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CROMOLYN INHIBITS 7, 12-DIMETHYLBENZ (A) ANTHRACENE INDUCED ORAL CANCER THROUGH APOPTOTIC INDUCTION AND SUPPRESSION OF CELL PROLIFERATION

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ABSTRACT

The present study evaluated the effect of cromolyn on the expression pattern of genes involved in the process of apoptosis (P^{53}, Bcl-2, Bax, Bcl-xL and Bad) and cell proliferation (PCNA, Cyclin D1, CDK4, CDK6 and Survivin) during 7,12-dimethylbenz(a)anthracene (DMBA)induced hamster buccal pouch carcinogenesis. Hamsters buccal pouches exposed to 0.5% DMBA in liquid paraffin, three times a week for 14 weeks, developed well differentiated squamous cell carcinoma. Administration of cromolyn at a dose of 80mg/kg b.w orally to hamsters treated with DMBA not only prevented the tumor formation but also induced apoptosis and suppressed cell proliferation. Thus, the apoptotic and anti-cell proliferative efficacy of cromolyn might have suppressed DMBA induced tumorgenesis in the buccal pouches of golden Syrian hamsters.

KEYWORDS: **Oral cancer, apoptosis, cell proliferation and cromolyn**

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INTRODUCTION

Cancer, a life threatening global burden, is characterized by clonality, autonomy, anaplasia invasion and metastasis. It comprises a large group of diseases and affects all age groups. Though 100 different types of cancers reported so far worldwide, oral cancer, skin cancer, mammary cancer, lung cancer, and cervical cancer are the most predominant cancers. Each and every year, the incidence of cancer is increasing worldwide. Most of the cancers arise due to changes in lifestyle, including tobacco smoking and alcohol abuse[1,2]. Oral carcinoma, the cancer of the oral cavity, has multifactorial aetiology and arises mainly due to tobacco and alcohol addiction, which cause divergent biochemical and molecular abnormalities in the oral cavity[3]. This form of cancer not only imposes a significant health burden, but also affects the overall life quality of the oral cancer patients. Lack of awareness, delay in diagnosis and patient's delay is attributed to the high incidence of oral cancer, despite the easy physical examination of the oral cavity[4]. Most of the oral cancer patients' survival outcome depends on the location and stages of the tumors. It has been focused that the 5 year survival outcome is still at 50% for oral cancer patients. Thus, the only key criteria, which could able to reduce the morbidity and mortality of oral cancer is early diagnosis[5]. Epidemiological studies from various countries pointed out that around 300,000 peoples are newly diagnosed with oral cancer every year worldwide and of these two - thirds are from developing countries[6]. India has recorded increasing annual incidence of oral cancer due to immense population as well as most of the populations were habituated to tobacco smoking, chewing and alcohol consumption[7].7,12-dimethylbenz(a)anthracene (DMBA) is one of the most preferred chemical carcinogens to develop oral cancer in the buccal pouch of golden Syrian hamster. This is due to the fact that histopathological, biochemical and a molecular abnormality induced by this chemical carcinogen in the buccal pouches of golden Syrian hamsters closely mimic or resembles cancer of the human oral cavity[8]. The present study thus utilized this experimental model to study the apoptotic and anti-cell proliferative potential of cromolyn. Cromolyn (disodium1, 3-bis[(2'-carboxylatochromon-5'-yl)oxy]-2 hydroxypropane) plays a pivotal role in mast cell stabilization via inhibiting the release of inflammatory mediators. Cromolyn is widely used as a safe anti-inflammatory drug for the treatment of bronchial asthma and allergic rhinitis. Radley et al[9] showed that the administration of cromolyn to mdx mice significantly reduced the necrosis of dystrophic muscle. Motawi et al[10] reported that cromolyn exhibited antidiabetic property by inhibiting the activity of glycogen synthase kinase - 3β. Hemmati et al[11] reported that cromolyn significantly inhibited the fibrogenic effect of paraquat, a non - selective herbicide, in rat lungs. Cromolyn inhibited the inflammatory process via inhibition of neutrophils activation and neutrophil chemotaxis. The inhibitory role of cromolyn in the release of cytokine could be suggested as one of the major mechanism to prevent pulmonary fibrosis in rats[11]. Cromolyn at a dose of 80mg/kg bw significantly prevented ethanol - induced gastric damage in rats[12]. It has been reported that inhaled cromolyn significantly reduced lung cancer - related cough[13]. It has been pointed out that cromolyn induced blood clotting and enhanced hypoxia in murine mammary carcinogenesis[14]. Ionov[15] suggested that cromolyn might have inhibited tumorigenesis via inhibition of mast-cell activity. Cromolyn showed potent antitumor efficacy in *in vivo* pancreatic cancer model[16]. Cromolyn downregulated basal NFκB expression in BXPC-3 pancreatic cancer cell. Arumugam et al[17] pointed out that the antitumor effect of cromolyn in pancreatic cancer could be attributed to its interaction with S100 proteins. The present study takes an effort to explore the anticell proliferative and apoptotic potential of cromolyn in 7,12-dimethylbenz(a)anthracene induced oral carcinogenesis.

