Phytomedicine 33 (2017) 69–76

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

Original Article

Gramine inhibits angiogenesis and induces apoptosis via modulation of TGF-β signalling in 7,12 dimethylbenz[a]anthracene (DMBA) induced hamster buccal pouch carcinoma

Arunkumar Ramu[a], Suresh Kathiresan[a,*], Bakrudeen Ali Ahmed[b,c]

[a] Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalai Nagar 608002, Tamil Nadu, India
[b] Faculty of Applied Science, Ton Duc Thang University, Ho Chi Minh, Vietnam
[c] University of Malaya, Institute of Biological Sciences, Faculty of Science, Kuala Lumpur, Malaysia

ARTICLE INFO

Keywords:
Oral cancer
Gramine
TGF-β
Angiogenesis
Apoptosis

ABSTRACT

Background: Transforming growth factor-β (TGF-β) and its receptors are considered as a novel target in cancer chemotherapy. Gramine, an indole alkaloid, possesses various pharmacological properties including anti-proliferative and anticancer. However, the anti-angiogenic property remains unexplored.
Purpose: The present study was designed to evaluate the anti-angiogenic and apoptosis induction properties of gramine through inhibiting TGF-β on DMBA induced oral squamous cell carcinoma (OSCC) in the hamster buccal pouch (HBP).
Methods: The effects of gramine on TGF-β signalling in DMBA induced carcinogenic events such as angiogenesis and apoptosis were analysed by studying the mRNA expression using RT-PCR, protein expression by western blot and histopathological analysis using haematoxylin and eosin (H & E) staining.
Results: Gramine significantly inhibited phosphorylation and nuclear translocation of Smad2 and Smad4 by blocking activity of the TGFβ-RII, RI and activation of inhibitory Smad7. Gramine inhibited angiogenic markers such as MMP-2, MMP-9, HIF-1α, VEGF, and VEGF-R2 as well as increased TIMP-2 expression. Furthermore, gramine induced apoptosis in DMBA induced tumour bearing animals by up regulating the pro apoptotic proteins Bax, cytochrome *C*, apaf-1, caspase-9 caspase-3 and PARP.
Conclusion: In this study, we clearly demonstrated that gramine treatment diminishes angiogenesis and induces apoptosis in hamster buccal pouch (HBP) carcinogenesis by modulating TGF-β signals.

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer. Approximately 300,000 new patients are diagnosed with OSCC and 145,000 deaths occur annually worldwide (Torre et al., 2015; Ferlay et al., 2015). Alcohol and tobacco consumption, unhealthy diets, and viral infections have been identified as risk factors for OSCC (Ram et al., 2011). Although multifarious medicines are used for OSCC treatment, the survival rate remains very low because cancer cells generate surplus blood vessels from the existing vasculature and metastasize to distant organs (Jung et al., 2015; Noguti et al., 2012). Researchers have attributed these hallmark abilities of cancer cells to the dysregulation of multiple intracellular signalling pathways, and among them, abnormal TGFβ/Smad functions have been identified as one of the key signal (Neuzillet et al., 2015).

The activated TGF-β binds to serine/threonine kinase receptors that phosphorylate Smad2 which forms heterodimeric complexes with common Smad4; thus Smad2/4 translocates into the nucleus, resulting activation of several genes involved in various stages of cancer development (Randall et al., 2004). Subsequently, Smad7, an intracellular receptor antagonist of TGF-β, prevents the phosphorylation and activation of Smad2 (Jin et al., 2015). However, over expression of TGF-β stimulates proliferation, invasion, and angiogenesis, thereby promoting tumour progression (Ikushima and Miyazono, 2010). Recent studies have indicated that deregulated of TGF-β and Smads functions are associated with the malignant progression of OSCC (Jensen et al., 2015;

