



Onset of the lymphocytic infiltration and hyperplasia preceding the proliferation in F1 mouse lungs from the N-ethyl-N-nitrosourea exposed mothers: Prevention during the lactation period by inositol hexaphosphate[☆]

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ABSTRACT

Maternal exposure to a carcinogen is associated with increased risk of different cancers in the offspring. The foetus is highly sensitive to carcinogens and this contributes to the foetal basis of the onset of disease. The better understanding of the molecular mechanisms involved in the early stage of lung tumourigenesis in the offspring is needed for the newer preventive strategies. We evaluated the effects of N-ethyl-N-nitrosourea (ENU) given on the 17th day of gestation and antitumour agent inositol hexaphosphate (IP6) to the mothers at the early stage of lung tumourigenesis in F1 mice. There was no treatment related effects on the litter size or body weight of the F1 mice at the PND12 or 24. Analysis of PCNA, NF- κ B (p50), IL-6, COX-2, pSTAT3, STAT3, caspase-3, caspase-9, PARP, Akt signalling and downstream cyclin D1 along with miR-155, suggested the modulation of proliferation, inflammation and apoptosis at PND12 and 24. IP6 administration to the predisposed mothers prevented the proliferation, inflammation and enhanced apoptosis in F1 lung as showed by a reduction in PCNA, NF- κ B (p50), IL-6, COX-2, pSTAT3, STAT3, miR-155 and increase in caspases, cleavage of poly (ADP-ribose) polymerase. IP6 administration also inhibited the activation of Akt and cyclin D1. Our study shows that tumourigenic changes take place in the lungs of the F1 generation from the carcinogen predisposed mothers even before the onset of tumours and the simultaneous intake of chemopreventive agent during the gestation or lactation period could prevent the lymphocytic infiltration and hyperplasia preceding the tumourigenesis.

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1. Introduction

Exposure of carcinogen during pregnancy is associated with impacts on offspring diseases like cancer [1].

N-ethyl-N-nitrosourea (ENU), a mutagen related to the family of N-nitroso compounds, is found in fumigants, biohazard and interacts with DNA forming adducts before causing the cancer in the developing foetus [2,3]. The foetus is highly sensitive to toxicity from chemical carcinogen that contributes to the onset of different cancer like liver, lung and kidney in offspring [1,4]. Use of ENU in mouse model for the transplacental tumour development has been used to evaluate the risk or effects associated with mutagens and carcinogens in the human population [3,5–7]. We have also shown that maternal ENU exposure

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during pregnancy results in lung tumours in the offspring in adulthood [3]. However, the molecular mechanisms remain to be explored in the early stages in the offsprings from the carcinogen predisposed mothers.

Emerging studies demonstrated that an early induction of cell proliferation, inflammation and inhibition of apoptosis may lead the development of a tumour at a later stage [8,9]. PCNA, an early cell proliferation marker expressed during S and G2 phases of the cell cycle, is found to be highly expressed during carcinogenesis and plays a central role in DNA replication, repair and cell cycle progression [10]. Nuclear factor κ B (NF- κ B), a family of transcription factors, has been showed to regulate growth, inflammation and apoptosis in different tissues [11]. Cyclooxygenase-2 (COX-2), involved in inflammation, cell proliferation and cell death, is also regulated by the activation of NF- κ B [12] resulting in the regulation of pro-inflammatory cytokines (IL-6) and chemokines gene expression. Further, activation of transcription factor STAT3 by phosphorylation in turn activates the transcription of genes implicated in inflammation and cancer [13]. Targeted inhibition of inflammatory mediators at the initial phase is valuable for not only alleviating inflammation, but also preventing the onset of cancer.

Enhanced cellular proliferation and deregulated apoptosis are prominent early cellular features after carcinogen exposure [14]. In apoptosis, caspase-3 gets activated through caspase-8 or caspase-9 and leads to the cleavage of poly (ADP-ribose) polymerase (PARP) causing DNA fragmentation [15] and suggesting the onset of apoptosis. Akt signalling is a critical in cancer because it contributes to tumourigenesis, tumour growth and therapeutic resistance. Activation of Akt is an early response to carcinogen exposure that promotes cell survival by activating the NF- κ B signalling pathway and inhibiting apoptosis [16–18]. Activated Akt, the serine 473 phosphorylation, results in the activation of a cascade of different protein targets including NF- κ B and cyclin D1 [19–21]. MicroRNAs (miRNAs), around 21 nucleotides long non-coding RNA, play an important role in regulating the expression of multiple genes [22,23]. Recent studies have shown that miRNA could be among the potential biomarkers for various cancers [24]. Overexpressed miR-155 promotes cell proliferation and inhibit apoptosis in breast, lung and colon cancers, but the specific mechanism of action of miR-155 remains to be understood [25,26]. The downregulation of miR-155 expression is also associated with induction of apoptosis through the activation of caspases in non-small-cell lung cancer [24,26,27].

Chemopreventive approaches are more effective in preventing and controlling the onset of diseases [28]. Chemopreventive agents including plant products and dietary constituents are shown to prevent the development of tumours [29].

A chemopreventive agent effectively regulates cell proliferation, inflammation and inducing apoptosis [28–30]. However, clinical trials with some agents like retinoid, alpha-tocopherol and carotenoid are not successful instead; adverse tumour promoting effects were observed. In view of above, we tried to look for the early changes in F1 mouse lungs from the carcinogen predisposed mothers

and the effects of inositol hexaphosphate (IP6), a naturally occurring nontoxic sugar phosphate mostly present in high fibrous food sources such as cereals, legumes, nuts and soybean. It is also present in almost all the plant and mammalian cells [36]. Others and we have used the 2% IP6 in drinking water in the chronic animal experiments to study the tumourigenesis and chemoprevention without showing any toxic effects [3,31,33]. IP6 has been shown to inhibit cell proliferation and induce apoptosis in different model [32]. However, the understanding of the potential molecular mechanism responsible for its anti-proliferative and apoptotic activity is limited [3]. Present study is aimed to elucidate the early changes during the lactation period in F1 mouse lungs from the predisposed mothers to be used for the cancer control.

