



Modulating effect of cromolyn on Akt, MAPK, ERK and DNMT expression pattern in 7,12-dimethylbenz (a) anthracene induced hamster buccal pouch carcinogenesis

Shanmugam Manoharan*, Sekar Karthikeyan, Asokan Manimaran, Raju Kowsalya, Krishnamurthy Vasudevan
Annamalai University, Annamalai Nagar, Tamil Nadu, India

ABSTRACT

The present study explores the modulating efficacy of cromolyn on the expression pattern of a serine/threonine-specific protein kinase (Akt), mitogen activated protein kinase (MAPK), extracellular signal regulated kinase (ERK) and DNA methyl transferase (DNMT) in 7, 12-dimethylbenz (a) anthracene (DMBA) induced hamster buccal pouch carcinogenesis. Oral tumors were developed in the hamster buccal pouches of golden Syrian hamsters using topical application of 0.5% DMBA in liquid paraffin (three times a week for 14 weeks). Though cromolyn completely inhibited the formation of DMBA induced tumors in the buccal pouches of hamsters, precancerous lesions such as hyperplasia, hyperkeratosis and dysplasia were evident. Cromolyn also modulated the expression pattern of Akt, MAPK, ERK and DNMT towards tumor inhibition as evidenced by the absence of tumors in DMBA + cromolyn treated hamsters (Western blotting analysis). Thus, the antitumor potential of cromolyn might be partly due to its modulating effect on the expression pattern of above mentioned cancer related biomarkers.

Keywords: Akt; DNMT; DMBA; ERK; MAPK; Oral cancer

INTRODUCTION

Cancer of the oral cavity is one of the predominant form of cancers worldwide and arises mainly due to habits such as tobacco smoking and chewing, betel nut chewing and excessive alcohol consumption (Anand *et al.*, 2014). While cancer of the oral cavity constitutes about 3-5% of all neoplasms in Western countries, this form of cancer accounts for 40-50% of all malignant neoplasms in developing nations including India (Jemal *et al.*, 2010; Gupta *et al.*, 2013). Oral cancer, at an advanced stage, reduces the survival outcome as well as the lifestyle and quality of patients. Despite the easy physical examination, surprisingly, this form of cancers is diagnosed at an advanced stage of tumors. Early diagnosis of oral cancer with promising biochemical or molecular markers should definitely help to reduce the annual incidence of oral cancer worldwide.

Golden Syrian hamsters, the best suited animal model for oral cancer research, on exposure to repeated topical application of 7,12-dimethylbenz (a) anthracene in their buccal pouches resulted in the formation of malignant tumors (Manoharan *et al.*, 2006). DMBA induced oral tumors exhibit close similarities to the hu-

man oral tumors with the histological, morphological, biochemical and molecular aspects (Nagini 2009). DMBA induced hamster buccal pouch carcinogenesis is thus utilized to study the cromolyn efficacy on the expression pattern of a serine/threonine specific protein kinase (Akt), mitogen activated protein kinase (MAPK), extracellular signal regulated kinase (ERK) and DNA methyl transferase (DNMT).

Cromolyn possesses diverse pharmacological effects and is used as a safe medication for Asthma (Radley *et al.*, 2006). *In vitro* and *in vivo* studies explored its anti-diabetic, antioxidant, anti-inflammatory and anticancer potential (Motawi *et al.*, 2013; Ionov *et al.*, 1991). The apoptotic potential of cromolyn has also been pointed out in various cancer cell lines (Motawi *et al.*, 2014). Cromolyn significantly reduced gastric damage (ulcer) in rats (Beck *et al.*, 1989). However, there are no scientific experimental animal studies to validate its efficacy on cancer prevention. The present study is thus designed to explore the anticancer property of cromolyn by analyzing the expression pattern of Akt, MAPK, ERK and DNMT in 7, 12-dimethylbenz (a) anthracene induced hamster buccal pouch carcinogenesis.

