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Modulation of p21, DAPK1 and COX-2 during the DMBA/TPA-induced mouse skin tumorigenesis and its prevention by phytic acid

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ABSTRACT

Chemoprevention by naturally occurring agents is gaining much attention as a newer dimension in the management of cancer. Many naturally occurring agents have shown cancer chemopreventive potential in a variety of bioassay systems and animal models, having relevance to human disease. Phytic acid or Inositol hexaphosphate (IP6), an antioxidant, is a naturally occurring polyphosphorylated carbohydrate that has shown a strong anticancer activity in several experimental models. We assessed the protective effects of Phytic acid against the 7, 12-dimethylbenz [*a*] anthracene (DMBA)/ 12-O-tetradecanoylphorbol-13-acetate (TPA) induced mouse skin tumorigenesis at 4 and 16 weeks, the time before and after the tumor development. At molecular level we studied expression and promoter CpG methylation status of p21, DAPK1 and COX-2. Our data suggests exposure of DMBA/TPA methylated the promoter region of p21 and DAPK1 genes in time dependent manner that could be the cause of down regulation of their expression with time, which were reversed by administration of phytic acid. But we did not observe methylation in COX-2 whereas upregulation of COX-2 was observed at protein level in mice treated with DMBA followed by TPA in time dependent manner. Administration of phytic acid prevented theses DMBA/TPA induced molecular changes. Study provides a rationale for cancer chemoprevention by natural occurring compounds like Phytic acid.

Introduction

The term 'cancer' represents a range of different type of malignant diseases; all of them exhibit uncontrolled cell division, tissue invasion and spreading as common characteristics. Skin cancer contributes approximately 30% of all newly diagnosed cancer in the world and solar ultraviolet radiation is an established cause of approximately 90% of all skin cancers [1]. Nonmelanoma skin cancer (NMSC) is the most common form of human cancer, with incidence rates dramatically rising during the last decade, perhaps as a result of increased sun exposure and the continuous depletion of the ozone layer [2, 3]. It is widely accepted that squamous cell carcinomas (SCCs), which are responsible for the majority of NMSC-related deaths, result from the accumulation of genetic alterations [4].

Different types of animal models of cancer have been developed to understand the cellular and molecular alterations associated with the process of carcinogenesis. Chemically induced two-stage mouse skin carcinogenesis represents one of the well-established *in vivo* models for study the sequential and step wise development of tumors [5]. In this system, tumor initiation is accomplished through a single topical application of a carcinogen, typically 7, 12-dimethylbenz (a) anthracene (DMBA). Tumor promotion takes place when the initiated cells are expanded due to repeated applications of 12-Otetradecanoylphorbol- 13-acetate (TPA), a hyper proliferative stimulus that promotes the generation of papillomas.

Chemoprevention by natural compound is a novel approach against cancer. Chemoprevention through the consumption of natural dietary compounds such as lycopene, resveratrol,

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genistein may reduce both morbidity and mortality from cancer by affecting various signalling pathways suggesting the novel cancer preventive strategy [6]. Phytic acid is a complex carbohydrate that is a naturally occurring compound found in grains, cereals, nuts, and foods that are high in fiber content. Some study demonstrated the antitumor effect of phytic acid [7]. In a continuing effort to evaluate safer and more effective forms of therapy, we investigated the molecular effect of phytic acid.

Epigenetics encompasses the wide range of heritable changes in gene expression that do not result from an alteration in the DNA sequence itself. DNA methylation, the reversible post-translational modification define the epigenetic landscape of a cell [8]. In cancer cells, increased *de novo* methylation (hypermethylation) of many promoters of tumor suppressors genes have been reported [9]. Genes can be transcriptionally silenced when their promoter region(s) CpG islands are hypermethylated [9]. The p21 protein has been suggested to mediate p53-induced growth arrest triggered by DNA damage [10]. The presence of hypermethylation of the p21 gene was associated with many cases of acute lymphoblastic leukemia and was also associated with unfavourable clinical outcome [11]. DAPK1 has been shown to suppress the metastatic ability of carcinoma cells [12]. Its expression has been shown to be absent or decreased in various cancers and cancer cell lines [13]. DAPK1 has been reported to be suppressed by promoter methylation in malignant tissue but its methylation was not found significant in benign tumors or at early stages [13]. COX-2 plays central roles in the inflammation associated cancer development and act as interface between inflammation and cancer. Up-regulated expression of COX-2 is found in multiple human cancers including colon, breast, prostate and skin [14-16]. In the mouse skin model, COX-2 is constitutively overexpressed papillomas and carcinomas [17].

