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Anti-invasion and anti-tumor growth effect of doxycycline treatment for human oral squamous-cell carcinoma – *In vitro* and *in vivo* studies

Ling-Chang Shen [a], Yuk-Kwan Chen [b,c,*], Li-Min Lin [b,c], Shyh-Yu Shaw [a,*]

[a] *Institute of Biotechnology, National Cheng Kung University, Tainan, Taiwan*
[b] *Department of Oral Pathology, Faculty of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[c] *Division of Dentistry, Department of Oral Pathology, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan*

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SUMMARY

Regional lymph node and distant organ metastasis of oral squamous-cell carcinoma (OSCC) has been associated with increased production of matrix metalloproteases (MMPs), and scientific data showed that doxycycline (Dox) could down-regulate the expression of MMPs. The objective of this study was to evaluate the effect of Dox on the expression of MMPs *in vitro* using the SCC-15 cell line and *in vivo* SCC-15 xenografted nude mice. SCC-15 cells maintained under distinct culture conditions expressed high levels of pro-MMP-2 and pro-MMP-9; however, as determined by zymography and Western blot analysis, Dox significantly reduced the production of pro-MMP-2 and pro-MMP-9 after 24 h of treatment in a dose-dependent manner (2.5–40 μg/ml). Dox (10 μg/ml) decreased the expression of MMP-9 mRNA but did not alter the level of MMP-2 mRNA after 24 h of treatment. In addition, this drug significantly inhibited the invasive and migration activities of SCC-15 cells *in vitro* (>75% inhibition at 10 μg/ml). On the other hand, daily administration of Dox (3 mg/mice) restrained tumor growth in SCC-15 xenografted nude mice, with an inhibition rate of 85.6%. Compared with the control group (treated with normal saline), MMP-9 mRNA levels in the fresh tumor tissue decreased upon Dox treatment ($P < 0.01$) while MMP-2 mRNA levels were unchanged. In conclusion, reduced expression of MMP-9 at the transcriptional level and MMP-2 at the post-transcriptional level caused by Dox was found to be associated with decreased invasion of oral SCC *in vitro*. Moreover, Dox exerted a significant suppressive effect on tumor growth in an *in vivo* nude mice model. Taken together, these results, to our knowledge, may first imply that Doxycycline has an adjuvant therapeutic effect on OSCC that is associated with inhibition of MMPs expression.

Introduction

Survival of oral squamous-cell carcinoma (OSCC) patients has not improved significantly despite recent advances in treatments.[1] One of the major prognostic factors for OSCC patients is regional lymph node metastases.[2] Cascade of tumor cell invasion and metastasis involves alterations in cell–cell and extracellular-cell matrix (ECM) adhesion capacity.[3] Matrix metalloproteases (MMPs) degrade ECM and basement membrane, which are critical steps for invasion and metastasis.[4] Elevated production of gelatinases (MMP-2 and MMP-9) has been observed in pulmonary adenocarcinoma,[5] ovarian carcimoma,[6] and OSCC.[7,8] Therefore, inhibition of gelatinases expression by agents may be a potential way of enhancing the prognosis of OSCC patients.

Doxycycline hyclate (Dox) is a semi-synthetic tetracycline. Besides its antibiotic activity, it inhibits MMP synthesis, and is the only MMP inhibitor approved by Food and Drug Association for dental applications.[9] Besides treatment for periodontitis,[10] Dox reduced proliferation, bone metastasis and gelatinolytic activity of MMP-2 and MMP-9 in breast cancer.[11,12] Moreover, Dox inhibited tumor cell proliferation, invasion, and metastasis in prostate cancer.[13,14] Rubins et al.[15] reported malignant mesothelioma proliferation could be inhibited by Dox, and Onoda et al.[16] found that Dox inhibited invasion of colorectal cell line. Hence, accumulated data revealed that Dox is a well-accepted MMP inhibitor, which chiefly inhibits MMP-2 and MMP-9.[10–16] However, mechanism of anti-invasive effect of Dox on MMPs in OSCCs remains to be elucidated.[17]

We used an *in vitro* model of a human tongue SCC cell line to investigate the inhibitory action of Dox on the expression of MMP-2 and MMP-9. Anti-invasive and anti-migration effects were tested under such condition. *In vivo* anti-tumor effect of Dox was also investigated in a xenograft nude mice model.

* Corresponding authors. Addresses: Department of Oral Pathology, Faculty of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan. Tel.: +886 6 2757575x65600; fax: +886 6 2766505 (Y.-K. Chen), Institute if Biotechnology, National Cheng Kung University, No. 1, University Raod, 701, Tainan, Taiwan (S.-Y. Shaw).
E-mail addresses: k0285@ms22.hinet.net (Y.-K. Chen), syshaw@mail.ncku.edu.tw (S.-Y. Shaw).

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Table 1
Oligoprimers for real-time quantitative reverse transcriptase polymerase chain reaction.

	Sense	Antisense
MMP-2	5′-CCGTGCCTTCAGCTCTACA-3′	5′-CGCCTGGGAGGAGTACAG-3′
MMP-9	5′-GCTTGCCCTGGTGCAGTAC-3′	5′-ATTGCCGTCCTGGGTGTAG-3′
GAPDH	5′-TGCACCACCAACTGCTTAGC-3′	5′-GGCATGGACTGTGGTCATGAG-3′

Materials and methods

Cell line and culture

Human lingual SCC cell line (SCC-15) was cultured as previously described.[18]

Drug treatment

Dox–HCl powder (purity 99.99%) (#D9891; Sigma) was dissolved in double-deionized water to make a 50 mg/ml stock solution. A concentration of 20 mg/ml was used in the xenograft nude mice model.

