Scutellarin inhibits the growth and invasion of human tongue squamous carcinoma through the inhibition of matrix metalloproteinase-2 and -9 and αvβ6 integrin

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Abstract. Scutellarin can inhibit the growth and migration of tongue cancer cells *in vitro* and can regulate cell adhesion; such agents will be the next generation anticancer therapeutics. In this study, we evaluated the antitumor effects of the scutellarin on human tongue squamous carcinoma (SAS) cell line and investigated its molecular mechanism. To explore the possible mechanisms underlying the antitumor effect of scutellarin, we studied its impact on the growth and invasion of SAS cells xenografted into nude mice, on the expression of MMP-2, MMP-9, integrin αvβ6, and c-JUN, and also on changes in collagen fibers. We observed that the growth of xenograft SAS tumors in nude mice was significantly inhibited by the administration of scutellarin without major adverse effects. Results showed that scutellarin mediated inhibition of tumor cell proliferation, induced apoptosis, and regulated expression of matrix metalloproteinase (MMP)-2 and -9, and integrin αvβ6 at the mRNA and protein levels *in vivo*. Moreover, scutellarin regulated the expression of collagen fibers in the tumor microenvironment (surrounding the tumor), thereby inhibiting cell invasion and metastasis. Our *in vitro* and *in vivo* studies have shown that scutellarin reduces the expression of MMP-2 and -9, and integrin αvβ6 in SAS cells, possibly through the regulation of expression of transcription factor AP-1. These results suggest that scutellarin can have an anti-tumor therapeutic effect by inhibition of the ability of SAS cells to invade and metastasize.

Introduction

Oral squamous cell carcinoma (OSCC), the 8th most common cancer worldwide, is a common cancer of the head and neck region (1). Despite advances in diagnostic methods and treatment strategies in recent years, the overall survival rate for patients with OSCC has not significantly improved. The poor prognosis of OSCC patients, with the 5-year mortality rate currently remaining at 53%, is due to its local invasion and distant metastasis (2). Tumor cell invasion and metastasis are regarded as multi-step phenomena involving the proteolytic degradation of the basement membrane (BM) and extracellular matrix (ECM), altered cell adhesion, and physical movement of tumor cells. Invasion and metastasis are important factors that impact OSCC patient outcomes and prognosis (1), and complicate cancer treatment.

Among the many steps involved in invasion and metastasis, excessive degradation of the ECM is a hallmark of this process (3). Many proteases can degrade ECM components, matrix metalloproteinases (MMPs), particularly the combination of MMP-2 and -9, are the main enzymes involved in degradation of type IV collagen (4). Studies have shown that MMP-2 and -9 are overexpressed in OSCC (5). Overexpression of the genes encoding MMP-2 and -9 is closely related to OSCC tumor angiogenesis, local tumor invasion, and lymph node metastasis, and indicates a poor prognosis (4,6,7). Therefore, blocking the expression of MMP-2 and -9 can inhibit tumor cell degradation of collagen IV in the ECM and BM, thereby suppressing tumor development, invasion, and metastasis.

Integrins are a family of heterodimeric cell surface receptors and are the major extracellular matrix receptors. Integrins regulate diverse processes, including proliferation, tumor invasion, and apoptosis (8). Unlike most epithelial integrins, integrin αvβ6 is not expressed in healthy adult epithelia, but is upregulated during wound healing and in cancer (9). Integrin αvβ6 may have multiple regulatory functions in oncogenesis. Integrin αvβ6, via its extracellular and transmembrane domains, activates TGF-β, leading to adhesion and epithelial-mesenchymal transition, while its cytoplasmic domain affects proliferation, generation of MMPs, and cell migration and survival (10,11). Studies have shown that integrin αvβ6 induces epithelial to mesenchymal transition in oral cancer (12), promotes invasion of squamous carcinoma cells (13), and is closely linked to liver metastasis of colon cancer cells (14). Therefore, targeted inhibition of integrin αvβ6 expression should reduce the malignant potential of tumors, and inhibit the ability of tumor cells to invade and metastasize.

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Collagen fibers, the main components of the ECM, are widely distributed in different layers of the oral mucosa. The ECM not only provides a protective screen for the tissues, organs, or cells it supports, but also participates in cell proliferation, survival, and apoptosis (15,16). Clinically, many diseases of the oral mucosa are related to the remodeling of collagen fibers. Therefore, pathological changes of the oral mucosa fiber structure and the relationship of oral cancer have recently gained attention (17). Recent studies have focused on the relationship between collagen fibers and carcinogenesis, but few reports have investigated the change in collagen fibers during the course of treatment.

The activator protein-1 (AP-1) participates in multiple aspects of tumor metastasis. AP-1 can induce cell proliferation and cancer, and can promote degradation of the tumor-surrounding matrix, changes in cell adhesion, and enhanced cell motility; it can promote angiogenesis and lymphangiogenesis through the regulation of downstream target genes, thus promoting tumor invasion and metastasis (18). AP-1 cis-regulatory elements exist in multiple members of the integrin family, and AP-1 influences the effect of tumor cells on ECM through integrins (19). Many metastasis-promoting factors can enhance the expression of MMP-2 and -9 through AP-1 signaling pathways, thus contributing to degradation of the basement membrane (20,21).