Abbreviations: OSCC, oral squamous cell carcinoma; HBP, hamster buccal pouch; DMBA, 7,12-dimethylbenz[*a*]anthracene; TGF-β, transforming growth factor- β; HIF-1α, hypoxia-inducible factor-1 α; VEGF, vascular endothelial growth factor; VEGF-R2, vegf receptor-2; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; TIMP-2, tissue inhibitors of metalloproteinases-2; Bcl-2, B-cell lymphoma 2; Bax, Bcl2-associated X protein; Apaf-1, apoptotic protease activating factor-1; Caspase-9, cysteine-aspartic protease-9; Caspase-3, cysteine-aspartic protease-3; PARP, poly (ADP-ribose) polymerase

* Corresponding author.
E-mail address: suraj_cks@yahoo.co.in (S. Kathiresan).

http://dx.doi.org/10.1016/j.phymed.2017.05.008
Received 8 July 2016; Received in revised form 21 April 2017; Accepted 12 May 2017

Table 1
Incidence of oral neoplasm and histological changes in the buccal mucosa of control and experimental animals in each group (n = 10). Tumour volume was measured using the formula $v = 4/3\ \pi\ [D_1/2]\ [D_2/2]\ [D_3/2]$ where D_1, D_2, and D_3 are the three diameters (mm) of the tumour. Tumour burden was calculated by multiplying tumour volume and the number of tumour/animals. () indicates total number of animals bearing tumours.

Parameters/Groups	Control	DMBA (0.5%)	DMBA + Gramine	DMBA + Taxol	Gramine alone
Tumour incidence	0	100%	11.5%	9.8%	0
Total number of tumours/animals	0	37(10)	2(2)	2(2)	0
Tumour volume (mm^3)/animals	0	374.68 ± 36.11	26.76 ± 2.54	24.62 ± 2.19	0
Tumour burden (mm^3)/animals	0	1338.37 ± 129.12	56.41 ± 5.17	53.38 ± 4.24	0
Hyperkeratosis	Not observed	Severe	Moderate	Moderate	Not observe
Hyperplasia	Not observed	Severe	Moderate	Moderate	Not observed
Dysplasia	Not observed	Severe	Mild	Moderate	Not observed
Squamous cell carcinoma	Not observed	Well differentiated	—	—	Not observed