2. Material and methods

2.1. Animals

Female and male, Balb/c mice (6–8 weeks of age) were procured from the inbred colony of CSIR-IITR, Lucknow. Male mice were used for the purpose of mating. Mice were housed under a 12-h light:dark cycle and were kept on solid pellet diet (M/S Provini Animal Nutrition India Pvt. Ltd., Bangalore) and water ad libitum. They were acclimatized for one week before the start of the experiments.

2.2. Chemicals and reagents

N-ethyl-N-nitrosourea and inositol hexaphosphate were procured from Sigma Co. (USA). [methyl-³H] thymidine (specific activity 17.20 Ci/mmol) was provided by BRIT, Mumbai, India. Caspase-3 or -9 enzyme activity assay kit was procured from R&D system, USA. The Super Signal West Femto Max Substrate was procured from Thermo Fischer Scientific, USA. Primary antibodies for PCNA, cyclin D1, NF- κ B (p50), IL-6, STAT3, pSTAT3, pAkt, PARP, caspase-3 and caspase-9 were purchased from Santa Cruz Biotechnologies, USA and antibodies for COX-2, Akt and β -actin were from Cell Signalling Technology, USA. The rest of the chemicals were purchased from local commercial sources and were of analytical grade.

2.3. Study design and treatment schedule

After one week of acclimatization, the breeding process was initiated by placing female and male mice in a ratio of 2:1. The day of the appearance of the vaginal plug was considered as day zero of gestation. Sixteen pregnant mice were randomly divided into four groups consisting of four mice each. Each mouse was housed individually. The four groups of studies were control, IP6, ENU, and ENU + IP6. Group I pregnant mice (control) received only 50 mM citrate buffer (pH-6.0) i.p. on the 17th days of gestation. Group II pregnant mice (IP6) received 2% IP6 in drinking water from the 17th days of gestation. Group III pregnant mice (ENU) received ENU at a dose of 40 mg/kg body weight dissolved in 50 mM citrate buffer pH-6.0 on the 17th days of gestation. Group IV pregnant mice (ENU + IP6) received ENU at a dose of 40 mg/kg body weight dissolved in 50 mM

Table 1
Treatments schedule.

Treatment group	No. of pregnant mice	Treatment on 17th days of gestation	No. of pups/group
Control	4	50 mM citrate buffer pH-6.0 (i.p.)	15–20
IP6	4	2% IP6 in drinking water	15–20
ENU	4	ENU in 50 mM citrate buffer pH-6.0 (i.p.)	15–20
ENU + IP6	4	ENU in 50 mM citrate buffer pH-6.0 (i.p.) and 2% IP6 in drinking water	15–20

citrate buffer pH-6.0 on the 17th days of gestation along with 2% IP6 in drinking water. Dose selection of ENU and IP6 was based on earlier studies from our and other laboratories [3,5,31,33]. All the treatments were continued for postnatal day (PND) 12 or 24 after the pups were delivered. Pups ($n = 15–20$) from each group were sacrificed at PND12 or 24 (Table 1). The lungs were harvested. Lungs from one third of the mice from each group were fixed in buffered formalin for histopathological analysis and lungs from the rest of the mice were taken out and processed for molecular studies.

2.4. Histopathology

At the time of termination, lungs from each mouse were perfused, washed in phosphate buffered saline (PBS) and fixed in 10% buffered formalin. Tissues were embedded in paraffin wax and 5 μ m thick median transverse sections were cut serially. Sections were de-paraffinized using xylene and graded concentrations of ethanol. Sections were then stained and counterstained with haematoxylin and eosin (H&E) to be examined under the light microscope for microscopic examination and photographed (100 \times) [33].

2.5. [methyl-³H] Thymidine incorporation in F1 mouse lung DNA

The [methyl-³H] thymidine incorporation assay was performed to assess the DNA synthesis in the F1 mouse lungs from the predisposed mothers following the method as described [34]. Here, a radioactive nucleoside is incorporated into new strands of chromosomal DNA during cell division. Pups from the four groups as described earlier were given intra-peritoneal injection of [methyl-³H] thymidine (33.0 μ Ci/animal in 0.1 ml normal saline) 1 h prior to the termination of the experiment at PND12 or 24. At the end of 1 h, animals were sacrificed; lungs were taken out and processed to prepare the acid hydrolysate for the DNA analysis as described. Acid hydrolysate, thus obtained, was used for DNA estimation and radioactivity counting. The concentration of DNA was read at 600 nm in spectrophotometer (BioRad, USA).

2.6. Real-time polymerase chain reaction for the status of miR-155 expression

Total RNA enriched with micro RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. For the evaluation of miR-155, 1 μ g of total RNA was reverse transcribed using a miScript II RT kit (Qiagen, The Netherlands) and micro RNA levels were quantified in ABI Step One plus thermo cycler (Applied

Biosystems, USA) using gene specific forward primer 5'-TTAATGCTAATTGTGATAGGGGT-3' for miR-155. A primer for reference control U6 was 5'-TGGCCCTGCGCAAGGATG-3' and miScript universal reverse primer was as provided with the kit. Briefly, 1 μ l (10 ng) of cDNA was incubated with 1 \times QuantiTect SYBR PCR master mix, 1 \times miScript universal primer, 0.5 pmol gene specific forward primer. The reaction was run for 15 min at 95 $^{\circ}$ C, followed by 40 cycles of 15 s at 94 $^{\circ}$ C, 30 s at 55 $^{\circ}$ C and 30 s at 70 $^{\circ}$ C. U6 was used as an endogenous control to normalize the gene expression. Relative expression of miR-155 was analyzed by using 2 Δ Ct values [35].