MATERIALS AND METHODS

(i) Tumor induction

Well differentiated squamous cell carcinoma was developed in the buccal pouches of golden Syrian hamsters using the site and organ specific carcinogen 7,12-dimethylbenz(a) anthracene (DMBA). Topical application of this carcinogen three times a week for 14 weeks resulted in tumor formation in the buccal pouches.

(ii) Administration of cromolyn

Cromolyn was orally administered at a dose of 80mg/kg bw to the experimental animal. To increase the bioavailability of cromolyn, it was administered along with the delivery agent N-cyclohexanoyl-L-leucine as suggested by Leone-Bay et al[18].

(iii) Experimental design

For the present ethically approved (Register number 160/1999/CPCSEA) experimental study, forty hamsters were procured from National Institute of Nutrition, Hyderabad, India and were categorized into four groups. Each group contained 10 animals and received food and water ad libitum. The animals were maintained in the Central Animal House, Annnamalai University as per ethical guidelines. Group I animals (vehicle treated control) received topical application of liquid paraffin alone (left buccal pouches) for 14 weeks (three times a week). Group II and III animals received topical application of DMBA (left buccal pouches) for 14 weeks (three times a week). Group III animals in addition to DMBA, also received cromolyn (80 mg/kg b.w) orally for 14 weeks (three times a week).Group IV animals received cromolyn alone (80 mg/kg b.w) orally for 14 weeks (three times a week). The experimental animals were sacrificed by cervical dislocation at the end of the experimental period.

(iv)Immunohistochemical analysis

Buccal mucosa tissue section from control and experimental animals in each group was incubated with primary antibodies (Bcl-2, Bax, PCNA, Cyclin D1: Dako, Carprinteria, CA, USA) at 4^{o} C. After incubation, the

slides were treated with secondary antibody labeled with horseradish peroxidase for an hour at room temperature. The slides were then treated with diaminobenzdiine to detect the immune complex. The slides were counterstained with hematoxylin and eosin after attaining acceptable colour intensity.

(v) Western blotting

Buccal mucosa tissues from control and experimental animals was homogenized, extracted and the protein concentrations were measured in the cell lysate. Then, the appropriate proteins were separated by polyacrylamide gel electrophoresis. The separated proteins were transferred to PVDF membrane by electroblotting. The membrane was then treated with corresponding primary antibodies (P^{53}, Bcl-xL, Bad, Survivin, CDK4, CDK6: Cell Signaling Technology, Danvers, MA, USA). It was then incubated with secondary antibodies labeled with horseradish peroxidase (Santa Cruz Biotechnology, USA). The protein bands were detected by DAB (diaminobenzidine) method. Further, densitometric analysis was performed using Bio-Rad Image Lab™ software version 4.1. β-actin was used for normalization and verification of protein loading.