In this study we have shown the chemopreventive efficacy of phytic acid against DMBA/TPA induced skin tumorigenesis via modulation expression of p21, DAPK1 or COX-2 and epigenetic status of p21 and DAPK1. 4 weeks end point was selected to know molecular changes before the tumor development and 16 weeks end point was to know the molecular changes that appeared after the tumor development

Material and method

Animals

Female Swiss albino mice (4-6 weeks old and 10–12gm body weight) were used for the study. All the animals were kept in 12/12 hour light/dark cycle and were fed pellet diet (consisting of protein, 21.53%; fat, 5.24%; crude fiber, 5.3%; carbohydrate, 57.59%; and caloric value, 363.64 kcal/100 g from M/S Ashirwad Pvt. Ltd., Chandigharh, India) with water ad libitum. Study was approved and animals were handled according to the norms of institutional animal ethics committee.

Chemicals

Phytic acid, 7,12-dimethylbenz[a]anthracene (DMBA), phorbol 12-myristate 13-acetate (TPA), sodium metabisulfite, and hydroxy quinone were procured from Sigma Co. (St. Louis, MO, USA). Ampli gold Taq DNA polymerase, dNTPs, MS-PCR primers were procured from Banglore Genei (India). The rest of the chemicals were procured from local commercial sources and were of analytical grade.

Animal treatment and chronic animal bioassay

Mice were shaved on the interscapular region of the back and only animals in the resting phase of the hair growth were used for treatment. DMBA/TPA induced two stage mouse skin tumorigenesis model was used in this study. In brief, single topical dose of 50 µg DMBA /0.1ml acetone was applied and after one week followed by topical treatment of 5 µg TPA /0.1ml acetone twice weekly, for 4 or 16 weeks. 2% Phytic acid is given in drinking water ad libitum. Animals were divided randomly in 4 groups consisting of 10 animals each. All treatments were given topically on the shaved back. Groups are divided as follows.Gr.1-Acetone, Gr.2- Phytic acid, Gr.3- DMBA+TPA, Gr.4- DMBA + TPA + Phytic acid. All the animals were sacrificed at the end of 4 or 16 weeks. Skin from the painted area was excised, cleaned, snap frozen in liquid nitrogen and stored at - 80^0C till further use.

DNA isolation and bisulfite modification

DNA was obtained from frozen whole epidermis by proteinase digestion followed by phenol–chloroform–isoamyl alcohol extraction and ethanol precipitation as described [18]. DNA methylation in the CpG islands of the gene promoters was determined by chemical modification of genomic DNA with sodium bisulfite and subsequent methylation-specific PCR (MSP) as described [19, 20]. In brief, 5 µg of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega Corp., Madison, WI), desulphonated by NaOH and neutralized by NH4-acetate. DNA was precipitated by ethanol using Salmon testis DNA (Sigma Co.) as carrier and dissolved in 20 µl of water.