OSCC with local invasion and successive transfer of biological characteristics are the main factors affecting success of treatment and prognosis (1). Therefore, identifying new drugs that inhibit invasion and metastasis has become critical in the cancer treatment process. Our previous studies have shown that scutellarin can inhibit the proliferation and migration of tongue cancer cells *in vitro* and has the ability to regulate cell adhesion (22). To further explore the anticancer effects of scutellarin, we studied the impact of scutellarin on the growth and invasion of tongue cancer cells (SAS) xenografted in nude mice, and evaluated the effect of scutellarin on the expression of MMP-2, -9, integrin αvβ6, and c-JUN, as well as on changes in collagen fibers, to explore the possible mechanisms underlying the antitumor effect of scutellarin.

Materials and methods

Reagents and cells. Scutellarin (purity 99%, HPLC) was purchased from Beidouxing Pharmaceutical Co. Ltd. (Tianjin, P.R. China). SAS cells were cultured as previously described (22). Briefly, SAS cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, and maintained at 37˚C in a humidified incubator (Hanau, Germany) containing 5% CO_2.

Animal treatment with scutellarin. Athymic Balb/ca nude mice (4-week-old males) were obtained from Shanghai Laboratory Animal Center (Shanghai, P.R. China). The care and treatment of the experimental animals complied with the Harbin Medical University guidelines for animal experiments. To create an animal xenograft model of human tongue squamous carcinoma, a 0.2-ml suspension of SAS cells ($1x10^5$ cells/ml) in a serum-free medium was subcutaneously injected into the left dorsal flank of each mouse. Seven days after implantation, induced tumor dimensions were recorded. Six mice in each group received oral gavage with scutellarin (5, 10, 20 mg/kg body weight) or with 0.9% normal saline solution, every other day. Length and width of each tumor were measured every other day. Following 3 weeks of administration of scutellarin or normal saline, the mice were sacrificed and the tumors removed for hematoxylin and eosin staining, immunohistochemical staining, Masson's trichrome staining, and transmission electron microscope observation. Tumor volume was determined by direct measurement with calipers and calculated by the formula: $\pi/6$ x (large diameter) x (small diameter)^2. Inhibition rate (IR) of the xenograft tumor was calculated as follows: IR (%) = [1 - (tumor volume of scutellarin-treated group/tumor volume of normal saline-treated group)] x 100%.

Masson's trichrome staining. Formalin-fixed, paraffin-embedded tissues were sectioned (5 μm thick), deparaffinized, and rehydrated. Masson's trichrome staining (23) was used to observe the content and distribution of collagen fibers.

TUNEL assay for apoptotic cells in vivo. Apoptosis was assessed in xenograft tumors using the terminal deoxyribonucleotide transferase-mediated nick-end labeling (TUNEL) method in combination with an *in situ* apoptotic cell detection kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The extent of apoptosis was evaluated by counting the number of TUNEL-positive (brown-stained) cells. The apoptotic index was calculated as the number of TUNEL-positive cells divided by the total number of cells in 10 randomly selected high-power fields.

Transmission electron microscopy (TEM). Xenografts were dissected and fixed with 2.5% glutaraldehyde for 2 h, post-fixed in 1% osmium tetroxide (OsO_4) at 4˚C for 2 h, and embedded with Epon-812 (EM Sciences, USA) for 72 h at 60˚C. Ultrathin sections (50 nm thick) were cut and stained with uranium acetate, followed by lead citrate, and then observed under a transmission electron microscope (JEOL Ltd., Tokyo, Japan).

Immunohistochemical staining. Deparaffinized sections taken from each tumor were incubated with 1% BSA for 30 min and then stained with mouse monoclonal anti-MMP-2 (Santa Cruz Biotechnology, Inc., CA, USA; sc-13595, 1:200 dilution), goat polyclonal anti-MMP-9 (Santa Cruz Biotechnology; sc-6840, 1:200 dilution), mouse monoclonal anti-PCNA (Santa Cruz Biotechnology; sc-25280, 1:200 dilution), or rabbit polyclonal anti-integrin αvβ6 (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, P.R. China; bs-5791R, 1:1000 dilution). The extent of cell proliferation was evaluated by counting the number of PCNA-positive cells in 10 randomly selected high-power fields and the proliferation index was calculated as the number of PCNA-positive cells divided by the total number of cells in the field. For MMP-2, -9, and integrin αvβ6 analysis, 10 areas were randomly selected under a microscope at a magnification of x200. Image Pro Plus 6.0 (Media Cybernetics, Inc., Bethesda, MD, USA) was used to quantify the extent of immunopositive expression in cells with integrated optical density (IOD) values.

Western blot analysis. The effect of scutellarin on proteins was assessed by western blotting. Cells were cultured to at least 80%

Table I. Primers used for RT-PCR analysis.