MATERIALS AND METHODS

A total number of forty golden Syrian hamsters were procured from National Institute of Nutrition, Hyderabad and divided into four groups of ten hamsters in each. The animals were maintained in the Central Animal House of Annamalai University, according to the

* Corresponding Author

Email: sakshiman@rediffmail.com

Contact: +91-4144 239141. Fax: +914144238080

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guidelines of an Institutional animal ethics committee. The animals were categorized as follows.

Group I – Hamsters treated with liquid paraffin alone on their left buccal pouches (three times a week for 14 weeks).

Group II – Hamsters treated with 0.5% DMBA in liquid paraffin alone on their left buccal pouches (three times a week for 14 weeks).

Group III – Hamsters treated with DMBA (three times a week for 14 weeks) + cromolyn (80mg/kg body weight orally three times a week for 14 weeks on alternate days of DMBA treatment). Oral administration of cromolyn was followed according to the procedure of Leone-Bay *et al.*, (1996).

Group IV – Hamsters treated with cromolyn alone (80mg/kg body weight orally three times a week for 14 weeks)

Animals were sacrificed by cervical dislocation and the oral tissues (normal and tumor tissues) were excised and subjected to Western blotting analysis to assess the expression pattern of Akt, MAPK, ERK and DNMT.

Western blotting

Proteins were measured in the tissues that were excised from the control and experimental animals in each group. The proteins were then separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were transferred into PVDF membrane and probed with respective primary antibodies (Akt, P³⁸ MAPK, ERK and DNMT: Cell Signaling Technology, Danvers, MA, USA) by incubating at 4°C overnight. The membrane was then treated and incubated with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology, USA) for 2h. The protein bands were visualized by treating the blot with diaminobenzidine (DAB), the substrate of horseradish peroxidase. The bands were scanned and quantified densitometrically using Bio-Rad Image Lab™ software version 4.1.

Statistical Analysis

The data for molecular markers are expressed as mean ± SD (n=10). One way analysis of variance (ANOVA) followed by a Duncans multiple range test (DMRT) was used to analyze the statistical significance between two groups. The two different groups were considered statistically significant if the p values was less than or equal to 0.05 between them.

RESULTS

The expression pattern and densitometry analysis of p-Akt, p-ERK and p-P³⁸ MAPK are depicted in figures 1 and 2 respectively. Western blot analysis revealed over expression of p-Akt, p-ERK and p-P³⁸MAPK in the buccal mucosa of tumor bearing hamsters (hamsters treated with DMBA alone) as compared to control hamsters. Cromolyn treatment to DMBA treated hamsters pre-

vented the abnormal expression of the above mentioned biomarkers. The densitometry analysis for the expression pattern of p-Akt, p-ERK and p-P³⁸ MAPK was done after normalization to total Akt, ERK and P³⁸ MAPK respectively.

The expression pattern and densitometry analysis for DNMT1, DNMT3a and DNMT3b are depicted in figures 3 and 4 respectively. Western blot analysis revealed over expression of DNMT1, DNMT3a and DNMT3b in the buccal mucosa of tumor bearing hamsters as compared to control hamsters. Cromolyn treatment to DMBA treated hamsters prevented the abnormal expression of the above mentioned biomarkers. The densitometric analysis for the expression pattern of DNMT was done after normalization to β-actin.