2.7. Analysis of protein expression by SDS-PAGE and western blotting

Lung tissue lysate was prepared in the 20 mM Tris-HCl (pH 7.5) buffer containing 2 mM sucrose, 2 mM EDTA, 0.5 mM EGTA, 2 mM MgCl₂, 2 mM PMSF, 1 mM DTT, 0.03 mM Na₃VO₄ and protease inhibitor cocktails (Sigma Co., USA). Protein concentration was quantified by Bradford's method. Tissue lysate equivalent to 50 μ g of protein was resolved on to 8–12.5% Tris-glycine gel and transferred onto an Immobilon-P-membrane (PVDF) (Millipore, USA) for 1 h at 90 V. Subsequently, membranes were blocked with 3% BSA in TBST (100 mM Tris-HCl, 150 mM NaCl and 0.1% Tween-20) for 1 h and probed with the primary antibodies for PCNA, cyclin D1, NF- κ Bp50, IL-6, STAT3, pSTAT3, pAkt, PARP, caspase-3, caspase-9, COX-2 and Akt at a dilution 1:3000. Further incubation was done with appropriate secondary antibodies conjugated to horseradish peroxidase (Bangalore Genei, India) at a dilution 1:10,000. The immunocomplex, thus formed, was visualized with the help of Super Signal West Femto Max Substrate in Versa Doc Gel Imaging System (Bio-Rad, Hercules, CA) and quantified using Gene Tool Syngene software. Each membrane was stripped and re-probed with β -actin as a loading control.

2.8. Immunohistochemical analysis of protein expression

To assess the localization of PCNA and caspase-3 (17 kDa), immunohistochemistry (IHC) was performed in F1 lung tissue sections following the earlier reported method with slight modifications [35]. 5 μ m paraffinized sections were dewaxed with xylene, hydrated in a descending series of alcohol and rinsed with water and blocked with horse serum for 30 min. Primary antibodies for PCNA and cleaved caspase-3 were used at a dilution 1:300 overnight in a moist chamber. ABC reagent was used with HRP conjugated secondary antibodies according to Vecta stain kit (Vector Lab). DAB was used as HRP substrate

for protein localization. Random micrographs were taken from DAB stained lung tissue section of the magnification of 400× and signal intensity was analyzed using open source ImageJ1.47v software, (National Institutes of Health, USA).

2.9. Estimation of the enzyme activity of the caspase-9 and -3

Enzyme activities of caspase-9 and caspase-3 were assessed in cytosolic fractions using a colorimetric assay kit from R&D system, USA. Briefly, fresh tissue lysate was prepared in lysis buffer from the kit and 100,000 × g fraction was collected. 50 μl cytosolic fractions containing 100–150 μg proteins were added to 50 μl 2× reaction buffers containing 10 mM DTT in a 96-well plate. 5 μl of 4 mM substrate (DEVD-pNA for caspase-3 or LEHD-pNA for caspase-9) was added to each sample and incubated at 37 °C for 2 h in dark condition. At the end, coloured complex was read at 405 nm in a microplate reader (BMG Labtech). Enzyme activity has been expressed in terms of optical density/mg protein/h in figures [3].

2.10. Detection of DNA fragmentation by TUNEL assay

DNA fragmentation, a hallmark of apoptosis was detected by terminal deoxynucleotidyl transferase (Tdt) mediated dUTP nick end-labelling (TUNEL). The procedure was carried out using a TACS 2Tdt DAB kit from Trevigen, USA, according to the manufacturer's protocol. TUNEL positive signals were visualized using the horseradish peroxidase-mediated diaminobenzidine (DAB) stain. Cell nuclei were counterstained with a methyl green solution as described elsewhere [3]. Random micrographs were taken from DAB stained lung tissue section from each group at the magnification of 400× and signal intensity was analyzed using open source ImageJ1.47v software (National Institutes of Health, USA) [35].

2.11. Statistical analysis

Data were expressed as mean ± SE. Statistical analysis of gene expression was performed using the Graph Pad Prism (version 5.0, Graph Pad Software Inc., USA). ANOVA was performed taking mean values for comparing between groups and statistical significance was determined in terms of 'P' values with the help of Newman–Keuls test analysis.

3. Results

3.1. Gross examination

There were no significant differences in the male:female ratio, litter size and body weight of the F1 mice between any of the treatment groups at the end of PND12 and 24. There was no abnormality like any lesion or tumourous growth in the F1 lungs from any group of treatment.

Administration of 2% IP6 in drinking water did not show any effect on the water consumption by the mice in any group in the present study.

3.2. Microscopic examination of F1 mouse lungs

H&E staining of paraffin-embedded tissue sections could not show any alteration in the histology of lungs from the control or IP6 treated group. Lungs from F1 mice from ENU exposed mother showed distinct histopathological changes in terms of lymphocytic infiltration at PND12. Lymphocytic infiltration was increased further and hyperplasia started appearing at PND24. Whereas, F1 mice from the ENU exposed mothers drinking IP6 during this period showed reduced lymphocytic infiltration at both the PND12 and 24. Under this condition, an inhibition was also observed in the incidence of hyperplasia at the PND24 (Fig. 1-I).

3.3. Incorporation of [methyl-³H] thymidine in the DNA of F1 mouse lungs

At the end of the treatment, [methyl-³H] thymidine incorporation into lung DNA and the status of DNA synthesis was evaluated at PND12 or 24. The [methyl-³H] thymidine incorporation in lung DNA was increased to 63 or 142% at the end of PND12 or 24 in F1 mice from ENU exposed mothers. In F1 mice from the ENU exposed mothers drinking IP6, [methyl-³H] thymidine incorporation into DNA was reduced (Fig. 1-II) and showed only 15 or 5% at PND12 or 24 as compared with control.

3.4. Status of the PCNA expression

In order to support the increased [methyl-³H] thymidine incorporation into DNA as a marker of proliferation, we analyzed the expression of PCNA. PCNA protein was overexpressed by 35 or 69% at PND12 or 24 in F1 mice from ENU exposed mothers as compared to control. But in F1 mice from the ENU exposed mothers drinking IP6, ENU induced expression of PCNA protein was very significantly reduced and showed only 2% increase or 7% decrease at PND12 or 24 as compared with control (Fig. 1-IIIb). Further, the overexpression of PCNA was supported by immunohistochemical analysis in F1 lung sections. ENU exposure increased the number of PCNA positive nuclei increased by 3.36 or 3.9-fold at PND12 or 24 as compared to control. In F1 mice from the ENU exposed mothers drinking IP6, the ENU induced PCNA positive nuclei were increased only to 2.21 or 1.36-fold at PND12 or 24 as compared with control (Fig. 1-IVb). Higher intensity of DAB stain in the nucleus suggested the nuclear localization of the overexpressed PCNA protein.