Methylation specific PCR (MS-PCR)

Methylation of CpGs in p21, DAPK1 and COX-2 gene promoter was studied by MS-PCR as reported earlier [21, 22]. Modified DNA, thus, obtained was used for hot start PCR (Ampli Taq DNA polymerase) with specific primers for methylation and unmethylation sequences (Table.1). The PCRs were performed with 50 ng bisulfite-modified genomic DNA template in 20 µl reaction mixture containing 1 X PCR buffer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 10 pmol each of forward and reverse primer, and 1 U Ampligold Taq DNA polymerase. The mixture was subjected to denaturation at 95◦C for 10 min followed by 35 cycles (95◦C for 50 s, 55◦C for 60 s, and 72◦C for 60 s) and a final extension at 72◦C for 7 min in a My-Cycler Thermal cycler from Bio-Rad. PCR products were electrophoresed in a 1.5% agarose gel and visualized using ethidium bromide. A methylation vs unmethylation band intensity ratio was compared among the

groups. Quantification was done using Gene Tool Syne gene software.

Table 1 : Primer sequences and PCR product sizes for MS-PCR

Gene	Primer Sequence	Product size	Annealing temp.(C^0)
p21 Methylated	F 5'-GTTAGCGAGTTTTCGGGATC-3' R 5'-CTCGACTACTACAATTAACGTCGAA-3'	111	55^0C
p21 Unmethylated	F 5'GGTTAGTGAGTTTTTGGGATTG-3' R 5'-CTCAACTACTACAATTAACATCAAA-3'	112	55^0C
DAPK1 Methylated	F 5'-TGTATGAGGGGTTCGTAGTAGC-3' R 5'-GATATCGTAATAATCGTCCACGTT-3'	136	55^0C
DAPK1 Unmethylated	F 5'-TGTATGAGGGGTTTGTAGTAGTGA-3' R 5'-CAATATCATAATAATCATCCACATT-3'	140	55^0C
COX-2 Methylated	F 5'-TTTGTCGTTGCGGTTTTTGC-3' R 5'-AAAACGAACTCCACGTAACG-3'	118	55^0C
COX-2 Unmethylated	F 5'-AAAGTTTGTTGTTGTGGTTTTTGT-3' R 5'-CTATAAAACAAACTCCACATAACA-3'	126	55^0C

Western Blotting

Skin epidermal tissue lysate (10%) was prepared in lysis buffer (pH 7.6) containing 20 mM Tris, sucrose (250 mM), $MgCl_2$ (2mM), EDTA (2mM), EGTA (0.5mM), DTT (1mM), Na_3VO4 (0.03mM), PMSF (2mM) and protease inhibitor cocktail (Sigma). Tissue lysate equivalent to 50 µg proteins was resolved on SDS-PAGE using 7.5-12.5% Tris-Glycine gel. [23]. The separated proteins were transferred onto nitrocellulose membrane (Bio-Rad) followed by blocking with 5% non fat milk powder (w/v) in Tris buffered saline containing 0.1% Tween 20 (TBST) for 1 h at 4ºC. Separate membranes were probed with antibodies of p21, DAPK1 and COX-2 (Santa Cruz Biotech) for overnight at 4ºC followed by washing with TBST. Washed membranes were incubated with peroxidase-conjugated appropriate secondary antibody (Bangalore Genei, India) for 1 h at 4ºC, and signals were visualized by Chemiluminescence HRP detection system from Millipore on Versa Doc (Bio-Rad). Membranes were stripped and reprobed with β-actin antibody (Sigma Co). Quantification was done using Gene Tool Syne gene software.

Statistical analysis

All the data were subjected to statistical analysis. Mean values and ± SE of all the groups were calculated. One way ANOVA following Student-Newman-Keuls tests for post hoc analyses was employed to assess the statistical significance of the difference between different treatment groups. Significance was determined in terms of 'p' values. A 'p' value of <0.05 was considered to be statistically significant.

Results

In order to evaluate the molecular basis of anti tumor effects of phytic acid, we studied the status of p21, DAPK1 and COX-2 expression at protein level before (at 4weeks) and after the appearance of tumors (at 16 weeks). We also tried to evaluate the promoter methylation status of these genes.