STATISTICAL ANALYSIS

The data are represented as mean ± SD. Statistical significance was analyzed using one way analysis of variance (ANOVA) followed by DMRT. P values less than 0.05 between two groups were considered statistically significant.

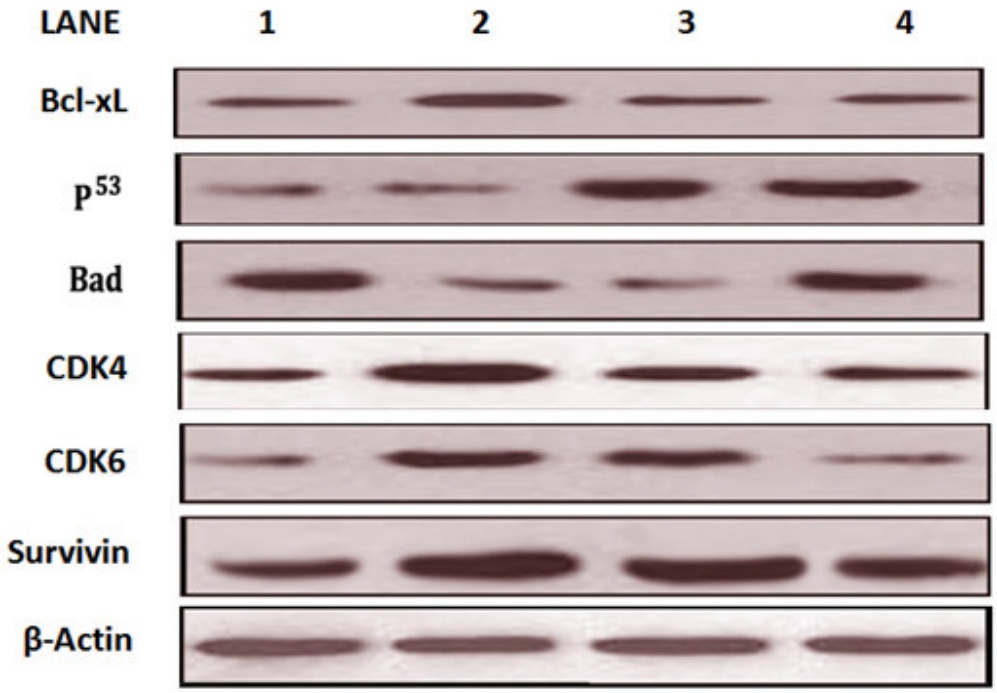
RESULTS

Figures 1 and 2 show the cromolyn efficacy on the expression pattern of P^{53}, Bcl-xL, Bad, CDK4, CDK6 and Survivin (Western blot analysis) in control and experimental hamsters in each group. Up-regulation of Bcl-xL, CDK4, CDK6, Survivin and down-regulation of P^{53} and Bad were noticed in the buccal mucosa of hamsters treated with DMBA alone. Cromolyn administration to DMBA treated hamsters brought back the expression pattern of the above said biomarkers towards control. The Bcl-2, Bax, PCNA, and Cyclin D1 expression pattern was analyzed using immunohistochemical methods (Figure 3). While Bcl-2, PCNA, and Cyclin D1 were over expressed, Bax expression was decreased in hamsters treated with DMBA alone. Cromolyn administration to DMBA treated hamsters brought back the expression pattern of above molecular markers to the near normal expression pattern (i.e. observed in control hamsters).