3.5. Status of NF-κB (p50), IL-6 or COX-2 expression

Expression of NF-κB(p50), IL-6 or COX-2 protein was increased at PND12 or 24 in F1 mice from the ENU exposed mothers as compared to control. IP6 administration to the mothers tried to prevent this overexpression. The increase in expression of NF-κB (p50), IL6 or COX-2 was 50, 28 or 37% at PND12 and 60, 43 or 46% at PND24 in F1 mice from the ENU exposed mothers as compared with control. In F1 mice from the ENU exposed mothers drinking IP6 during this period, ENU induced expression of NF-κB (p50), IL-6 or COX-2 was reduced to 25, 14 or 36% at PND12 and 16, 15

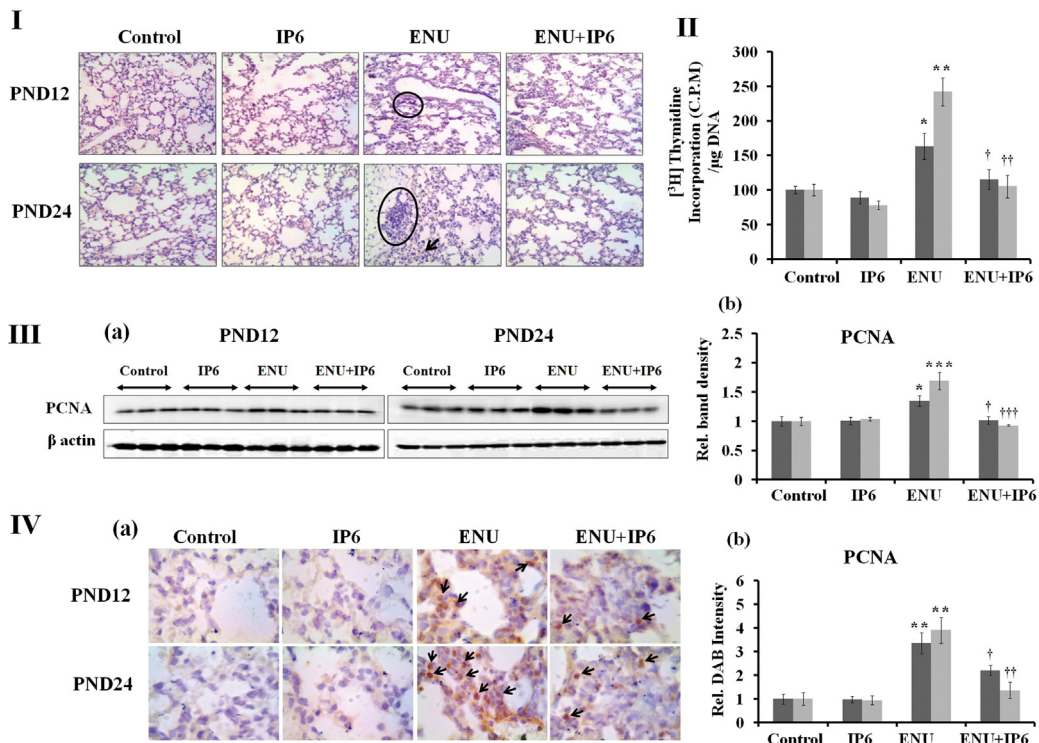


Fig. 1. Histopathology, [methyl-³H] thymidine incorporation and PCNA expression. Round circle indicates lymphocytic infiltration and arrow indicate hyperplasia in F1 lung from ENU exposed mothers (I). Quantitative analyses of [methyl-³H] thymidine incorporation (II). Western blot analysis of PCNA protein and quantitative analysis are shown in (IIIa) and (IIIb). Qualitative and quantitative analyses of IHC for PCNA are shown in (IVa) and (IVb). Arrows point out the higher DAB intensity in F1 mice from ENU exposed mothers is showing PCNA positive nuclei in F1 lung tissues section. Bars represent mean \pm SE of triplicate. * \dagger $P < 0.05$; ** $\dagger\dagger$ $P < 0.01$; *** $\dagger\dagger\dagger$ $P < 0.001$; *,** compared with control; \dagger , $\dagger\dagger$, $\dagger\dagger\dagger$ compared with ENU. ■ PND12; □ PND24.

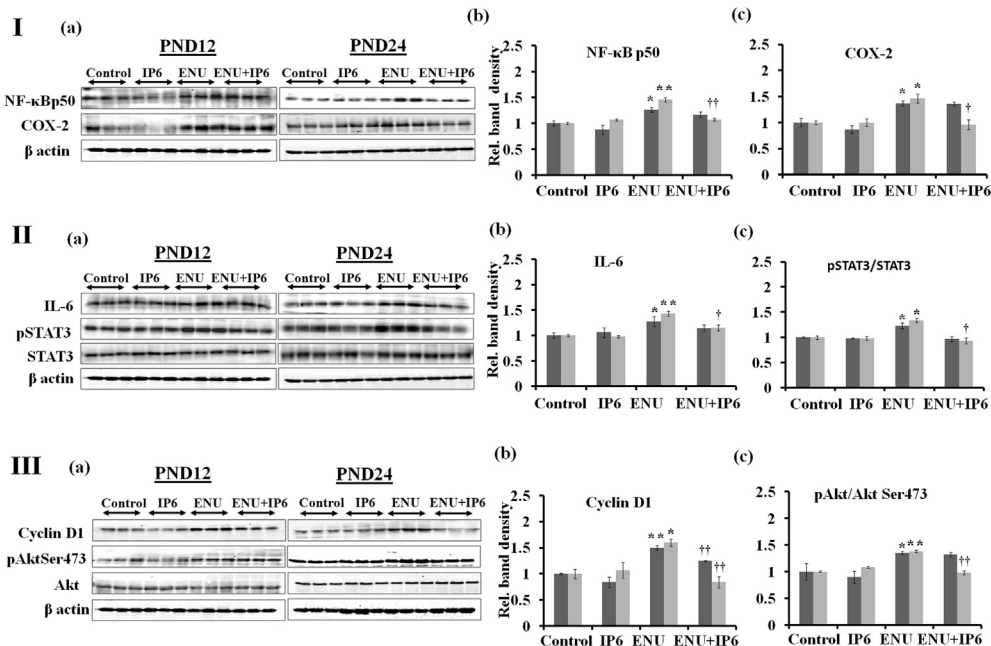


Fig. 2. Expression of inflammatory and cell survival genes. Western blot and quantitative analysis of COX-2, NF- κ B (p50), IL-6, pSTAT3, STAT3, pAkt Ser⁴⁷³, Akt, and cyclin D1 proteins are shown in (Ia), (IIa), (IIIa), (Ib,c), (IIb,c) and (IIIb,c). Bars represent mean \pm SE of triplicate. * \dagger $P < 0.05$; ** $\dagger\dagger$ $P < 0.01$; *** compared with control; \dagger , $\dagger\dagger$ compared with ENU. ■ PND12; □ PND24.