MTS assay[19]

SCC-15 cells were seeded into 96-well plate and cultured for 2-day, following which culture medium was replaced with serum-free medium containing various dosages of Dox. After further culturing for 24 h, MTS reagent (G3582; Promega, Madison, WI, US) was added to each well followed by incubation at 37 °C for 1 h. Cell viabilities were determined by measuring absorbance at 490 nm with a spectrophotometer (Multiskan RC, Labsystems, Finland).

Gelatin zymography[20]

A 15 μl of condition medium was treated with SDS–PAGE sample buffer with neither a reducing agent nor boiling, and samples were fractionated in 8% polyacrylamide gel containing 1 mg/ml of gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 and incubated in an activation buffer (10 mM Tris–HCl, pH 7.4, 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 1.25% Triton X-100). Then

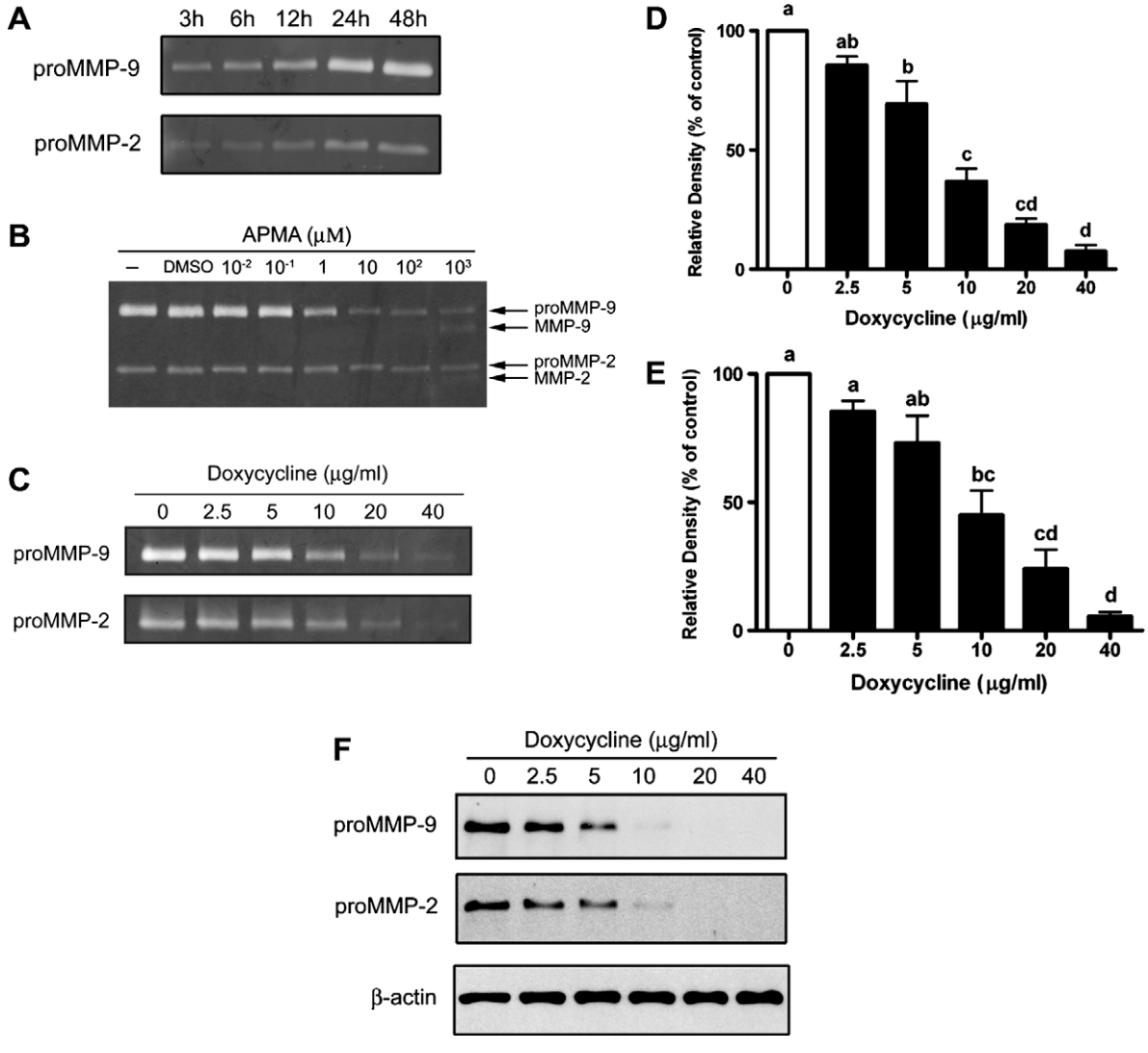

Figure 1 Gelatine zymography of gelatinase secreted by SCC-15 cells. (A) Gelatinolytic bands and molecular weight positions can be clearly observed for pro-MMP-2 (72-kD) and pro-MMP-9 (92-kD) after various periods (3–48 h) of incubation. (B) APMA-dependent reduction of gelatinase activity in conditioned media after incubation for 30 min. Effect of Dox on pro-MMP-2 and pro-MMP-9 production by cultured SCC-15 cells. Cells were treated with Dox at 0–40 μg/ml for 24 h, following which culture media samples were collected and subjected to gelatin zymography (C) and Western blot analysis (F). Bar graphs (D and E) show the dose–response effect of Dox on pro-MMP-2 and pro-MMP-9 production. The expression level of -actin protein in equal amounts of total cell lysate was measured at the same time as that of the internal control. Means and standard errors were obtained from three independent experiments. Different letters represent significant differences between groups by Tukey's analysis of $P < 0.05$.