Expression pattern of P^{53}, Bcl-xL, Bad, CDK4, CDK6 and Survivin in the buccal pouch tissues of control and experimental animals

Figure 1

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



Densitometric analysis of protein expression after normalization to β-actin in the buccal pouch tissues of control and experimental animals. Data are expressed as mean ± SD (n=10)

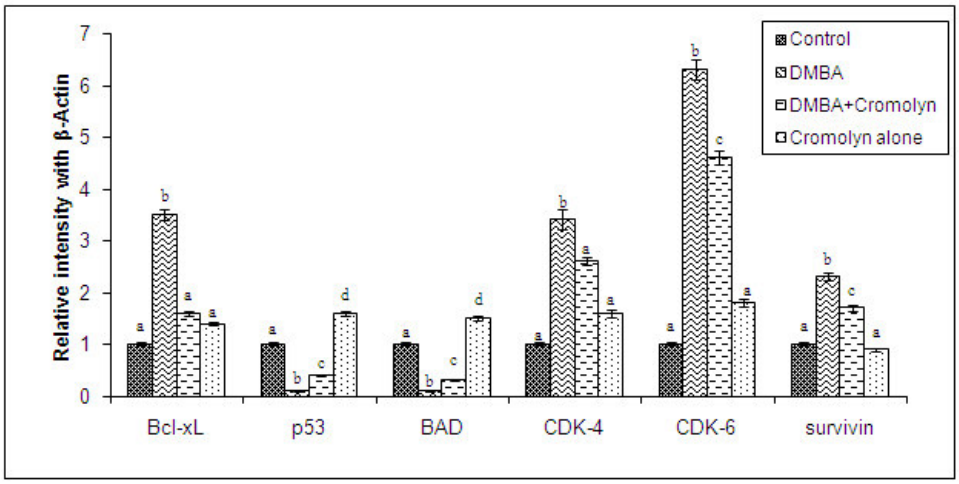


Figure 2

Common superscripts between two groups - not significant. Different superscripts between two groups - significant p<0.05.

Immunoexpression Pattern of Bcl-2, Bax, PCNA and Cyclin D1 proteins observed in the buccal mucosa of control and experimental hamsters in each group

