EMODIN EFFICACY ON THE AKT, MAPK, ERK AND DNMT EXPRESSION PATTERN DURING DMBA-INDUCED ORAL CARCINOMA IN GOLDEN SYRIAN HAMSTERS

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Abstract

Background: The present study has evaluated the Emodin efficacy on the Akt, MAPK, ERK and DNMT expression pattern during 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinoma in golden Syrian hamsters, in order to explore its antitumor potential.

Materials and methods: Oral tumors were developed in the buccal pouches of golden Syrian hamsters using the carcinogen, DMBA.

Results: While the incidence of tumor formation was 100% in hamsters treated with DMBA alone, the tumor formation was not noticed in DMBA + Emodin treated hamsters. Also, Emoidin reduced the severity of precancerous pathological lesions such as dysplasia, in the hamsters treated with DMBA. Emodin administration corrected the abnormalities in the expression pattern of Akt, MAPK, ERK and DNMT in the buccal mucosa of hamsters treated with DMBA.

Conclusions: The present study thus suggests that the tumor preventive potential of Emoidin is partly related to its modulating effect on the Akt, MAPK, ERK and DNMT expression pattern, as these molecular markers have a pivotal role in the process of cell proliferation, inflammation, invasion, and apoptosis.

Keywords: Oral carcinoma, Akt, MAPK, DNMT.

Introduction

Oral squamous cell carcinoma, a cancer of the oral cavity, is one of the ten most common malignant neoplasms and is the 5th most frequent cancer worldwide (Markopoulos, 2012). The development of oral cancer is usually preceded by distinct precancerous lesions such as leukoplakia, erythroplakia and oral submucous fibrosis. However, the potential or degree of malignant transformation varies from one another (Carnello et al., 2011). Oral carcinoma has a spectrum of risk factors, which include tobacco, alcohol, diet and nutrition, betel quid, viruses, immune deficiency and poor oral hygiene (Khyani et al., 2015). Tobacco chewing together with tobacco smoking, betel quid chewing and alcohol abuse is recognized as the strongest risk factor of oral cancer (Sridharan, 2014). Several epidemiological and aetiological studies pointed out the synergistic efficacy of tobacco and alcohol in the development of oral carcinoma (Patil et al., 2013). Although oral cancer is common in several countries, the highest incidence is reported every year from India, Pakistan, Sri Lanka, France and Hungary (Vigneswaran and Williams, 2014). The behavioral risk factors, tobacco and alcohol, accounts for 75-95% of all oral cancers in India (Gupta et al., 2013). Creating awareness on the risk factors of oral cancer to the public could help to reduce the annual incidence, since the risk factors are avoidable one (El Rhazi et al., 2014). It has also been pointed out that early diagnosis will improve the survival outcome of the patients (80%) and quality of life than diagnosis at late stage [30%] (Baykul et al., 2010).

Golden Syrian hamsters serve as an ideal model to study the tumor inhibitory potential of natural products, especially in oral carcinoma (Steele and Lubet, 2010). Hamsters possess buccal pouches (a pocket like anatomy) on their oral cavity, which can retain the carcinogen for the longest time when applied topically and thus tumors develop within a shorter duration (Chen et al., 2012). DMBA induces buccal pouch carcinogenesis in the hamsters through inducing chronic inflammation, causing extensive oxidative DNA damage and by inducing DNA mutations (Ismail et al., 2016). Oral cancer chemoprevention researchers prefer DMBA induced hamster buccal pouch carcinogenesis model, as this model exhibits close resemblance and similarities with human oral tumor in many aspects, including at histopathological and molecular levels (Manoharan et al., 2016; Casto et al., 2013).

P13K/Akt and ERK/MAPK pathways perform a pivotal role in the regulation of tumor growth, progression, invasion and metastasis (Gan et al., 2010). As P13K/Akt pathways play crucial and critical role in both cell survival and apoptotic pathway, their abnormal regulation or expression could mediate the neoplastic transformation in various cancers including oral carcinoma (Chang et al., 2013). The status of p-Akt has been focused as an independent prognostic marker in various cancers. Inhibition of P13K/Akt pathway was resulted in the induction of apoptotic cascade in various cancers (Matsuoka and Yashiro, 2014). P13K/Akt pathway was significantly activated during oral carcinogenesis. Tumor growth inhibition could thus be achieved by blocking the activation of Akt or ERK signaling pathways (Chappell et al., 2011).