Deregulation of p21, DAPK1 and COX-2 expression after DMBA/TPA application and its prevention by phytic acid

DNA damage and deregulated cell cycle are the critical early changes and preceded tumor development. Since we aimed to evaluate the molecular events altered even before the onset of the tumor and progresses with time, we assessed the status of the representative genes, p21, DAPK1, and COX-2. Expression of p21, DAPK1, and COX-2 remained unchanged in mice exposed to only acetone or phytic acid at any end point of study. But at the end of 4 weeks, in mice exposed to DMBA followed by TPA application, p21 and DAPK1 expression were downregulated by 25% and 10% respectively in comparison to acetone treated mice. This downregulation was prevented by phytic acid and reduced to 2% and 1% respectively in comparison to acetone treated mice. Whereas at the end of 4 weeks, in mice exposed to DMBA followed by TPA application, COX-2 expression was upregulated by 61% in comparison to acetone treated mice. This downregulation was prevented by phytic acid and reduced to 1% in comparison to acetone treated mice. At the end of 16 weeks, DMBA treated followed by TPA exposed mice showed downregulation of p21 and DAPK1 expression by 44% and 35% respectively in comparison to acetone treated mice. This downregulation was prevented by phytic acid and reduced to 1% and 7% respectively in comparison to acetone treated mice. Whereas at the end of 16 weeks, in mice exposed to DMBA followed by TPA application, COX-2 expression was upregulated by 85% in comparison to acetone treated mice. This upregulation was prevented by phytic acid and reduced to 31% in comparison to acetone treated mice (Fig. 1).

So downregulation of p21, DAPK1 and upregulation of COX-2 expression appeared even before tumor develop and get more with the time during DMBA/TPA induced skin tumorigenesis and chemopreventive agent phytic acid tried to recover these alterations (Fig.1).