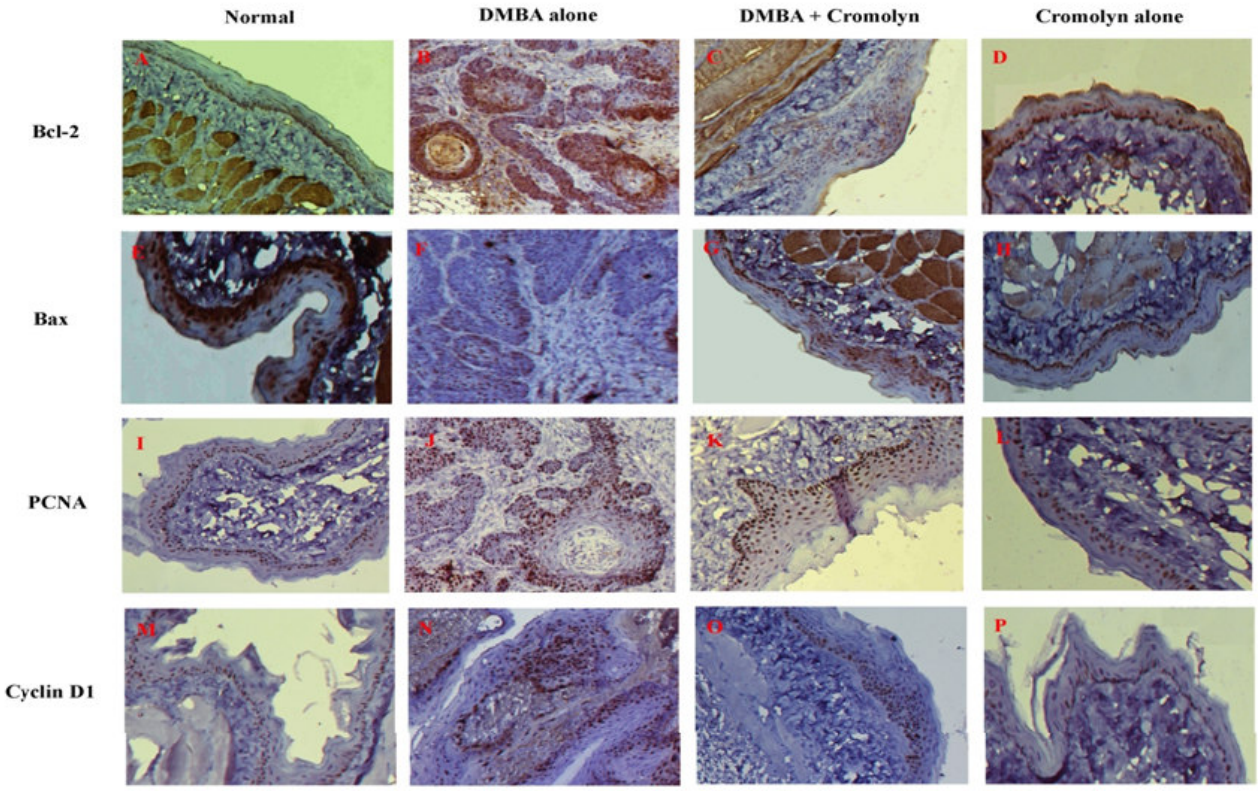


Figure 3 (A)

Bcl-2: A and D - Control and Cromolyn alone (expression not detectable); B - DMBA alone (over expression); C - DMBA + Cromolyn (down regulated). (B) Bax: E and H -Control and Cromolyn alone (nuclear expression positive), F - DMBA alone (nuclear expression negative), G - DMBA + Cromolyn (nuclear and cytoplasmic expression positive). (C) PCNA: I and L - Control and Cromolyn alone (expression not detectable), J - DMBA alone (over expressed), K - DMBA + Cromolyn (down regulated). (D) Cyclin D1: M and P - Control and Cromolyn alone (expression not detectable), N - DMBA alone (overexpressed), O - DMBA + Cromolyn (down regulated).

DISCUSSION

The goal of molecular medicine is to predict the exact therapeutic entities by developing valuable diagnostic criteria to the carcinogenesis. Investigation and identification of ideal biomarkers for oral cancer could only improve the treatment modalities as well as the quality of life of the patients. DMBA is always preferred to