DISCUSSION

A serine/threonine specific protein kinase (Akt) has a pivotal role in various cell signaling pathways and was found to be over-expressed in several carcinomas (Roy *et al.*, 2011; Chin *et al.*, 2009). In the present study, the mechanistic pathway for the antitumor potential of cromolyn was elucidated by analyzing the status of signal transduction pathways such as Akt, P³⁸ MAPK, ERK and DNMT during DMBA induced oral carcinogenesis. Cromolyn administration at a dose of 80mg/kg body weight to the DMBA treated hamsters (on alternate days of DMBA painting) prevented the tumor formation in the buccal pouches, which explores its potent chemopreventive potential during DMBA induced hamster buccal pouch carcinogenesis. Akt and MAPK signaling pathways play critical role in the regulation of genes that have a pivotal role in the cell growth, apoptosis, inflammation and invasion. The pathway of Akt has been associated with the cell survival and suppresses the apoptosis via phosphorylating the proapoptotic protein BAD. It has been pointed out that Akt inhibition stimulated ROS mediated apoptosis in the mitochondria (Han *et al.*, 2014). Activation of Akt resulted in the activation of transcription factors such as NFκB and inactivation of key apoptotic proteins such as p53 and Bax (Ozes *et al.*, 1999). Akt activation has been associated with prognostic significance of oral squamous cell carcinoma (Lim *et al.*, 2005). It has been reported that activation of Akt blocked the apoptotic events in various cancer cell lines (Gottlob *et al.*, 2001). Exposure of oral fibroblast to betel nut extracts resulted in Akt and NFκB activation (Lu *et al.*, 2008). The activity of Akt is regulated by its two phosphorylation sites located at threonine 308 and serine 473 (Kuo *et al.*, 2013). Akt phosphorylation could induce the molecular pathway that favors neoplastic transformation (Xu *et al.*, 2012). Over expression of p-Akt noticed in the buccal mucosa of hamsters treated with DMBA alone might have helped in the tumor progression.

Mitogen - activated protein kinase (MAPK) gene family perform a vital and critical role in cell signaling cascade by regulating the transcription of several genes in the

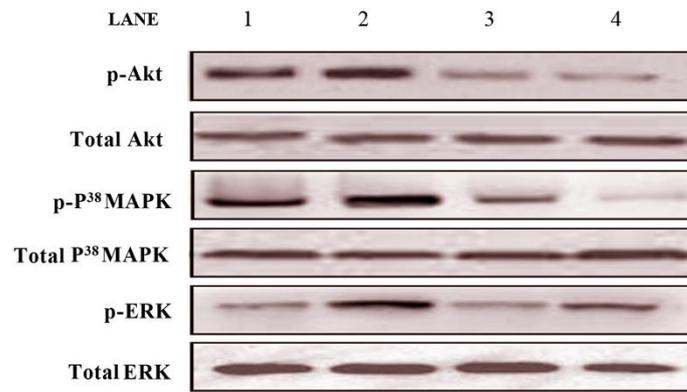


Figure 1: Expression pattern of p-Akt, Akt, p-P38 MAPK and p-ERK in the buccal pouch tissues of control and experimental animals

Lane 1: Control, Lane 2: DMBA alone, Lane 3: DMBA + Cromolyn, Lane 4: Cromolyn alone