Sequence name	Primers
MMP-2	
Forward	5'-GTGGATGATGCCTTTGCTCG-3'
Reverse	5'-CCATCGGCGTTCCCATACTT-3'
MMP-9	
Forward	5'-GCTTGCCCTGGTGCAGTAC-3'
Reverse	5'-ATTGCCGTCCTGGGTGTAG-3'
αvβ6	
Forward	5'-AGAGAAGAAGCAGGCACATTATC-3'
Reverse	5'-AGGTAGGACATCGTTCACAGG-3'
GAPDH	
Forward	5'-AACGGATTTGGTCGTATTGG-3'
Reverse	5'-TGGAAGATGGTGATGGGATT-3'

confluence, and then exposed to different concentrations (0, 3, 15, 75 nM) of scutellarin for 24 h. Collected cells were lysed with RIPA buffer (Beyotime, Nantong, P.R. China) on ice. Protein concentration in conditioned media samples was determined (Beyotime). Cell lysates (50 μg total protein) were separated by 10% SDS-PAGE and electrophoretically transferred onto Hybond PVDF membranes. After blocking in TBS-T containing 5% non-fat dry milk, the membranes were incubated overnight at 4˚C with primary antibodies against the target proteins. After washing twice with 0.01 M PBS, the membranes were incubated with secondary anti-rabbit IgG antibody linked to horseradish peroxidase, and protein levels were detected using an ECL detection system (Amersham, Uppsala, Sweden).

RT-PCR analysis. Total RNA was isolated from experimental cells using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed into cDNA and amplified using an RT-PCR system (Promega, Madison, WI, USA). The primer sequences and product sizes are listed in Table I. Amplified products were fractionated using 1% agarose gel electrophoresis. GAPDH was used as an internal control.

Statistical analysis. Experiments were independently performed at least 3 times. Data are presented as the mean ± SEM (standard error of the mean). Data were analyzed using one-way ANOVA and Student's t-test. Statistical evaluation was performed using SPSS 16.0 software. Statistical significance was established at $P<0.05$.

Results

Scutellarin inhibited SAS xenograft tumor growth in vivo. Our previous studies show that scutellarin can inhibit the proliferation of tongue carcinoma SAS *in vitro*. This study investigated the effects of scutellarin on SAS xenograft tumor growth. Fig. 1 shows the growth of nude xenografts in different treatment groups. Macroscopic appearance of xenografted tumors revealed a smaller tumor mass in the groups treated with 10 or 20 mg/kg scutellarin than in the normal saline-treated group (Fig. 1A).

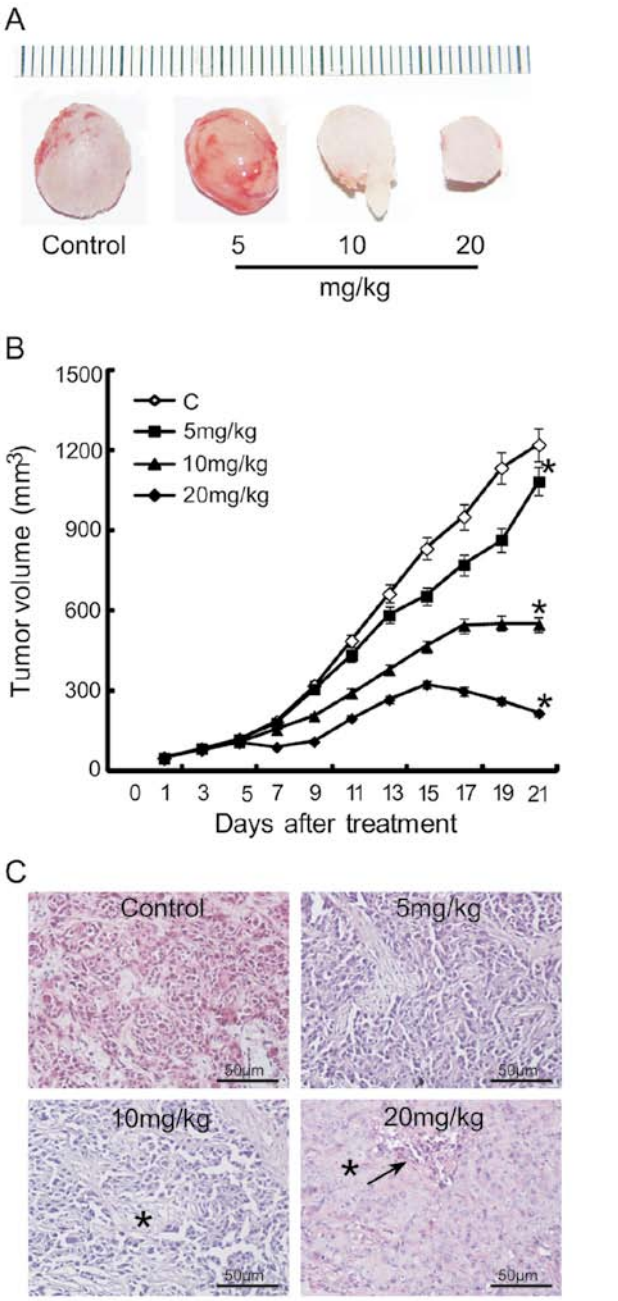

Figure 1. Effect of scutellarin on xenografted SAS tumor growth in nude mice. A, Representative tumors from mice from control and scutellarin-treated (5, 10, or 20 mg/kg) groups. B, Growth curve of SAS tumors indicating means and the standard error. *P<0.01 vs. control. C, Representative hematoxylin and eosin (H&E) staining of SAS tumors; an increase in ECM (asterisk) can be observed as lightly-stained acellular areas after treatment with scutellarin; tumor necrosis (arrow) was also observed in the 20 mg/kg scutellarin-treated group. Bar, 50 μm.