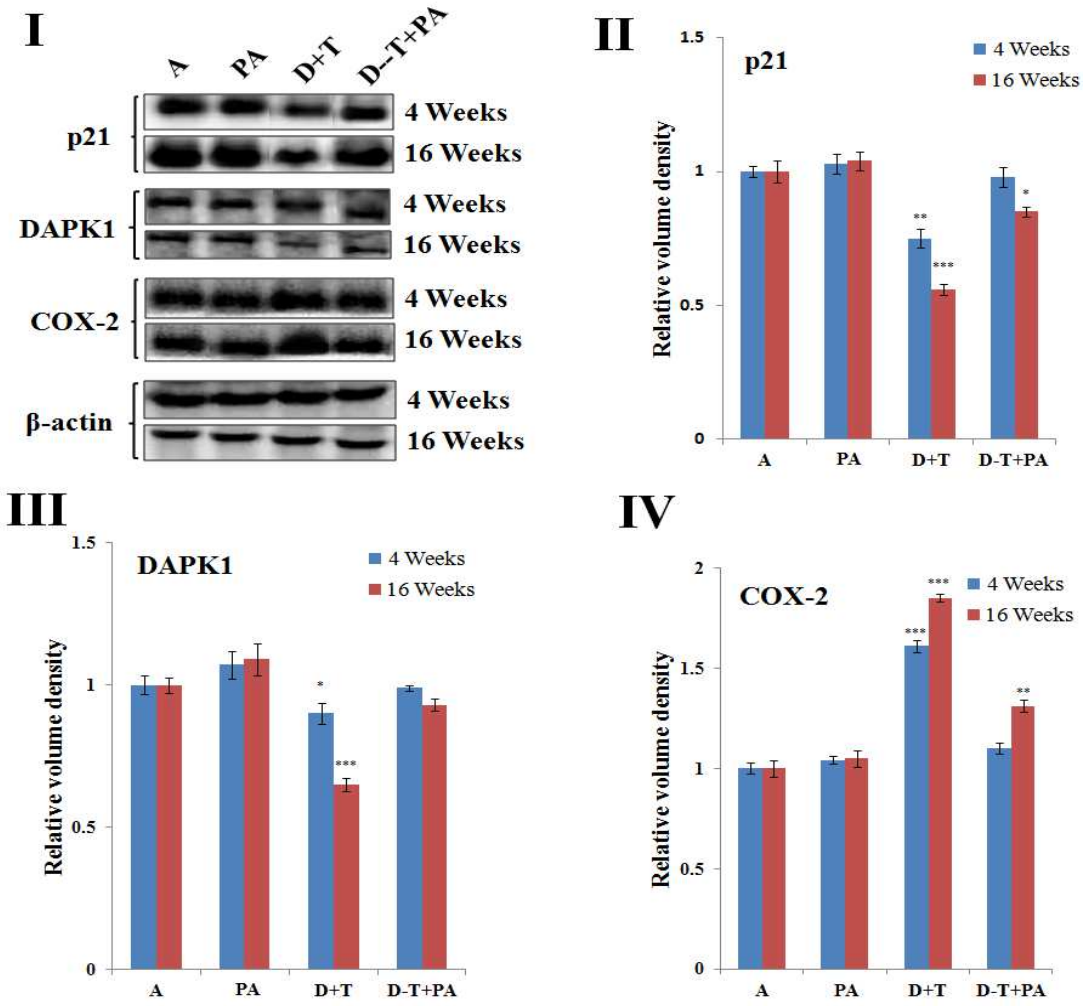

Figure-1

Fig.1: Downregulation of p21, DAPK1 and upregulation of COX-2 protein expression in response to DMBA followed by TPA exposure and its prevention by phytic acid. Qualitative analysis of their expression is shown at protein level in I and quantitative analysis of their expression is shown in II, III and IV. Bars represent the standard error.* p<0.05, ** p<0.01, *** p<0.001 in comparison to acetone treated group.

Promoter CpG Methylation of p21, DAPK1 and COX-2 after DMBA/TPA application and its prevention by phytic acid

CpG methylation in the gene promoter is involved in the transcriptional silencing of the gene and has been implicated in cancers. Having shown the status of the expression of p21, DAPK1, and COX-2 at the protein level, further we tried to assess promoter CpG methylation status of these genes. Promoter CpG methylation of p21, DAPK1, and COX-2 was not observed in mice exposed to only acetone or phytic acid at any end point of study. At the end of 4 weeks, DMBA treatment followed by TPA application resulted into the promoter CpG hypermethylation of p21 and DAPK1 by 24% and 26% respectively in comparison to acetone treated mice. This hypermethylation was prevented by phytic acid and reduced to 1% and 2% respectively in comparison to acetone treated mice. Whereas at the end of 16 weeks, DMBA treated followed by TPA exposed mice showed increased promoter CpG methylation of of p21 and DAPK1 by 96% and 58% respectively in comparison to acetone treated mice. This downregulation was prevented by phytic acid and reduced to 9% and 15% respectively in comparison to acetone treated mice. Whereas Promoter CpG methylation of COX-2 was not observed in mice exposed to DMBA followed by TPA expression both at the end of 4 and 16 weeks end point. Thus phytic acid administration tried to reduce the hypermethylation of promoter CpG of these genes very effectively (Fig.2).

