caspase-3/-7 in Leuk1 and WSU-HN4 cell lines. To verify that the SLPI protein can induce apoptosis in Leuk1 and WSU-HN4 cell lines, we performed a quantitative caspase-activity assay. Caspase-3 and -7 are known as caspase effectors and induce apoptosis. Our results revealed that the activation of caspase-3/-7 was increased in a dose- and time-

dependent manner (Fig. 5A and B). In addition, after 6 h, caspase-3/-7 was constantly activated and lasted for >24 h.

Inhibition of SLPI on NF-κB activity in Leuk1 and WSU-HN4 cell lines. NF-κB, an early response gene associated with SCC progression, is implicated in the regulation of proinflammatory and anti-apoptosis pathways. A previous study (31) has revealed that SLPI inhibited the activation of NF-κB in macrophages. In the present study, in order to explore the hypothesis that SLPI may promote oral precancerous lesion and OSCC cell apoptosis by hindering the NF-κB-mediated anti-apoptosis pathway, we used the NF-κB luciferase reporter assay to investigate whether the SLPI-protein treatment decreased the activation of the NF-κB gene *in vitro*. Our
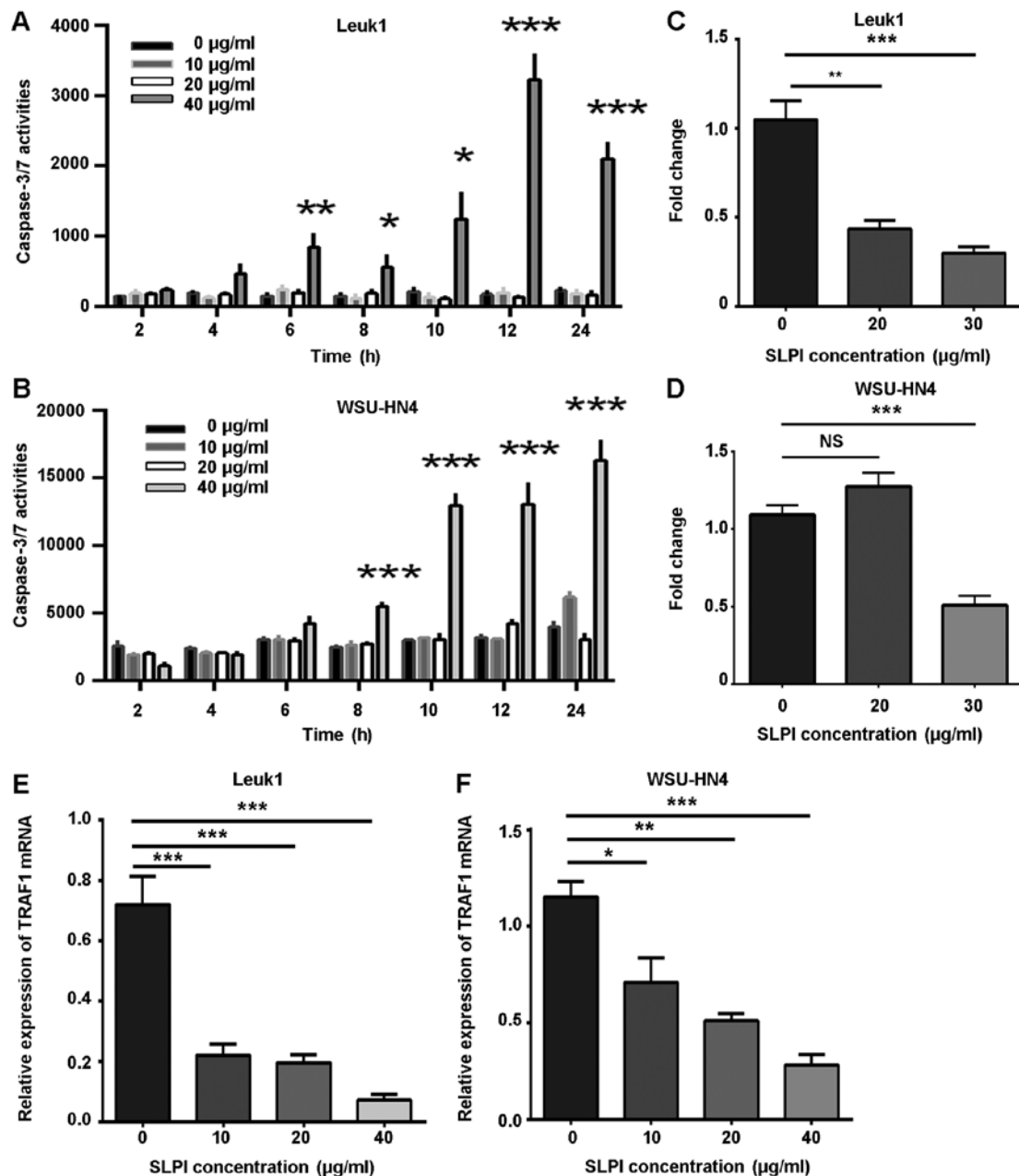
Figure 5. Exogenous SLPI protein induces apoptosis *in vitro*. (A and B) SLPI activated caspase-3/-7 in (A) Leuk1 and (B) WSU-HN4 cell lines. (C and D) SLPI inhibited activation of the NF-κB pathway in Leuk1 and WSU-HN4 cell lines in a dose-dependent manner. (E and F) After incubation with different concentrations of SLPI, the mRNA expression levels of TRAF1 decreased both in Leuk1 and WSU-HN4 cell lines. The data are shown as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001; NS, not significant.