Our results showed that the growth of SAS xenografts was significantly affected by scutellarin treatment. After treatment for 3 weeks, the average tumor size in the 5, 10, and 20 mg/kg scutellarin-treated groups was 1085, 550, and 385 mm^3, respectively; however, that in the normal saline-treated group was 1223 mm^3 (Fig. 1B). The rate of inhibition by scutellarin treatment, as assessed by tumor volume, was determined to be 11.3, 55.0, and 68.5% in the 5, 10, and 20 mg/kg scutellarin-treated groups, respectively.

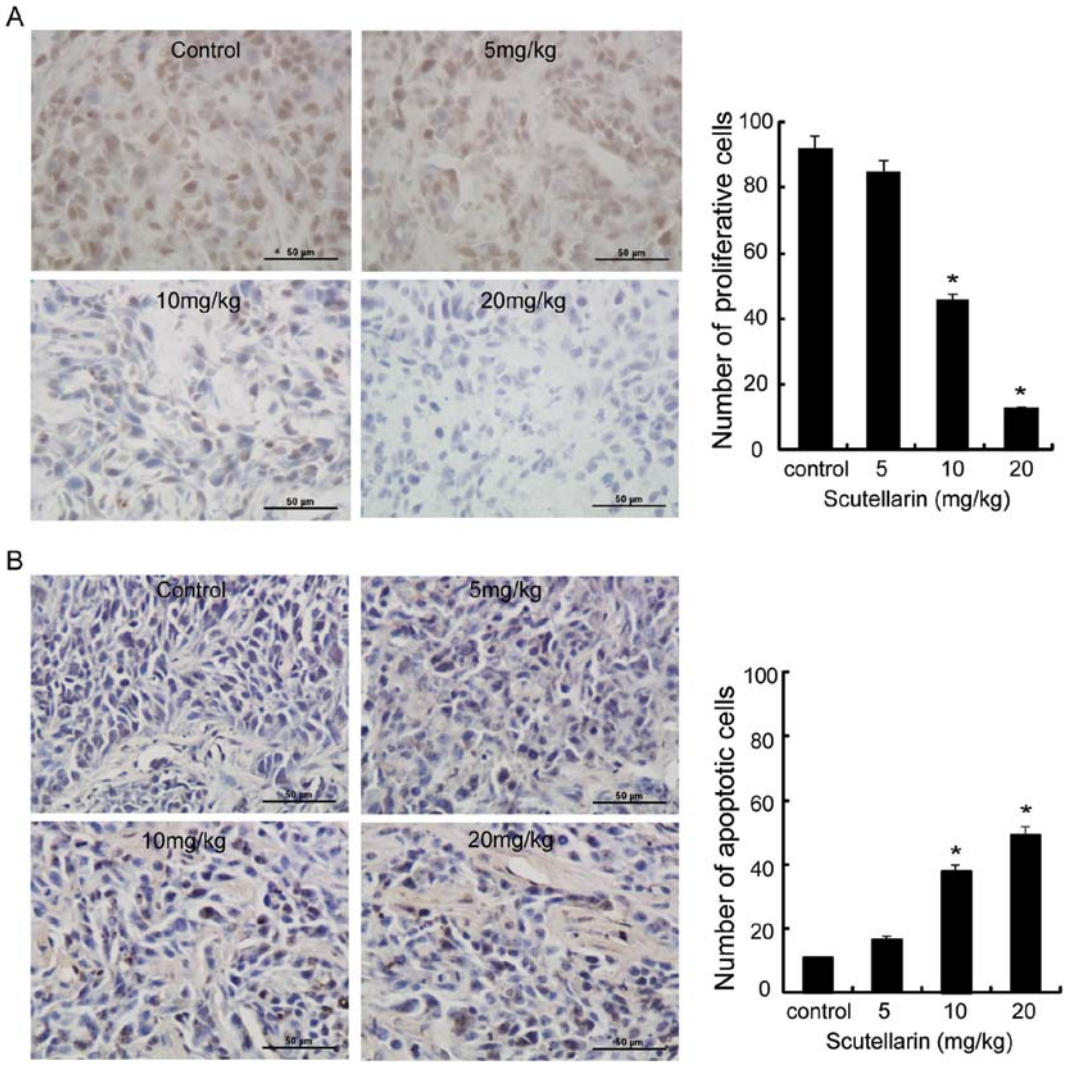

Figure 2. Histological analysis of proliferation and apoptosis of SAS tumors grown in nude mice for 3 weeks. A, PCNA expression and B, TUNEL staining in tumors from different treatment groups. Numbers represent mean ± SEM values. *P<0.01 vs. control group. Bar, 50 μm.