or 4% at PND24 as compared with control (Fig. 2-Ia–c and IIa–c).

3.6. Modulation of STAT3 as a result of IL-6 overexpression

IL-6 mediated activation of STAT3 transcription factor by its phosphorylation is critically involved in inflammation. We did not observe any treatment related change in the expression of STAT3 at PND12 or 24, but the overexpression of phosphorylated STAT3 (pSTAT3) protein was observed in F1 mice from the ENU exposed mothers. The expression of p-STAT3 was upregulated by 24 or 40% at PND12 or 24 in F1 mice from ENU exposed mothers. F1 mice from the ENU exposed mothers drinking IP6 showed only 1 or 6% downregulation of STAT3 at the PND12 or 24 as compared with control. The ratio of pSTAT3/STAT3 expression was evaluated and found to be 23 or 34% upregulated at PND12 or 24 as compared to control. In F1 mice from the ENU exposed mothers drinking IP6 during this period showed the ratio of pSTAT3/STAT3 upregulated by 3 or 7% at PND12 or 24 as compared with control (Fig. 2-IIa, c).

3.7. Activation of Akt pathway

As observed, ENU could induce the proliferation as well as inflammatory markers in F1 lung during the lactation period. Akt pathway could also play important roles in the regulation of cell proliferation, inflammation and apoptosis. To test our hypothesis whether Akt pathway is also involved in the regulation of cell proliferation, inflammation and apoptotic machinery in F1 mice from ENU exposed mothers, we evaluated the status of Akt and the activation of Akt by analysing phosphorylated serine 473 Akt residues. Akt expression remained unaltered at PND12 or 24 in F1 mice from ENU exposed mothers in the presence or absence of IP6 as compared to control. However, the expression of pAkt Ser473 was upregulated by 47 or 48% at PND12 or 24 in F1 mice from ENU exposed mothers. F1 mice from ENU exposed mothers drinking IP6 showed only 2 or 1% downregulation at PND12 or 24 as compared with control. The ratio of pAktSer473/Akt expression increased by 35 or 38% at PND12 or 24 in F1 mice from ENU exposed mothers as compared with control. In F1 mice from the ENU exposed mothers drinking IP6; this ratio did not vary at PND12. But at PND24, this ratio showed 2% decrease as compared with control (Fig. 2-IIa, c). These results indicate that IP6 inhibits the activation of Akt at an early stage of carcinogen exposure.

3.8. Status of the cyclin D1 expression

In order to show the relationship between Akt activation and the status of cyclin D1, we analyzed the expression of cyclin D1 and showed a significant increase by 50 or 60% at PND12 or 24 in F1 mice from the ENU exposed mothers as compared with control (Fig. 2-IIIa, b). F1 mice from the ENU exposed mothers drinking IP6 showed the reduced overexpression of cyclin D1 which remained 25% higher or 16% lower at PND12 or 24 as compared with control.

3.9. Expression of the apoptosis related proteins

We tried to evaluate the effectors pathway of apoptosis and showed that the expression of caspase-9 (35 kDa) or caspase-3 (17 kDa) was decreased by 24 or 22% at PND12 and 40 or 39% at PND24 in F1 mice from ENU exposed mothers as compared to control (Fig. 3-Ia, b). F1 mice from the ENU exposed mothers drinking IP6 showed a significant increase in the expressions of caspase-9 (35 kDa) or caspase-3 (17 kDa). The increase was 11 or 1% at PND12 and 4 or 6% at PND24 in F1 mice as compared with control.

In order to confirm the activation of one of the major executors of apoptosis, we analyzed the expression of the caspase-3 (17 kDa) by IHC (Fig. 3-IIa, b). The intensity of the DAB stain, an indicator of the protein expression, for the caspase-3 was decreased to 0.56 or 0.43-fold at PND12 or 24 respectively in F1 mice from ENU exposed mothers as compared with control. In F1 mice from the ENU exposed mothers drinking IP6, DAB intensity of activated caspase-3 was increased to 1.07 or 1.12-fold at PND12 or 24 respectively as compared with control.

3.10. Enzyme activity of the caspase-9 or caspase-3 protein

Having shown the changes in the expression of the caspase-9 or caspase-3 protein, we performed the enzyme assay to show the functional activities of the altered proteins. Enzyme activity of caspase-9 or caspase-3 was decreased to 18 or 20% at PND12 and 25 or 32% at PND24 in F1 mice from the ENU exposed mothers. In F1 mice from the ENU exposed mothers drinking IP6, enzyme activities of caspase-9 or caspase-3 significantly increased to 13 or 10% at PND12 and 25 or 22% at PND24 as compared with control. Taken together, these results suggest that IP6 drinking mothers during lactation period regulate the functional activities of both caspases that lead to the apoptosis in F1 mice (Fig. 3-III).

3.11. Expression of the miR-155

In order to explore the responsible factors for inhibiting the apoptosis, we evaluated the status of miR-155 that could target the apoptosis genes like caspase-3. MiR-155 was upregulated by 2.26 or 2.74-fold at PND12 or 24 in F1 mice from ENU exposed mothers. But F1 mice from the mothers drinking IP6 showed only 1.32 or 1.72-fold upregulation in miR-155 expression at PND12 or 24 as compared with control (Fig. 4-I).