induce oral, mammary and skin carcinogenesis in animal models. DMBA metabolite, the diol epoxide, binds and forms adducts with cellular DNA and thereby leads to neoplastic transformation. DMBA mediated carcinogenesis resembles human cancers in many biochemical, histopathological and molecular aspects[19,20]. The aim of the present study is thus to explore the apoptotic and anticell proliferative property of cromolyn in experimental oral carcinogenesis. The fundamental characteristics of tumor biology include cell differentiation, proliferation, apoptosis and angiogenesis. Imbalance in apoptotic or cell proliferative mechanistic pathways would favour malignant transformation. Extensive studies pointed out genetic alterations that occur in apoptotic, cell proliferative and angiogenic pathways during oral carcinogenesis[21,22]. Immunohistochemistry and Western blotting are commonly used to find out the expression of proteins that are related to carcinogenic processes. While tumor formation was 100% in the buccal mucosa of hamsters treated with DMBA alone, we observed only hyperplasia and mild dysplasia in hamsters treated with DMBA + cromolyn. Our results thus reveal the tumor - preventive potential of cromolyn in DMBA induced hamster buccal pouch carcinogenesis. To validate its antitumor property, the present study evaluated its effect on apoptotic and cell proliferative pathways in DMBA induced hamster buccal pouch carcinogenesis. Cancer proceeds through multistages (initiation, promotion and progression) and each stage is finely tuned and regulated by multiple genes. Extensive studies targeted cell cycle regulatory proteins as prognostic markers in oral carcinoma due to the fact that the cell cycle dysregulation is a hallmark in the pathogenesis of oral carcinogenesis[21,22]. Tumor progression is associated with evasion of apoptosis and abnormal cell proliferation[21]. PCNA, a cell cycle related protein, plays a pivotal role during cell proliferation. Since PCNA is expressed in cells undergoing DNA repair, its prognostic significance is limited. Extensive evidences exhibited an abnormal expression of this protein in various cancers including oral carcinogenesis[23]. Measurement of the PCNA status could provide definitive information about nodal metastasis in oral cancer patients. An ascending expression of PCNA has been documented from normal to precancerous condition and to cancerous lesions[24]. Our results corroborate these findings. Cyclin proteins perform their pivotal role in the cell cycle progression. Cell cycle regulation is precisely maintained by “cyclins” and dysregulation in the expression of these cyclin proteins could lead to defects in cell-cycle mechanism, which in turn results in carcinogenesis. Cyclin D1, a protooncogene, performs its pivotal role and critical role in the G1→S phase cell cycle transition. Abnormal expression of Cyclin D1 may shorten the G1 phase during cell cycle regulation[25,26]. Cyclin D1 over expression is always pointed out as significant molecular alterations in oral carcinogenesis[27]. The present results lend credence to these findings. Survivin, a bifunctional protein, exists physiologically as a functional homodimer on the human 17q25 chromosome. Survivin, a biomarker usually appears during embryonic development, has been regarded to have potential diagnostic and prognostic significance in various cancers. Though survivin disappears during normal cell differentiation, it reappears in malignant tissues[28,29]. Survivin plays critical role in the G2/M cell cycle progression and was found to be over expressed in myriad of clinical cancers, including, breast, colon, esophagus oral and renal cell cancers[30,31]. Previous studies were reported that survivin expression should be an essential event in the early stages of carcinogenesis and its expression was independently correlated with poor prognosis[30,32]. Liping et al[31] reported survivin over expression in oral cancer cell lines. The antiapoptotic function of survivin favours metastasis of oral carcinoma. CDK4 and CDK6 are involved in the G1 progression and G1→S transition occurring in the cell cycle. Cyclin D1 – CDK4/CDK6 complex plays pivotal role in cell cycle G1/S transition. Abnormal CDK4/CDK6 expressions should be inhibited to prevent tumor progression further. Over expression of CDK4 and CDK6 were well documented in various types of tumors[33]. The CDK4/CDK6 are utilized as valuable targets for chemoprevention studies. Bcl-2 protein, the gene product of Bcl-2 protooncogene, is an antiapoptotic protein. Over expression of Bcl-2 protein extends the survival of genetically damaged cells as well as facilitates neoplastic transformation. Increase in Bcl-2 expression observed in the present study suggests that buccal tissues from hamsters treated with DMBA alone escaped from the apoptotic cascade. It has been reported that the expression of Bcl-2 was higher in poorly differentiated squamous cell carcinoma than the well differentiated one[34]. Upregulation of Bcl-2 has been focused as a key event in the early stage carcinogenesis. Altered expression of Bcl-2 has been documented in oral epithelial dysplasia[35]. Over expression of Bcl-2 was earlier reported by Manoharan et al[22] in tumor bearing hamsters. Bcl-2:Bax ratio determines the fate of cell survival or cell death. In normal epithelium, Camisasca et al[36] observed Bcl-2 expression in lower epithelial cell layers and the Bax expression throughout the epithelium. Loro et al[37] suggested that the transcriptional or post transcriptional regulation might have accounted for the loss of Bax in oral squamous cell carcinoma. Previous studies[22] and the present study also confirm these findings. P^{53} is generally regarded as a guardian of the genome and as molecular policeman due to its role in cell cycle arrest or induce apoptosis during abnormal cell proliferation. Imbalance in programmed cell death favors the growth of solid tumors, which is mainly due to a reduction in cell death rather than abnormal cell proliferation. Tumor cells do not obey the exogenous growth regulatory signals and thus progress and proliferate abnormally. P^{53} exerts its apoptotic role in regulating Bax and Bcl-2 expression. P^{53} induces apoptosis by stimulating Bax, a proapoptotic protein, to abrogate the function of Bcl-2. Mounting evidence reported inverse correlation between P^{53} and Bcl-2 and positive association between P^{53} and Bax in carcinogenesis and this association could be responsible