Histologically, moderately differentiated SAS cells were noted in tumors from the normal saline-treated group and in tumor remnants of the scutellarin-treated groups (Fig. 1C). Tumor cells were sparsely distributed in the 10 mg/kg treatment group, displaying degenerative changes with vacuoles within the cytoplasm, while the visible cell mass was surrounded by a large amount of extracellular matrix. Furthermore, tumor necrosis was observed in the 20 mg/kg scutellarin-treated group.

Effects of scutellarin on cell proliferation and apoptosis in vivo. Histological sections of SAS tumors grown in nude mice for 3 weeks were analyzed for proliferation and apoptosis. Immunohistochemical analysis of cell proliferation (PCNA) showed no difference between the tumors from control and those from the 5 mg/kg scutellarin-treated mice; however, those from the 10 and 20 mg/kg scutellarin-treated mice demonstrated reduced proliferative ability of the transplanted tumor tissue; moreover, the positive cell number and intensity of PCNA expression was reduced with increasing drug concentration (Fig. 2A).

Fig. 2B shows that scutellarin significantly induced apoptosis. Statistical analysis of the cell proliferation index demonstrated that the cell proliferation index was significantly lower with increasing drug concentration (Fig. 2A; P<0.01); the proliferation index in the 10 mg/kg-treatment group was only half of that of the control group. Furthermore, the apoptotic index increased with the concentration of the drug used in treatment (Fig. 2B; P<0.01); in particular, the apoptotic index of the 10 mg/kg-treated group was 4-fold higher than that of the control group, indicating a direct killing effect of scutellarin on transplanted tumors.

In addition, we observed the ultrastructural changes of transplanted tumor tissue after scutellarin treatment using TEM. The results revealed tumor cell membrane integrity, normal cytoplasm, and organelles without enlargement, large nuclei, and uniform chromatin distribution in the control group; in addition inactive fibroblasts, and fewer fibers with a sparse distribution was seen in this group (Fig. 3A). This was compared to the transplanted tumor tissue in the scutellarin-treated group. In the latter group, cell membranes demonstrated integrity, but the nuclei

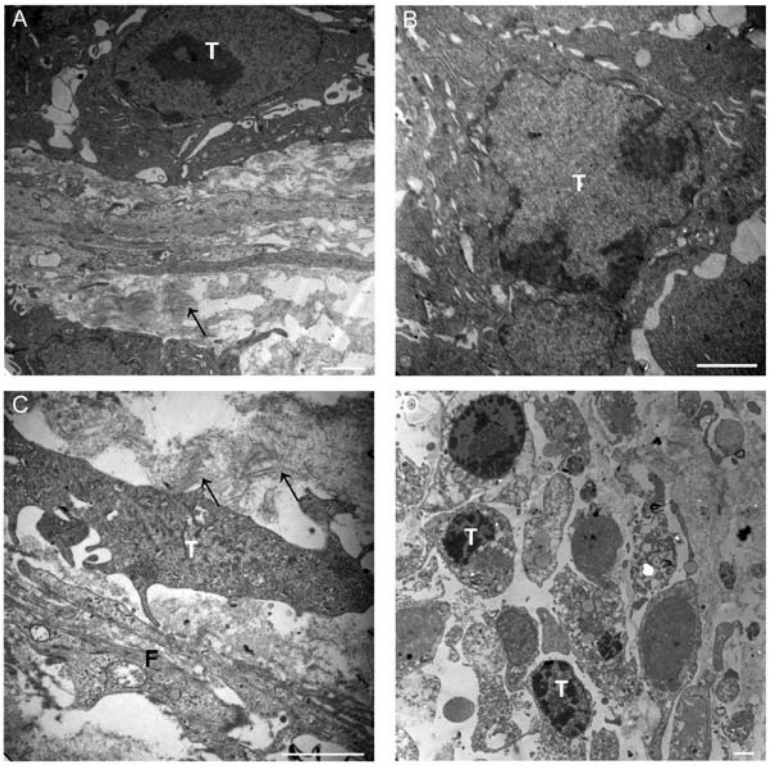

Figure 3. Transmission microscopy of ultrastructural changes in SAS cell xenograft tissue. A, Tumor cells (T) and fibers (arrow) in control. B, Cell nucleus showing aggregation; mitochondria and endoplasmic reticulum demonstrate mild swelling in the 5 mg/kg scutellarin-treated group. C, Fibroblasts (F) and fibers (arrow) surrounding tumor cells (T) in the 10 mg/kg scutellarin-treated group. D, Tumor cells necrosis in the 20 mg/kg scutellarin-treated group. Bar, 2 μm.

showed aggregation, mitochondria and endoplasmic reticulum demonstrated mild swelling in the 5 mg/kg scutellarin-treated group (Fig. 3B). Moreover, apoptosis was significant, and the numbers of fibers were increased and surrounded the tumor cells in the 10 mg/kg scutellarin-treated group (Fig. 3C). We also observed that many cell nuclei showed more aggregation, and tumor cell apoptosis and necrotic cells were frequently observed in the 20 mg/kg scutellarin-treated group (Fig. 3D); however, fibers were not significantly increased compared with control group.