3.12. Status of PARP cleavage supporting the apoptotic events

Expression of PARP, a substrate of activated caspase-3 was analyzed to show the downstream effects of caspase-3. The level of cleaved fragment PARP (85 kDa) was decreased to 35 or 42% in F1 mice from the ENU exposed mothers as compared with control at PND12 or 24 (Fig. 4-IIa, b). IP6 tried to prevent this decrease in F1 mice from the mothers drinking IP6 and showed only 1% decrease in the cleaved fragment PARP expression at PND12. But the cleaved

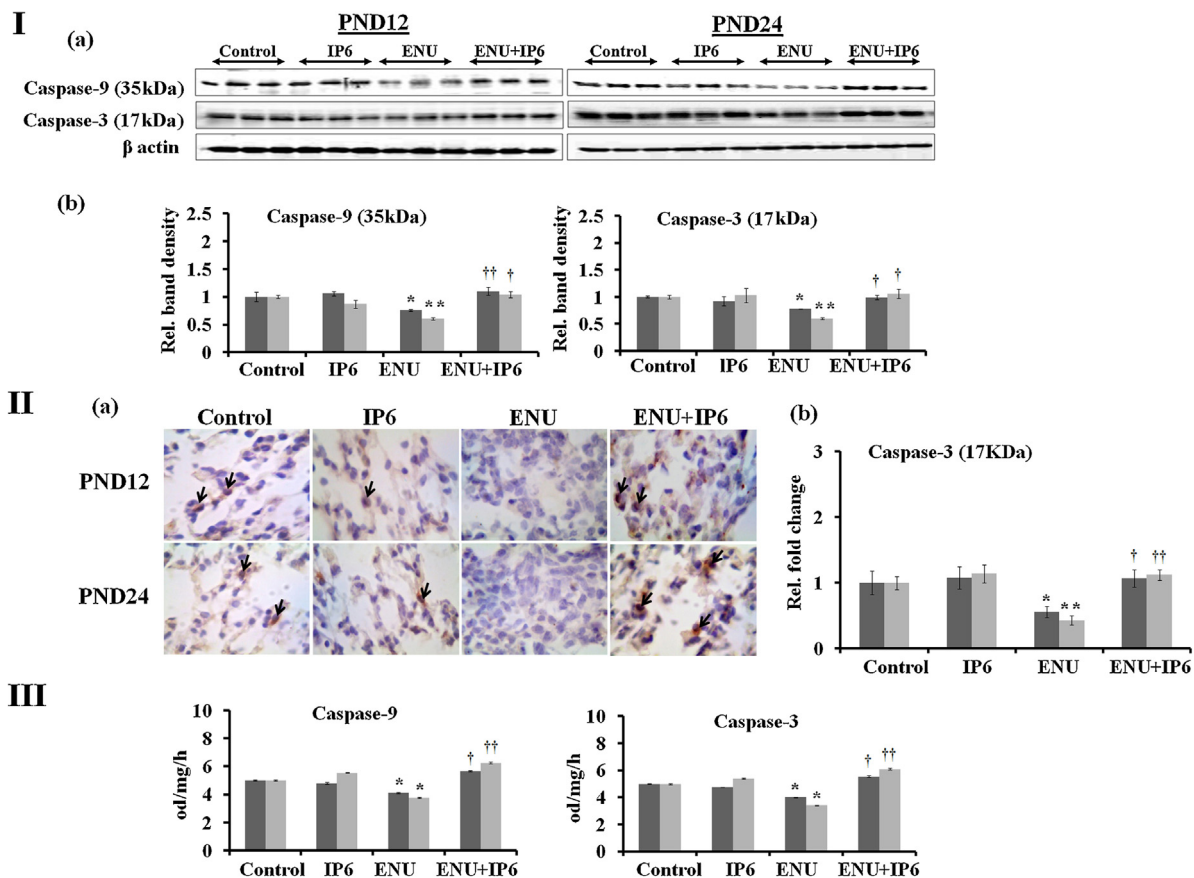


Fig. 3. Status of apoptotic molecules. Western blot and quantitative analysis of caspase-9 (35 kDa) and caspase-3 (17 kDa) are shown in (Ia) and (Ib). Qualitative and quantitative analyses of caspase-3 (17 kDa) done by IHC are shown in (IIa) and (IIb). Quantitative analysis of caspase-9 and caspase-3 enzyme activity is shown in (III). Bars represent mean \pm SE of triplicate. * \dagger $P < 0.05$; ** \dagger $P < 0.01$; ** compared with control; \dagger , \dagger compared with ENU. ■ PND12; □ PND24.

fragment PARP expression was increased by 6% at PND24 as compared with control.

3.13. *In situ* detection of DNA fragmentation to support the status of apoptosis

In order to support the alterations in the apoptosis, we determined the DNA fragmentation, a hallmark of apoptosis, by TUNEL assay in the F1 mice lungs from ENU exposed mothers in the presence or absence of IP6. Intensity of the DAB stain corresponds to the DNA fragmentation. DNA fragmentation as showed by DAB intensity was inhibited by 35 or 44% at PND12 or 24 in F1 mice from ENU exposed mothers as compared with control. In F1 mice from the mothers drinking IP6, the DNA fragmentation was enhanced by 8 or 18% at PND12 or PND24 as compared with control (Fig. 4-IIIa, b).

4. Discussion

There are growing evidences that carcinogen exposure to mothers during the gestation period could result in the adverse effects including cancer in the offspring [1]. Experimental studies have revealed that the process of

carcinogenesis can be modulated by timely intervention by the administration of chemopreventive agent or by intake of protective agents through regular food and drinks [7,29,36]. Numerous epidemiological as well as experimental studies have been shown that correlation between reduced risk of cancer and intake of dietary constituent. The intervention of carcinogenesis process in the post-initiation stage has to be the most appropriate and practical since this stage is reversible [37].

Appearance of lymphocytic infiltrations and hyperplasia at the end of PND12 or 24 in F1 mice from the ENU exposed mothers and its prevention by IP6 suggested the exploration of the molecular events of proliferation and inflammation as targets for the early management of tumour development. Earlier reports suggest that the alteration in proliferation and inflammatory molecules at an early stage facilitates the chemically induced tumour development in a later stage [3,13]. A time dependent increase in the [methyl- 3 H] thymidine incorporation in F1 mouse lung DNA and the overexpression of PCNA suggest the role of these molecules at an early stage of development during the lactation period. This observation is supported by the earlier studies on the expression of proliferation marker PCNA [10].