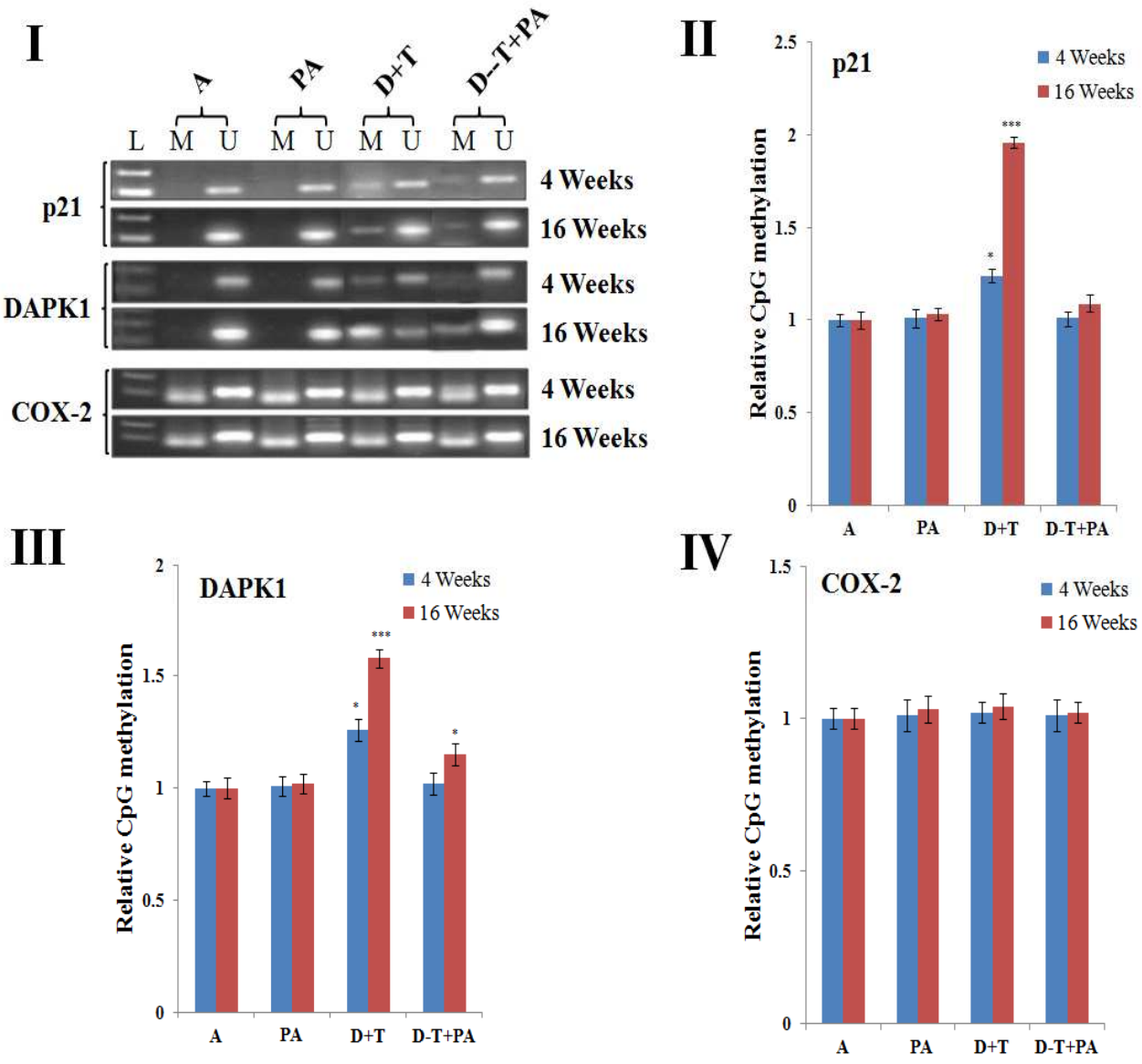

Figure-2

Fig.2: Promoter CpG methylation status of p21, DAPK1 and COX-2 in response to DMBA followed by TPA exposure and its prevention by phytic acid. Analysis of their promoter CpG methylation of are shown by MSPCR blots (I) and histograms for quantitation based on the ratio of methylated vs unmethylated bands (II, III, IV). Lane U=Amplification using unmethylated primer pairs. Lane M = Amplification using methylated primer pairs. Bars represent the standard error.* p<0.05, ** p<0.01, *** p<0.001 in comparison to acetone treated group.

Discussion

Cancer is the leading cause of death worldwide. Although, enormous progress made for the diagnosis and treatment of various types of cancer, still we do not have the single magic drug that can completely and selectively destroy cancerous cells. So there is a need to develop or identify the potent chemopreventive agent to overcome the poor clinical outcome of the current treatment avenues. Phytic acid posses anticancer potential that has been shown to skin tumor model [7]. But its molecular mechanism is not well understood. Here we suggested the chemopreventive efficacy of phytic acid against DMBA/TPA induced skin tumorigenesis via prevention of

deregulated expression of p21, DAPK1, COX-2 and promoter CpG hypermethylation of p21 and DAPK1.