Effects of scutellarin on collagen matrix. We observed a significant change in interstitial collagen fibers in transplanted tumor tissue between control and treated groups using TEM, as described above. To further observe the change in collagen fibers, we assessed the collagen matrix using Masson's trichrome staining. We found that the collagen fibers were sparsely dispersed in tumor cells in the control and 5 mg/kg scutellarin-treated group (Fig. 4A and B); in comparison, increased collagen fibers, and bundles of fibers that separates the tumor cells were seen in the 10 mg/kg scutellarin-treated group (Fig. 4C), while the tumor was divided into many small sections by collagen fibers in the 20 mg/kg scutellarin-treated group (Fig. 4D).

Scutellarin suppresses molecular events driving invasion and adhesion in xenografts. Furthermore, we studied the effect of scutellarin on tumor invasion-related factors. As MMPs degrade the tissue matrix and facilitate tumor invasion, we first analyzed their levels. We found that MMP-2 and -9 levels were decreased in tumors by scutellarin treatments, specifically at higher doses (Fig. 5). Immunohistochemistry revealed that the expression level of integrin αvβ6 was significantly decreased with an increase in the concentration of scutellarin (Fig. 5).

Effects of scutellarin on the expression of MMP-2, -9, integrin αvβ6, and c-JUN in SAS cells. We next evaluated the mechanism underlying the anti-tumor effect of scutellarin *in vitro* by measuring expression of the related factors; in particular, we investigated factors associated with cell adhesion and invasion. We examine the effect of scutellarin on the expression of MMP-2, -9, integrin αvβ6, and c-JUN in SAS cells treated with different concentrations of scutellarin (0, 3, 15, 75 nM). Western blot analysis showed that scutellarin reduced the expression of MMP-2, -9, integrin αvβ6, and c-JUN in a concentration-dependent manner (Fig. 6A).

RT-PCR was used to evaluate the expression levels of the relevant mRNAs after scutellarin treatment of SAS cells. As shown in Fig. 6B, we found that, compared to controls, mRNA levels corresponding to MMP-2, -9, and integrin αvβ6 decreased in cells treated with scutellarin in a dose-dependent manner. This demonstrated that scutellarin can directly modulate the expression of these factors.

Discussion

Scutellarin, also known as scutellarin 7-O-β-D-glucuronide, is a known flavone glycoside. Studies have shown that scutellarin has a variety of biological effects in numerous mammalian

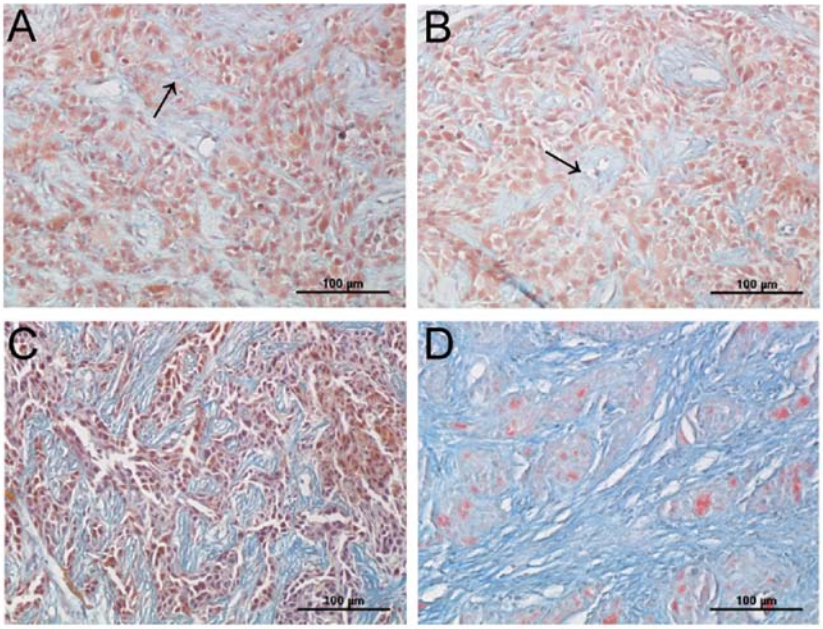

Figure 4. Changes in collagen fiber in different treatment groups with Masson's trichrome staining. A, Control; B, 5 mg/kg; C, 10 mg/kg; D, 20 mg/kg. Arrow, collagen fiber. Bar, 100 μm.