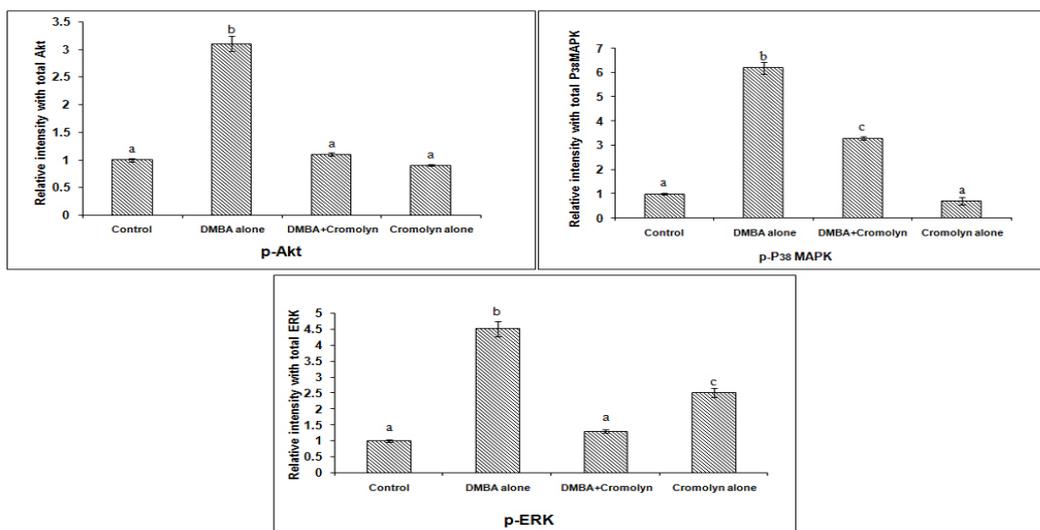


Figure 2: Densitometric analysis for p-Akt, p-P38 MAPK and p-ERK expression pattern

Data presented are the mean \pm SD (n=10). Common superscripts between two groups - not significant. Different superscripts between two groups – significant $p < 0.05$.

nucleus in response to cellular environmental changes. MAPK includes three protein kinases ERK, JNK, and P³⁸ protein kinase. MAPK, due to its diverse role in carcinogenesis, is considered as a major molecular target for cancer prevention. MAPK also plays a major role in the pro-inflammatory responses. This intracellular signaling cascade also regulates NF κ B activation (Weng et al., 2012). Up regulation of MAPK signaling cascade has been reported in skin inflammation (Kumar et al., 2015). It has been reported that activation of MAPK induced skin cancer via over expression of iNos and Cox-2 (Lee et al., 2014). MAPK pathway target proteins were up regulated in various cancers, including oral cancer (Wolfe et al., 2011).

Extracellular signal regulated kinase (ERK) pathway proteins play a vital role in the survival and proliferation of the cells. ERK signaling is one of the classical signaling pathways, which induce cell proliferation via phosphorylation of GSK-3 β and through c-myc mediated cell cycle signaling (Gkouveris et al., 2014). ERK and

P³⁸ MAPK were reported to have a crucial role in the migration of oral cancer cells (Wang et al., 2014). ERK pathway is involved in the apoptotic inhibition and induction of tumorigenesis (Brusevold et al., 2012). ERK activation is one of the characteristic feature observed in the apoptotic pathways (Roy et al., 2010). Activation of p-ERK, p-P³⁸ MAPK and p-Akt has been reported in oral cancer cells (Lu et al., 2012). Up regulation of p-ERK1/2 was reported in DMBA induced hamster buccal pouch carcinogenesis.

Kumar et al., (2012) reported over expression of MAPK pathway protein p-ERK and P³⁸ MAPK in hamster bearing oral carcinogenesis. P³⁸ MAPK is activated in response to cellular stresses and has a role in blocking abnormal cell proliferation or apoptotic induction. Lee-lahavanichkul et al., (2014) reported that P³⁸ MAPK inactivation inhibited the head and neck cancer cell growth both in vitro and in vivo. The activation of P³⁸ MAPK occurs in response to several stimuli including growth factors, cellular stress, and inflammatory cy-

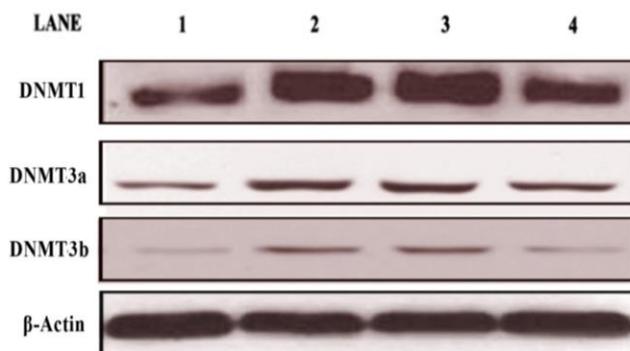


Figure 3: Expression pattern of DNMTs in the buccal pouch tissues of control and experimental animals

Lane 1: Control, Lane 2: DMBA alone, Lane 3: DMBA + Cromolyn, Lane 4: Cromolyn alone.