MAPK family plays an important role in cell differentiation, proliferation, inflammation and apoptosis. This family also consists of ERK and P38. Profound studies pointed out that P38 MAPK are activated by a bundle of stimuli such as growth factors, cytokines and chemical stresses, resulting in cell proliferation, migration and apoptosis (Gerthoffer et al., 2012). MAPK has also a pivotal role in the regulation of angiogenesis. ERK, a subfamily of the MAPK family, plays an important role in the regulation of cell differentiation and proliferation (Munshi and Ramesh, 2013). ERK has been identified as a positive regulator of cell cycle and it stimulates cyclic dependent kinases - cyclin D complex, which is essential for the progression of the cell cycle (Villanueva et al., 2007). Several studies have pointed out that the accumulation of activated ERK (pERK) in the nucleus could result in genomic result.
instability as well as promotes tumorigenesis. ERK also has a role in the stimulation of tumor suppressor pathways (Bobrovnikova et al., 2010). DNA methylation, a major factor in epigenetic phenomenon, plays prominent role in the process of carcinogenesis. The enzyme DNA methyl transferase (DNMT) regulates the process of DNA methylation (Jin et al., 2011). Profound studies on various cancers documented the over expression of DNMT (Estève et al., 2016). Thus, DNMT has been utilised as a major target for cancer treatment.

Emodin, an anthraquinone derivative, is present in the root and rhizome of several Chinese medicinal plants and used in traditional Chinese medicine to treat numerous illnesses (Yang et al., 2014). Profound studies documented the pharmacological, biochemical and therapeutic effects of Emodin using experimental animal models or via in vitro approach. Emodin explored its cytotoxic potential against various tumor cell lines including human lung squamous carcinoma cells, human cervical cancer cells, human hepatoma cells, human breast cancer cells and human cervix epithelioid carcinoma cells (Zu et al., 2015; Yaoxian et al., 2013; Huang et al., 2013; Huang et al., 2013). Emodin exerted apoptotic potential in various cancer cell lines. In vivo studies demonstrated its anti-diabetic, hepatoprotective and anticancer properties (Zhao et al., 2009; Lin et al., 2012). The present study explores the Emodin efficacy on the Akt, MAPK, ERK and DNMT expression pattern during 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinoma.

Materials and methods

Animals

We procured forty golden Syrian hamsters, (male; 80-120g) from National Institute of Nutrition, Hyderabad, India and were maintained according to the Institutional ethical committee guidelines in the Central Animal House, Annamalai University (Registration Number 160/1999/ CPCSEA).

Tumor induction

The present study utilized DMBA, the site specific and organ specific carcinogen to produce tumors in the buccal pouches of golden Syrian hamsters. Topical application of this carcinogen (0.5% in liquid paraffin) three times a week for 14 weeks developed well differentiated squamous cell carcinoma (confirmed by oral pathologist, Annamalai University)

Experimental design

The experimental hamsters were divided into four groups of ten in each and housed in the polypropylene animal cages (5 animals/cage). The experimental hamsters were provided with pellet diet and water ad libitum. The experimental protocol followed in the present study is given in the figure 1.

| Groups | 0 | 1 | 14 | 15 | Weeks |
|--------|---|---|----|----|-------|
| I      |   |   |    |    | Liquid paraffin alone |
| II     |   |   | DMBA alone |
| III    |   | DMBA + Emodin |
| IV     | Emodin alone |

- Liquid paraffin alone
- DMBA (0.5% in liquid paraffin)
- Emodin (50 mg/kg b.w.)

Figure 1: Experimental protocol

Group I hamsters received liquid paraffin alone on their buccal pouches (topical application, three times a week for 14 weeks) and served as a vehicle treated control. The buccal pouches of group II hamsters were exposed to 0.5% DMBA in liquid paraffin alone (Topical application; three times a week for 14 weeks) and served as carcinogen treated control. Group III hamsters were exposed to DMBA, as in group II, and were orally administered with Emodin (50mg/kg b.w), three times a week for 14 weeks;
on alternate days of DMBA painting. Group IV hamsters received Emodin alone orally (three times a week for 14 weeks). After the experimental period, the buccal mucosa was excised from all the experimental hamsters and subjected to Western blotting to assess the expression pattern of p-Akt, p-ERK, p-P38 MAPK, DNMT1, DNMT3a and DNMT3b.

**Western blotting**

The proteins were extracted from the buccal mucosa tissues and quantified (Bradford, 1976). The tissue extract was then subjected to polyacrylamide gel electrophoresis (PAGE) to separate the various proteins. Electroblotting was employed to transfer the separated protein bands onto PVDF membrane. The membrane containing protein blots were then incubated with the corresponding primary antibodies (p-Akt, p-ERK, p-P38 MAPK, DNMT1, DNMT3a and DNMT3b, Cell Signaling Technology, Danvers, MA, USA). After the incubation period the blot was treated with the secondary antibodies labelled with horseradish peroxidase (Santa Cruz Biotechnology, USA). The immune complex formed was then treated with diaminobenzidine, and the bands were scanned and analysed densitometrically (Bio-Rad Image Lab™ software version 4.1 software).

**Statistical analysis**

The statistical significance between the groups was assessed using One way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The results obtained were considered statistically significant if the p value was less than 0.05 between the two groups.

**Results**

Buccal mucosa p-Akt, p-ERK, p-P38 MAPK, DNMT1, DNMT3a and DNMT3b expression pattern obtained using Western blotting and their densitometry analysis in the control and experimental hamsters are given in the figures 2-5. Western blot analysis revealed over-expression of the above said molecular markers in the hamsters treated with DMBA alone when compared to control hamsters. Emodin administration orally at the dose of 50mg/kg b.w to the hamsters treated with DMBA brought back the expression pattern of all the markers to near normal range.