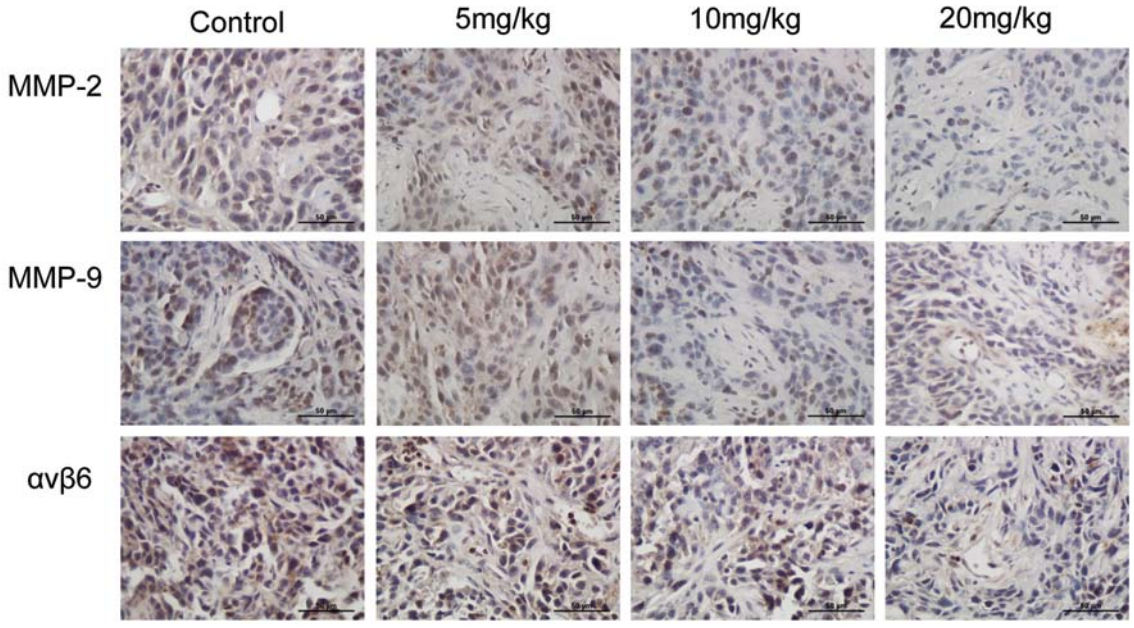

Figure 5. Immunohistochemistry staining of MMP-2, -9 and αvβ6 in xenografts tissue. Expression levels of MMP-2, -9, and integrin αvβ6 were lower in scutellarin-treated groups than in the control. Bar, 50 μm.

systems (24,25). Recently, studies reported that scutellarin inhibited the growth of the human breast cancer cell line MCF-7 (26). Scutellarin can also inhibit cell proliferation and induce cell apoptosis in cells of the human Burkitt lymphoma Namalwa cell line (27). Scutellarin promoted the apoptosis of cobalt chloride-mediated human prostate cancer cell line PC12 (28); in addition, scutellarin sensitized 5-fluorouracil (5-FU)-induced apoptosis of colon cancer cells (29). Previous studies by our research group have shown that scutellarin inhibits the growth of tongue cancer cells *in vitro* and has the ability to regulate cell adhesion (22). However, the effect of scutellarin on OSCC cells had not previously been fully examined. In this study, we found that scutellarin efficiently inhibited OSCC xenograft tumors *in vivo* in a dose-dependent manner.

We applied 3 different concentrations of scutellarin (5, 10, and 20 mg/kg) to treat SAS tumor-bearing mice, and found that the growth inhibition was weak in the 5 mg/kg-treated group, with an inhibition rate of only 11.3%. Inhibition of xenograft tumor increased significantly with an increase in dose, up to 68.5% in the 20 mg/kg-treatment group; however, we observed tumor necrosis under both light and electron microscopy in 20 mg/kg group. This shows that scutellarin can

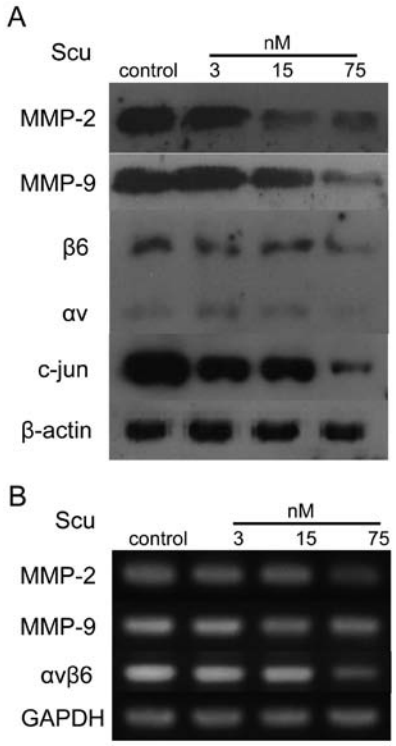

Figure 6. Scutellarin regulates the expression of proteins and the production of mRNA associated with cell adhesion and invasion in SAS cells. A, After incubation with scutellarin for 24 h, total cell lysates were prepared and subjected to SDS-PAGE, followed by western blotting. The membranes were probed with specific primary antibodies against MMP-2, -9, integrin αvβ6, c-JUN, and β-actin, followed by treatment with the appropriate peroxidase-conjugated secondary antibodies. B, mRNA expression related to genes encoding MMP-2, -9, integrin αvβ6, and GAPDH was examined by RT-PCR. β-actin and GAPDH were used as internal controls for western blot analysis and RT-PCR, respectively. Scu, scutellarin.

dose-dependently inhibit tumor growth, but that excessive drug concentrations may directly cause tumor cell necrosis, resulting in some side-effects. We believe that *in vivo* administration of a 10 mg/kg dose is the ideal concentration: 10 mg/kg scutellarin significantly inhibited tumor growth, while the animals did not undergo significantly abnormal deaths; deaths that occurred were mainly procedurally-related deaths.