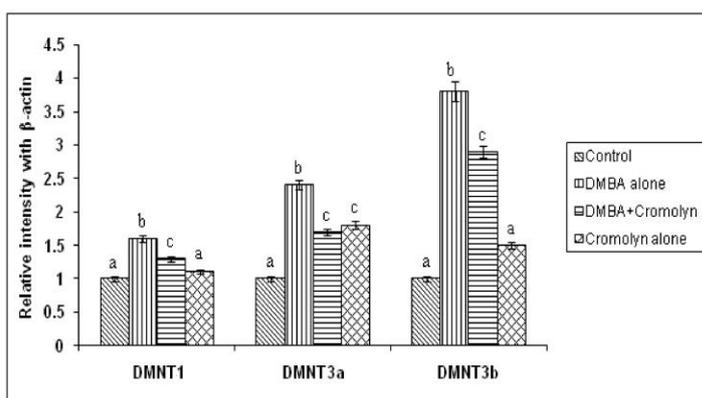


Figure 4: Densitometric analysis for DNMT1, DNMT3a and DNMT3b expression pattern

Data presented are the mean \pm SD (n=10). Common superscripts between two groups - not significant. Different superscripts between two groups - significant $p < 0.05$.

tokines. Profound studies pointed out the contribution of P^{38} MAPK in the growth and progress of breast, lung, prostate and head and neck cancer (Chen et al., 2009; Rodriguez et al., 2012). Previous studies also reported that P^{38} MAPK activation promoted tumor invasion and migration (Kumar et al., 2010). Over expression of p-Akt, p-ERK and p- P^{38} MAPK in hamsters treated with DMBA alone (tumor bearing hamsters) thus indicates their critical and crucial role in the initiation and progression of oral tumors.

DNA methyl transferase (DNMT) belongs to S-adenosyl methionine dependent methyl transferase superfamily, plays, critical and crucial role in the hypermethylation of genes that are involved in cell cycle regulation, apoptosis and DNA repair. Hypermethylation of these genes could lead to their silencing, which in turn could lead to carcinogenesis (Fang et al., 2003). DNMT methylates cytosine residue of CpG islands located in the promoter region of newly synthesized daughter DNA strands. While DNMT1 is involved in maintaining DNA methylation, DNMT3a and 3b are involved in the process of de novo methylation. Hypermethylation by DNMT potentially affects the transcription and translation of the target genes. Inhibition of DNMT by suitable inhibitors could help to re-express the desired proteins, which have a critical role in the prevention of carcinogenesis (Rice et al., 1998). DNA hypermethylation was

reported in both precancerous and cancerous lesions (Diez-Pérez et al., 2011). An increased activity of DNMT has been reported in various cancer cell lines (Bender et al., 1998). It has been reported that DNMT1 expression was increased in 36% of cancer specimens (Chen et al., 2014). DNMT3a and DNMT3b involvement has been reported in several cancers, including gastric and lung cancer (Su et al., 2010). The present study also highlighted the over-expression of DNMT in DMBA induced hamster buccal pouch carcinogenesis.

Extensive studies focused that the active constituents of medicinal plants inhibited the tumor formation and progression by modulating the Akt, MAPK and DNMT expression. Phytoconstituents that inhibit or block the activation of Akt and ERK phosphorylation and DNMT over-expression may emerge as a potent anticancer agent. We noticed down regulation of p-Akt, p-ERK, p- P^{38} MAPK and DNMT in DMBA + cromolyn treated hamsters. The results of the present study thus suggest that cromolyn might have modulated the expression of Akt, ERK, P^{38} MAPK and DNMT towards the inhibition or suppression or reversal of tumorigenesis in the buccal mucosa of hamsters treated with DMBA. Cromolyn could thus be considered as an anticancer agent, alone or in combination with existing chemotherapeutic drugs, for the treatment of cancer.

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