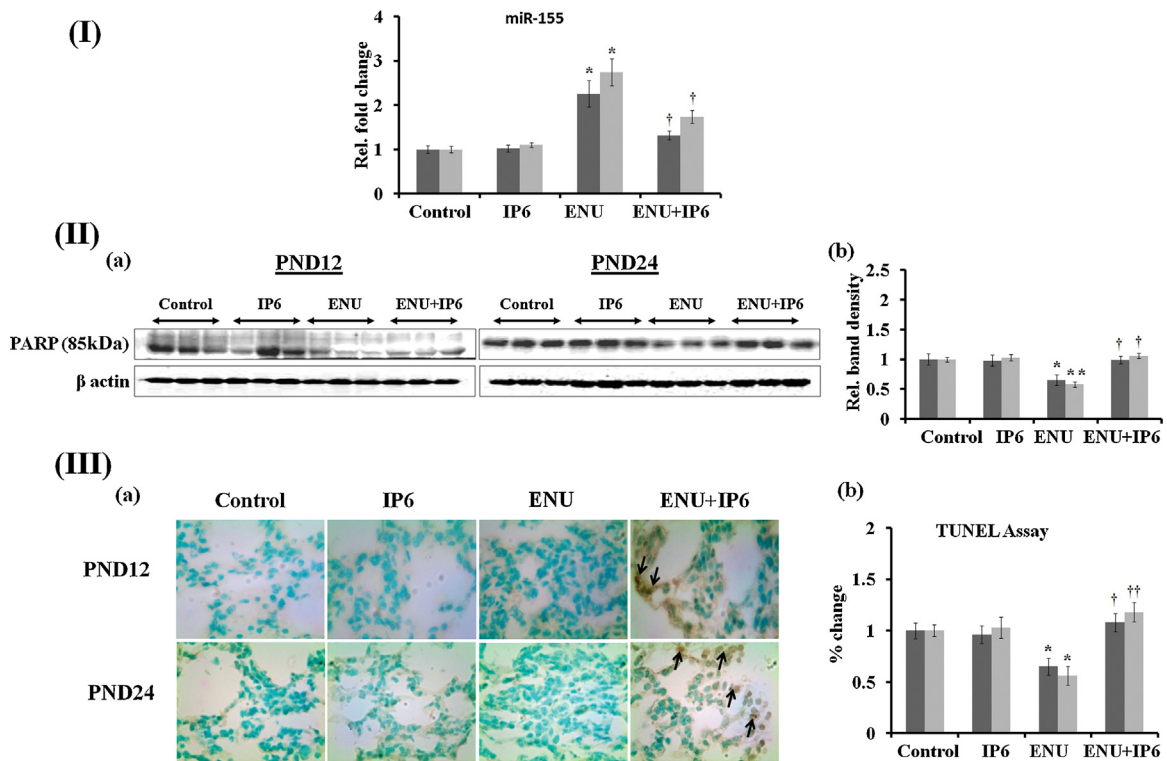


Fig. 4. Status of miR-155 and PARP. The relative fold change of miR-155 expression in F1 lung tissue is shown in (I). The result was normalized against the expression level of U6. Western blot and quantitative analysis of cleaved-PARP are shown in (IIa) and (IIb). Bars represent mean \pm SE of triplicate: * $\dagger P < 0.05$; ** $\dagger\dagger P < 0.01$; ** compared with control; $\dagger\dagger\dagger$ compared with ENU. Evaluation of apoptosis by TUNEL assay in F1 lung tissue sections from ENU exposed mothers at PND12 and 24 is shown in (IIIa). Fragmented DNA in apoptotic cells is stained brown with DAB and is shown by arrows at magnification 400 \times . Quantitative analysis of DNA fragmentation is shown in (IIIb). ■ PND12; □ PND24.

External stimuli are shown to enhance IL-6 and COX-2 via NF- κ B (p50) activation [13,38]. Similarly, we have also shown that the ENU exposed mothers can also trigger the inflammatory regulators and create microenvironment causing the lymphocytic infiltration and hyperplasia in offsprings. This is well supported by the earlier report [39] showing the accumulation of lymphocytic infiltration precedes the inflammation that could be inhibited by the chemopreventive agents [12,38]. Prevention of the ENU caused alterations by IP6 show the sensitivity of the molecules towards the antitumour agents. Beside this, the prevention of inflammation and hyperplasia in terms of IL-6, COX-2 and NF- κ B (p50) in F1 mouse from the ENU exposed mothers drinking IP6 suggests its chemopreventive effects as early as the time of lactation. Activation of NF- κ B (p50) and STAT3 is well known to contribute in the regulation of inflammation and cell proliferation [13]. Our analysis showed that the activated NF- κ B (p50) and STAT3 could regulate many of the genes involved in the regulation of inflammation, proliferation and the inhibition of the apoptotic machinery and their sensitivity towards the chemopreventive agent, IP6.

Akt pathway is an important target for the anticancer drug development as it plays an important role in the regulation of cell proliferation and inflammation, by regulating cyclin D1 and NF- κ B [20] and also in regulating the apoptosis. Our results showing the activation of the Akt pathway

by rapidly inducing the phosphorylation of Akt in the early stages of development of F1 mice from the predisposed mothers are in accordance with the previous reports [19].

Increasing interest in the strategies for cancer prevention, chemopreventive approaches have been used either to suppress or prevent the initial phases of tumourigenesis like cell proliferation, inflammation and induce apoptosis. Chemopreventive agents such as indole-3-carbinol and epigallocatechin gallate are known to suppress Akt activation and promote apoptosis [37]. IP6 supplementation to predisposed mothers prevented the Akt activation in the offsprings possibly by reducing the Akt phosphorylation suggesting that the related molecules could also be controlled by the IP6. As most of the chemopreventive agents inhibit the carcinogenesis by inducing the apoptosis [37], the induction of apoptosis is among the important strategies for the development of anticancer drugs [9,8,14]. In the same line of studies, we showed the downregulation of the expression of caspase-9 and caspase-3 in F1 mice from the ENU exposed mothers and its prevention by the IP6. Functional activity of the overexpressed genes supported the prevention and the onset of the apoptosis in the early stages after the exposure.