In order to observe the malignant state of cells in the transplanted tumor tissue after scutellarin treatment, we evaluated the proliferation and apoptosis of tumor cells. Our results showed that with 10 mg/kg scutellarin-treatment, the proliferation index was 50% of that of the control group, while the apoptosis index was 3 times that of the control group. These results confirmed that scutellarin inhibited SAS xenograft tumor cell proliferation and induced apoptosis *in vivo*. Moreover, we also observed scutellarin-medicated induction of tumor cell apoptosis in a dose-dependent manner under TEM. Therefore, scutellarin inhibited tumor cell proliferation and induced apoptosis; these effects may play an important role in tumor growth arrest.

Integrin αvβ6 is undetectable in normal oral tissues but is upregulated in carcinomas of the colon, cholangiocarcinoma, and ovarian, breast, endometrial, gastric, cervical, and oral squamous cell carcinoma (10). Expression of integrin αvβ6 promotes migration and invasion in SCC (11,30). Therefore inhibition of integrin αvβ6 expression may reduce cell invasion. Our results show that scutellarin reduces integrin αvβ6 expression in tumor cells, which is consistent with our *in vitro* results (22). Therefore, we speculate that scutellarin reduces the connection of the tumor cells with the extracellular matrix by inhibiting integrin αvβ6, thereby regulating the different signal transduction pathways that mediate tumor invasion and metastasis (8). This phenomenon is worthy of further study, and indicated that the effect of scutellarin is not confined to the induction of differentiation and apoptosis, and that it has anti-metastatic potential.

Invasion via degradation of the ECM and BM is one of the critical steps in the cascade of metastasis (31). Gelatinase (MMP-2 and -9) is the key regulatory enzyme in the degradation of the ECM and BM (4,32). In a variety of malignant tumors, the expression of MMP-2 and -9 is higher than in normal tissue (33). Some studies have shown that the expression of MMP-2 and -9 is increased in OSCC tissues (5); high expression of MMP-2 and -9 is closely related to the invasion and metastasis of OSCC (6,34). Therefore, reduction in expression of these MMPs can theoretically be used to treat this disease. We observed the expression of MMP-2 and -9 in scutellarin-treated transplanted tumor tissue by immunohistochemistry. The results showed that scutellarin significantly reduced expression of MMP-2 and -9. This indicated that scutellarin directly inhibited tumor invasion by reducing gelatinase activity.

To infiltrate into the surrounding tissue, it is necessary for tumor cells to puncture the surrounding matrix fibers. Collagen fiber is an important fiber component in the ECM. Collagen fiber content increased in scutellarin-treated transplanted tumor tissue; the collagen fibers showed great morphological changes: scutellarin promoted envelopment and attack of the tumor by collagen fibers. Thus, the content and distribution of collagen fibers affected tumor cell invasion. Increases in collagen content and reduced expression of MMPs are coherent results. This suggests that scutellarin may be involved in regulating collagen synthesis and distribution in the tumor microenvironment.

By using different doses of scutellarin for treatment of nude mice with SAS-transplanted tumors, we observed that scutellarin inhibited cell proliferation and induced apoptosis, and inhibited tumor invasion and metastasis by regulating the expression of adhesion molecules and MMPs. To further explore the mechanism of the anti-tumor effect of scutellarin, we assessed the expression of related molecules *in vitro*. The results showed that scutellarin reduced expression of MMP-2, -9, integrin αvβ6 at protein and mRNA levels in a dose-dependent manner.

Some studies have shown that the expression of MMP-2, -9, and integrin αvβ6 are regulated by AP-1 (20,35,36). Multiple members of the MMP family have promoter regions containing 1 or more AP-1 binding sites; AP-1 is a key factor regulating high expression of the relevant genes (37). The AP-1 signaling pathway is involved in tumor cells with high expression of MMP-2 and -9 (36). Moreover, there are AP-1 *cis*-regulatory elements in a the genes encoding a variety of members of the integrins; the AP-1 signaling pathway is closely related to the abnormal expression of these integrin molecules (38). Therefore, we detected the impact of scutellarin on the expression of c-JUN, and found that scutellarin inhibited the expression of c-JUN. This indicated that scutellarin may regulate the activity of c-JUN, and thereby the expression of the downstream genes, thus exerting an anti-tumor effect; however, the exact anti-tumor mechanisms require further research.

Our previous studies have shown that scutellarin does not exhibit any significant toxicity on human umbilical vein endothelial cells (39). In the xenograft model, we used the maximum dose of 20 mg/kg every other day. In our experiment, neither weight-loss nor poor appetite was noted in animals in the scutellarin-treated group. This showed that a low concentration of scutellarin may have no effect on normal cells and may target tumor cells (22,39).

In conclusion, our study showed that scutellarin inhibited xenograft tumor growth in nude mice and down-regulated expression of MMP-2, -9, and integrin αvβ6 in SAS cells. These data suggest the suitability of scutellarin as an adjuvant anti-invasive treatment for oral SCCs.

Acknowledgements

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