![Figure 2](image)

**Figure 2**: Expression pattern of p-Akt, p-P38 MAPK and p-ERK in the buccal pouch tissues of control and experimental animals in each group. Lane 1: Control, Lane 2: DMBA alone, Lane 3: DMBA + Emodin, Lane 4: Emodin alone.
Figure 3: Densitometric analysis for p-Akt, p-P38 MAPK and p-ERK expression pattern in control and experimental animals in each group. Data presented are the mean ± SD (n=10). Common superscripts between two groups - not significant. Different superscripts between two groups - significant p<0.05.

Figure 4: Expression pattern of DNMT1, DNMT3a and DNMT3b in the buccal pouch tissues of control and experimental animals. Lane 1: Control, Lane 2: DMBA alone, Lane 3: DMBA + Emodin, Lane 4: Emodin alone.
Deregulation of the MAPK pathway has been reported in several cancers, particularly in more than 50% of hepatocellular carcinomas. It has been reported that the mammary cancer metastasis can be inhibited by inhibiting P38 MAPK expression (Dhillon et al., 2007).

ERKs occupy the central position of diverse cellular signaling pathways that are involved in cell growth, proliferation and survival (Hong et al., 2015). Deregulation in the ERK / MAPK pathway is associated with the poor prognosis of oral cancer. ERK1/2 and P38, the subfamilies of MAPK, were deregulated in various carcinogenesis (Mebratu and Tesfai zig, 2009). Aberrant ERK signaling could therefore lead to malignant transformation. ERK activation resulted in the activation of the spectrum of genes including PCNA, which in turn favors malignant transformation (Jin and Robertson, 2013).

There are three classes of DNMTs in the mammalian cells, DNMT1, DNMT3a and DNMT3b. DNMT1 is a maintenance methyltransferase and DNMT3a and DNMT3b are de novo methyltransferases (Liang et al., 2002). All three types of DNMT were abnormally expressed in cancerous conditions (Skowronska et al., 2010). DNMT1 plays prominent and pertinent role for DNA methylation during DNA replication. DNMT1 over expression was reported in lung, colorectal, prostate, breast, gastric and oral cavity cancers (Delpu et al., 2013). It has been pointed out that DNMT inhibition might lead to reactivation of the silenced genes. Down-regulation of p53 has been associated with up-regulation of DNMT1 (Au Yeung et al., 2010).

Extensive studies reported the effect of Emodin on the Akt, MAPK, ERK and DNMT expression pattern in various cancer cell lines. Lin et al. (2016) pointed out that Emodin induced apoptotic process in the hepatic cancer cell lines via P13K/Akt and MAPK signaling pathways. Liu et al. (2015) explored the inhibitory effect of Emodin on the expression of P13K/Akt in the mechanical stress induced hypertrophic scarring. Emodin prevented the mammmary cancer cell proliferation via down-regulating P13K/Akt protein expression (Sui et al., 2014). It has been reported that Akt down-regulation by Emodin is due to the inhibition of the components of the P13K pathway (Olsen et al., 2007). Zheng et al., (2015) observed that Emodin downregulated Akt expression in K562/Adr cells. Way et al., (2014) demonstrated that Emodin effectively inhibited the epithelial-mesenchymal transition in head and neck cancer cells via inhibiting Akt pathways. Inhibition of Akt activation has been suggested as a major mechanism of antitumor effect of Emodin against pancreatic cancer in mice (Wei et al., 2011). It has been pointed out that Emodin attenuated the
phosphorylation of PI3K/Akt and ERK in HPG2 cells (Cui et al., 2016). Emodin exerted its antiinvasive property via downregulating ERK1/2 and Akt/PI3K activation in glioma cells (Kim et al., 2005). Inactivation of ERK and Akt has been demonstrated in human lung adenocarcinoma cells (Su et al., 2005). Emodin downregulated the expression of DNMT1 and DNMT3 in pancreatic cancer cells. (Pan et al., 2016; Zhang et al., 2015). We, for the first time, demonstrated the modulating effect of emodin on the Akt, MAPK, ERK and DNMT expression pattern in experimental oral carcinogenesis.

In the present study, we have explored the activation of Akt, ERK, P38 MAPK and DNMT, which have a crucial role in the process of cell proliferation, apoptosis and cell differentiation in the tumor bearing hamsters (DMBA alone treated hamsters). Emodin administration at a dose of 50mg/kg b.w to hamsters treated with DMBA inhibited the activation of all these molecular markers, as evidenced by the down regulation of these markers (Western blotting). The present study thus suggests that Emodin might have modulated the Akt, ERK, P38 MAPK and DNMT pathways towards suppression of tumor formation during DMBA induced oral carcinogenesis.

Conclusion

The present study concludes that the antitumor potential of Emodin might have partly attributed to its inhibitory effect on the activation of Akt, ERK, P38 MAPK and DNMT pathways during DMBA induced hamster buccal pouch carcinogenesis.

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