for the evasion of apoptosis by tumor cell[21,22]. P^{53}, in fact, transcriptionally activates Bax and downregulates Bcl-2 expression[38]. Recurrence and overall short survival of oral cancer patients were correlated with significant accumulation of mutant P^{53} protein. Mutant P^{53} was expressed in 60-80% of the patient with leukoplakia. P^{53} mutation was noticed in more than 50% of the oral cancer tissues[39, 40, 41, 42]. A very short half-life of tumor suppressor gene, P^{53}, makes more difficult to analyze its status immunohistochemically in the normal tissue. A large number of previous studies, including our reports have shown increased P^{53} expression in the buccal mucosa of hamsters treated with DMBA using immunohistochemical studies[21, 22]. This might be due to variation in monoclonal antibodies (mutant form) used. In the present study, Western blot analysis however revealed reduced P^{53} expression in tumor bearing hamsters. B-cell lymphoma – extralarge (Bcl-xL) proteins plays critical and crucial role in the apoptotic cascade and it serves as an antiapoptotic protein. Bcl-xL is involved in the progression of tumorigenesis by inhibited apoptosis in the tumor cells. Zhang et al[43] reported that Bcl-xL was over expressed in patients with tongue carcinoma and the expression was positively correlated with tumor staging. Tumor cells showed resistance against chemotherapeutic drugs if they over express Bcl-xL. It has been reported that Bcl-xL significantly contributes in the crosstalk between autophagy and apoptosis[44]. Bad is one of the pro apoptotic proteins of Bcl-2 family and the major function of Bad is to sequester Bcl-2 and thereby preventing the interaction of Bcl-2 with Bax and BAK. Downregulation of Bad was reported in oral carcinogenesis[45]. Dysregulation in apoptotic and cell proliferation cascade could lead to the cancer progression and thus apoptotic (pro and anti) proteins and proteins involved in cell proliferation are utilized as molecular targets for therapeutic intervention. In the present study, we noticed an interesting observation that the administration of cromolyn at a dose of 80mg/kg bw orally to hamsters treated with DMBA completely inhibited tumor formation in the buccal pouches of golden Syrian hamsters. Also, cromolyn modulated the expression of apoptotic and cell proliferative markers in favour of tumor inhibition. To the best our knowledge, this is the first report on the efficacy of cromolyn on apoptotic and cell proliferative markers expression pattern in *in vivo* experimental oral carcinogenesis. Cromolyn administration to DMBA treated hamsters' downregulated the expression of PCNA, Cyclin D1, CDK4, CDK6, Survivin, Bcl-2 and Bcl-xL and upregulated the expression of P^{53}, Bax and Bad. Cromolyn showed potent apoptotic activity in various cancer cell lines including MCF7, Caco2, HepG2, and Hep2[46]. Cromolyn administration to DMBA treated hamsters might have prevented P^{53} mutation or inactivation. Also, cromolyn might have enhanced the P^{53} expression by downregulating Cyclin D1 expression in DMBA treated hamsters. Downregulation of Bcl-2 and Bcl-xL accompanied by increased P^{53}, Bax and Bad expression in DMBA+cromolyn treated animals suggests that cromolyn might have induced the mitochondrial mediated apoptotic cascade. Cromolyn suppressed survivin expression and elevated caspase-3 expression in the above mentioned cancer cell lines [46]. The present study thus explores the apoptotic and anticell proliferative properties of cromolyn in DMBA induced hamster buccal pouch carcinogenesis. To conclude, the antitumor efficacy of cromolyn is attributed to its apoptotic and anticell proliferative potential. The overall results of the present study thus explore cromolyn as a promising candidature for cancer treatments.

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