Aberrant DNA methylation has been found in p21 genes, leading to their down-regulation in tumors as in case of tumor suppressor genes [24]. We also observed 24% more promoter CpG methylation of p21 than control at the end of 4 weeks of DMBA / TPA exposure. This suggests the early involvement of promoter CpG methylation even before the onset of tumors. In later end point (16 weeks), promoter CpG methylation of p21 was increased with time. Increased promoter CpG methylation at 16 weeks end point was well correlated with the p21 down regulation and suggested its relevance in the development of tumors from beginning of tumorigenesis.

In several malignant cell lines, studies have shown that expression of DAPK1 is decreased or absent [12]. Additionally, decreased expression of this gene imparts resistance to interferon-induced apoptosis in cells, and a link between the loss of DAPK1 expression and cellular apoptosis has been shown to facilitate metastasis, at least in one experimental system [25]. Promoter methylation of DAPK1 gene has been observed in multiple tumor types and may increase cellular resistance to programmed cell death [26]. We also observed the promoter CpG hypermethylation in DAPK1 genes after DMBA / TPA exposure from 4 weeks to at the time of well defined tumors 16 weeks end point in time dependent manner. Moreover, available report suggests frequent targeting of the DAPK1 promoter methylation by aberrant DNA methyltransferases [27].

We did not observe promoter CpG hypermethylation in the mut p53 and COX-2 gene as it is not frequently regulated via promoter CpG methylation. Moreover, available reports suggest COX-2 promoter CpG methylation is not a frequently occurring phenomenon in cancer [28]. At the protein level, we observed over expression of COX-2 after DMBA / TPA exposure in time dependent manner. That means level of COX-2 increased with time from 4 to 16 weeks of DMBA / TPA exposure. More over expression of COX-2 could be the reason of skin papilloma appearance in later end point of study i.e. 16 weeks. The continued over expression of COX-2 [29], is in agreement with the earlier reports that suggests COX-2 itself has been shown to be upregulated in various tumors and shown to be involved in tumor development [30]. Upregulation of COX-2 at all the time points in time dependent manner could be for its involvement in neoplastic transformation and establishment of tumors by altering the inflammatory and related processes. Hence, deregulation in inflammatory pathways corresponded to the altered epigenetic scenario after DMBA/TPA exposure.

We have provided the evidences about the status and the involvement of epigenetic and associated events during the course of the development of tumors from start to finish. Chemopreventive interventions by compound with any molecular change are likely to give an insight for targeted cancer therapies and prevention [7]. Phytic acid is a naturally occurring compound, exhibiting tumor suppressing effect through its anti-angiogenic, anti-oxidant and anti inflammatory properties [7]. The analysis of the effects of IP6 on the DMBA/TPA caused molecular modulations suggested the possible mechanisms of chemoprevention by these compounds. IP6 protected the DMBA/TPA induced deregulation of p21, DAPK1 and COX-2 at all the time. Prevention was more pronounced at the later end points at 16 weeks due to higher degree of molecular alterations leading to the development of the tumor.

Phytic acid also protected the p21 and DAPK1 gene promoter CpG hypermethylation caused by DMBA / TPA exposure thus ultimately restored the expression of p21 and DAPK1. In addition to this phytic acid also prevented the DMBA / TPA induced over expression of COX-2. its downstream effect on the methylation as well suggesting its chemopreventive action through the modulation of CpG methylation.

In summary, our study identified that p21 and DAPK1 promoter get methylated in DMBA with TPA exposed groups, which downregulate the protein expression of these genes and contributes in development of skin tumors. On the other hand DMBA / TPA exposure caused overexpression of COX-2 in time dependent manner. Phytic acid inhibits these DMBA/TPA induced molecular changes with restoration of p21 and DAPK1 expression in addition to dowregulation COX-2 expression. So use of natural compounds can provide an effective mean of cancer control.

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Conflict of interest statement

We declare that we have no conflict of interest.

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