results demonstrated that exogenous SLPI protein downregulated the NF-κB-gene activity in Leuk1 and WSU-HN4 cell lines, in a relatively dose-dependent fashion, as displayed in Fig. 5C and D. In addition, the Leuk1 cell lines were more sensitive to SLPI protein. This initial data indicated that SLPI may promote apoptosis in OPML and OSCC cells by inhibiting the NF-κB anti-apoptosis pathway.

SLPI suppresses the mRNA expression level of TRAF1. It is well-known that the transcription factor, NF-κB, regulates numerous antiapoptotic proteins. Therefore, in the present study, after incubating the Leuk1 and WSU-HN4 cell lines with different concentrations of SLPI, we collected the total RNA

and determined the mRNA expression levels of XIAP, BIRC2, BIRC3, TRAF1, TRAF2, BCL2L1 and BCL2, which are the downstream genes of the NF-κB pathway. Compared with the control group, the mRNA expression of TRAF1 in exogenous SLPI groups significantly decreased (P<0.05; Fig. 5E and F); the mRNA expression levels of the other genes were not statistically significant (data not shown). To some extent, these results may indicate that TRAF1 is likely to participate in the apoptosis regulation of SLPI in OPML and OSCC. In the future, we need to further investigate the relationship between the expression of SLPI and TRAF1 and explore whether the NF-κB pathway is the main pathway involved in the regulation of apoptosis.

Discussion

In our previous study (3), using non-invasive oral brush biopsies, it was revealed that SLPI was markedly reduced in the OPML tissues compared with normal oral mucosa. The abundance of SLPI protein in the saliva of patients with OPML revealed the same changing pattern. In addition, another study (18) reported that the content of SLPI protein in the OSCC tissues was less than its expression in the normal oral tissues. SLPI protein in well- and moderate-differentiated OSCCs (32) is more abundant than that in poorly differentiated ones. Based on these findings, we aimed to study the correlation between the abundance of SLPI protein and different histological stages of OL by IHC. Our results indicated that along with the progressive severity of dysplasia in OL tissues, the abundance of SLPI protein gradually decreased. Furthermore, there is a statistically significant difference in the expression of the SLPI protein between OPML and OSCC (P<0.05). According to these results, SLPI protein may be a potential tool to predict the possibility of malignant transformation of oral precancerous lesions and perhaps provide reliable evidence for early diagnosis and timely treatment to reduce the incidence of OSCC. To verify these results, more *in vivo* experiments are needed in the future.

The biological effects of the SLPI protein in OPML and OSCC cells are not yet fully understood. According to previous studies, SLPI has distinct functions in various types of tumors. For instance, it was reported that SLPI promoted cell proliferation and antagonized paclitaxel in ovarian cancer cells (17,33). In the Lewis lung carcinoma 3LL-S cell line (34), SLPI promoted cell proliferation and played an important role in the malignant progress. In addition, SLPI promoted cell survival and growth of castration-resistant prostate (35) and pancreatic cancer (8). Paradoxically, Nakamura *et al* observed that SLPI inhibited cell growth and induced apoptosis in ovarian cancer cells (36). Furthermore, previous studies (16,37,38) also reported that SLPI induced apoptosis of mammary tumor cells *in vitro* and decreased mammary tumor growth *in vivo*. Thus, the role of this protein in cancer metastasis is dual. It can promote metastasis in gastric (39) and ovarian cancer (40), however, in OSCC it may have a protective effect on the process of metastasis (5,18). It is well-known that abnormal apoptosis may induce the occurrence of cancer. The present study revealed for the first time, that SLPI promoted apoptosis in OPML and OSCC cell lines. Combined with its inhibition effect of metastasis in OSCC, SLPI may have a potential protective role in OSCC. In addition, many studies (41,42) have revealed that proteins which inhibit cell proliferation and induce apoptosis in OL can participate in the prevention of malignant progression. SLPI may be a potential therapeutic drug for patients with OPML and OSCC. In the present study, exogenous SLPI significantly inhibited cell proliferation at the concentration of 40 μg/ml. Using exogenous SLPI in Leuk1 and WSU-HN4 cell lines, the activation of caspase-3/-7 was increased in a dose- and time-dependent manner, especially at the concentration of 40 μg/ml and after 6 h and the activation lasted for >24 h. The changing patterns of SLPI expression and the dual effects of SLPI in various tumors are worth being further researched in depth.