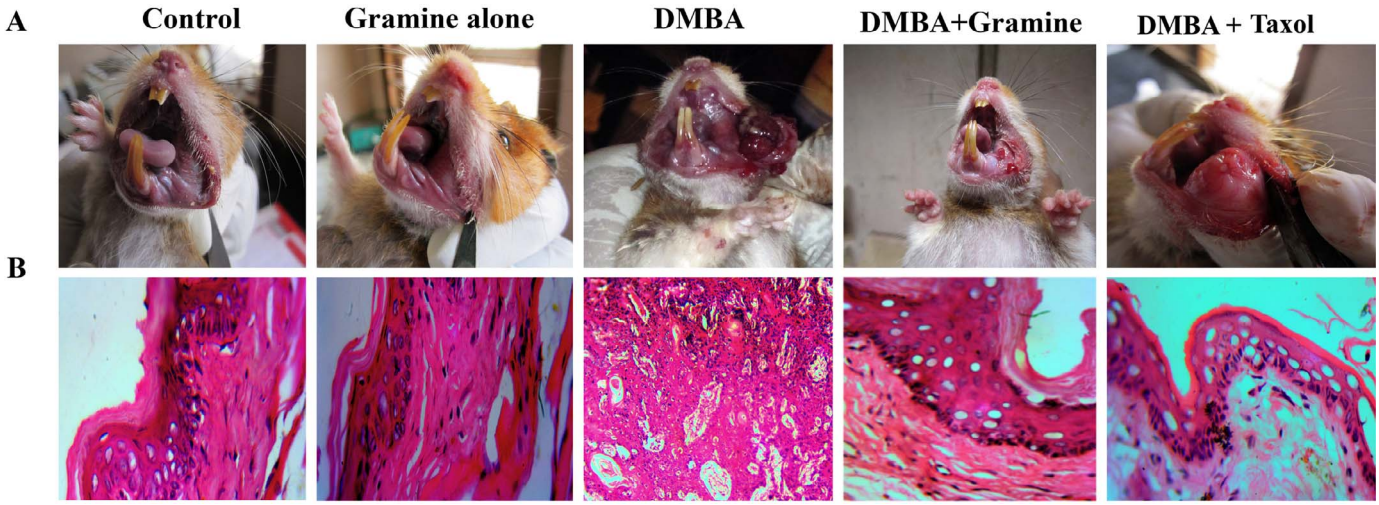

Fig 1. (A) Gross appearance of buccal pouch mucosa of control and experimental animals. Well defined tumour mass present in hamster buccal pouch painted with DMBA. Tumour mass was decreased in DMBA induced cancer animals treated with gramine or taxol. No significant abnormalities were noted in control and gramine alone animals. (B) H&E stained regions of buccal pouch epithelium of control and experimental animals (100X). Buccal pouch epithelium from DMBA group exhibiting well differentiated SCC. Buccal pouch epithelium of DMBA and gramine or taxol administrated exhibiting dysplasia. Control and gramine alone animals exhibiting normal buccal pouch.

Sivadas et al, 2014). Therefore, an agent that effectively blocks TGF-β signals for suppressing angiogenesis and triggering apoptotic genes to induce cell death in cancer cells may exhibit anti-tumour progression potential in OSCC.

The scientific communities consider phytochemicals from medicinal and dietary plants attractive options for the development of anticancer agents (Ali et al., 2012). Previous studies have revealed that young leaves of barley (*Hordeum vulgare*) and silver maple (*Acer saccharinum*) suppress cell growth and promote apoptosis in various carcinomas (Lahouar et al., 2015; Gonzalez-Sarrias et al., 2012). Gramine, one of the main indole alkaloid present in barley and silver maple, has various biological and pharmacological effects, such as antioxidant, vasorelaxant, and anti-cancer activities (Iwata et al., 2001; Hong et al., 2009; Kumar and Suresh, 2014; Argandona et al., 1987). However, the molecular mechanism of action of gramine on OSCC remains unclear. In this study, we investigated the effect of gramine on angiogenesis and apoptosis through the modulation of TGF-β signalling in DMBA-induced HBP carcinogenesis.

Materials and methods

Chemicals

Gramine (99%), DMBA and goat anti-rabbit IgG-HRP polyclonal antibody was purchased from, Sigma chemical Co., St. Louis, MO and Cayman Chemical. TriZol reagent was from GeNei, Bangalore, India. cDNA conversion kits were from Takara. Taxol (99%) and antibodies for TGFβ-RII, TGFβ-RI, Smad-2, pSmad-2, Smad-4, Smad7, HIF-1α, VEGF, VEGF-R2, MMP-2, MMP-9, TIMP, Bcl-2, Bax, cytochrome *C*, apaf-1, caspase-3, caspase-9 and PARP were obtained from Santa Cruz Biotechnology, USA and Cell Signaling Technology, USA.

Animals

Male golden Syrian hamsters aged 8–10 weeks weighing between 100 and 110 g were purchased from the Central Animal House, Annamalai University, Tamil Nadu, India. Animals were housed in well-ventilated room (temperature 23 ± 2 °C, humidity 65–70% and 12 h light/dark cycle) conditions for 16 weeks. All experimental studies were carried out in accordance with the Indian National Law of Animal Care and Use (Reg No./160/1999/CPCSEA).

Treatment schedule

Experimental animals were divided into 5 groups of 10 each. In group 1, the left buccal pouch of hamsters were painted with liquid paraffin alone and served as vehicle control. Group 2 received gramine only and served as untreated control. Groups 3, 4 and 5 animals were painted with 0.5% DMBA in liquid paraffin three times per week for 16 weeks at the left buccal pouch (Shklar, 1999). Groups 4 and 5 animals