Many pathways are involved in the regulation of cell apoptosis, such as the P13K/Akt, NF-κB, p53 and MAPK signaling pathways. Previous studies (31,43) revealed that SLPI inhibits LPS-induced NF-κB activation in monocytic cells, alveolar epithelial cells (20) and gingival squamous cell carcinoma-derived Ca9-22 cells (21). One underlying mechanism is that SLPI inhibited the degradation of IκBα without affecting the phosphorylation and ubiquitination of IκBα in monocytic cells. SLPI suppressed the NF-κB activation by preventing the IκBβ degradation in Ca9-22 cells. Another theory is that SLPI can enter cell nucleus and prevent p65 interaction with the NF-κB consensus region by binding directly to the NF-κB binding sites. In the present study, SLPI was localized in the nucleus of some cells and found in the cytoplasm in more cells. We hypothesized that SLPI inhibited the activation of NF-κB through the two ways aforementioned, especially by inhibiting the IκBα or IκBβ degradation. However, this hypothesis needs to be further investigated. Previous studies (23-25) found that constitutive activation of the NF-κB signaling pathway in HNSCC promoted survival, growth, migration and the secretion of pro-inflammatory and pro-angiogenic cytokines. According to these results, we assumed that SLPI may regulate cell apoptosis in OPML and OSCC by inhibiting the NF-κB signaling pathway. In the present study, due to the apoptosis effect of SLPI, it was used at a concentration of <40 μg/ml to ensure that sufficient cells could be collected to detect the activation of the NF-κB reporter gene. We observed that, in a relatively dose-dependent manner, SLPI significantly decreased the activation of the NF-κB reporter gene in oral pre-malignant cells and oral squamous carcinoma cells. And Leuk1 cell lines were more sensitive to SLPI irritation. In previous research it was demonstrated that the expression level of NF-κB in OSCC cells was significantly higher than that in OPML and normal oral epithelial tissues (44,45) and that NF-κB may be a valid target for therapeutic approaches in patients with OSCC and potentially malignant oral lesions. This may explain why SLPI is more pronounced in Leuk1 cells and may have the ability to prevent OL cancerization process. Subsequently, we analyzed the changes of the downstream anti-apoptotic genes of the NF-κB pathway. We observed that the level of TRAF1 mRNA in Leuk1 and WSU-HN4 cells obviously decreased in a dose-dependent manner. Previous studies have demonstrated that TRAF1 (46,47) exerts a direct anti-apoptotic effect. For instance, along with BIRC2 and BIRC3, the NF-kB-controlled expression of TRAF1 and TRAF2 (48) can provide maximum protection against the TNF induced-apoptosis. TRAF1 transgenic mice (49) have a defect in antigen-induced apoptosis of the CD8^{+} T cells. Previous research revealed that the expression of TRAF1 in response to *H. pylori* infection in gastric epithelial cells was mainly regulated by the activation of NF-kB. In addition, upregulation of TRAF1 plays an anti-apoptotic role in *H. pylori*-infected gastric cells (50). Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, we found that TRAF1 also promoted anti-apoptosis activity via inducing the NF-κB signaling pathway. These findings indicated that they can regulate each other. We hypothesized that by using exogenous SLPI protein, the NF-κB signaling pathway, which is suppressed by SLPI, inhibits the expression of TRAF1. In turn, the reduced TRAF1 may further inhibit NF-κB signaling pathway. However it is also possible that SLPI

firstly inhibits TRAF1 and then TRAF1 regulates NF-κB. To verify this assumption, we should use TRAF1 and NF-κB mutant types to observe the relevant changes of NF-κB and TRAF1 after using SLPI. In brief, it tentatively explored that SLPI protein may promote cell apoptosis through the inhibition of the NF-κB pathway. However, the detailed apoptosis regulatory mechanism of the SLPI protein in OPML and OSCC is not sufficiently clarified in the present study. This will be the focus of our next experimental study.

In conclusion, this is the first time to find that the abundance of SLPI protein is negatively correlated with the grade of OL and it may have a potential prediction role for the malignant transformation of OPML. The present study further revealed that the SLPI protein exerted a pro-apoptotic effect in OPML and OSCC cell lines. It promoted apoptosis, to some extent, through suppression of the NF-κB pathway. The present study about the apoptosis regulatory mechanism of SLPI is a tentative research. In the future, more research is needed to elucidate the detailed mechanism.

Acknowledgements

Competing interests

The authors declare that they have no competing interests.

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