Activation of PARP, a result of an activated caspase-3, causes DNA fragmentation [15]. Our results on the inhibition of DNA fragmentation suggest the reduced apoptosis in F1 mice from the ENU exposed mothers which appeared

to be reversed in the presence of IP6 possibly due to the increased DNA fragmentation as a result of PARP cleavage. We infer that the inhibition of lymphocytic infiltration and hyperplasia by IP6 could be due to the induction of apoptotic events as reported by others [32].

MicroRNAs, the short non-coding RNAs, influence a variety of cellular functions, including proliferation, inflammation and apoptosis [27]. MiR-155 has been shown to predominantly confine to endothelial and immune cells and gets upregulated to promote the cell proliferation [24–26]. We have shown that ENU promoted the lymphocytic infiltration, during the lactation and we also showed the relationship between the lymphocytic infiltration and the status of nir155. The downregulation of miR-155 expression is also associated with induction of apoptosis through the activation of caspase-3 which has been identified as a novel target of miR-155 [27]. In accordance with the earlier reports, our results show that the upregulation of miR-155 in F1 mice from the ENU exposed mother could be the cause of the downregulation of apoptotic events. This is supported by the inhibitory effects of IP6 on the expression of miR-155 and causing apoptosis possibly through a caspase-3 mediated mechanism in F1 mice from predisposed mothers.

5. Conclusion

Our results clearly showed that proliferation, inflammation, apoptotic molecules as well as miR-155 are affected in F1 mice from the mothers predisposed to carcinogen and could be the cause of the lymphocytic infiltrations and development of hyperplasia as early as at the PND12 and 24. However, predisposed mothers taking antitumour agents during this (lactation) period could prevent the lymphocytic infiltration and hyperplasia in F1 mice and effectively modulate the critical events involved in proliferation, inflammation and apoptosis.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

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References

- [1] M.S. Miller, K.M. Gressani, S. Leone-Kabler, A.J. Townsend, A.M. Malkinson, M.G. O'Sullivan, Differential sensitivity to lung

- tumorigenesis following transplacental exposure of mice to polycyclic hydrocarbons, heterocyclic amines, and lung tumor promoters, *Exp. Lung Res.* 26 (2000) 709–730.
- [2] V. Capilla-Gonzalez, S. Gil-Perotin, A. Ferragud, L. Bonet-Ponce, J.J. Canales, J.M. Garcia-Verdugo, Exposure to N-ethyl-N-nitrosourea in adult mice alters structural and functional integrity of neurogenic sites, *PLoS ONE* 7 (2012) e29891.
- [3] S. Sahay, D.S. Upadhyay, K.P. Gupta, N-ethyl-N-nitrosourea-induced transplacental lung tumor development and its control: molecular modulations for tumor susceptibility in a mouse model, *Toxicol. Res.* 4 (2015) 71–82.
- [4] E.J. Tokar, B.A. Diwan, M.P. Waalkes, Renal, hepatic, pulmonary and adrenal tumors induced by prenatal inorganic arsenic followed by dimethylarsinic acid in adulthood in CD1 mice, *Toxicol. Lett.* 209 (2012) 179–185.
- [5] J.M. Rice, Transplacental carcinogenesis in mice by 1-ethyl-1-nitrosourea, *Ann. N. Y. Acad. Sci.* 163 (1969) 813–827.
- [6] W. Slikker, N. Mei, T. Chen, N-ethyl-N-nitrosourea (ENU) increased brain mutations in prenatal and neonatal mice but not in the adults, *Toxicol. Sci.* 81 (2004) 112–120.
- [7] D.J. Castro, C.V. Löhr, K.A. Fischer, C.B. Pereira, D.E. Williams, Lymphoma and lung cancer in offspring born to pregnant mice dosed with dibenzo[a,h]pyrene: the importance of in utero versus lactational exposure, *Toxicol. Appl. Pharmacol.* 233 (2008) 454–458.
- [8] X. Yu, Y. Pan, H. Ma, W. Li, Simvastatin inhibits proliferation and induces apoptosis in human lung cancer cells, *Oncol. Res.* 20 (2013) 351–357.
- [9] Q. Dang, W. Song, D. Xu, Y. Ma, F. Li, J. Zeng, L. Li, Kaempferol suppresses bladder cancer tumor growth by inhibiting cell proliferation and inducing apoptosis, *Mol. Carcinog.* (2014), <http://dx.doi.org/10.1002/mc.22154>
- [10] D.R. Dietrich, Toxicological and pathological applications of proliferating cell nuclear antigen (PCNA), a novel endogenous marker for cell proliferation, *Crit. Rev. Toxicol.* 23 (1993) 77–109.
- [11] D.M. Brantley, C.L. Chen, R.S. Muraoka, P.B. Bushdid, J.L. Bradberry, F. Kittrell, F.E. Yull, Nuclear factor- κ B (NF- κ B) regulates proliferation and branching in mouse mammary epithelium, *Mol. Biol. Cell* 12 (2001) 1445–1455.
- [12] S. Shishodia, P. Potdar, C.G. Gairola, B.B. Aggarwal, Curcumin (diferuloylmethane) down-regulates cigarette smoke-induced NF- κ B activation through inhibition of I κ B α kinase in human lung epithelial cells: correlation with suppression of COX-2, MMP-9 and cyclin D1, *Carcinogenesis* 24 (2003) 1269–1279.
- [13] M. Pandey, K.P. Gupta, Involvement of STAT3, NF- κ B and associated downstream molecules before and after the onset of urethane induced lung tumors in mouse, *Environ. Toxicol. Pharmacol.* 34 (2012) 502–511.
- [14] A. Catassi, D. Servent, L. Paleari, A. Cesario, P. Russo, Multiple roles of nicotine on cell proliferation and inhibition of apoptosis: implications on lung carcinogenesis, *Mutat. Res.* 659 (2008) 221–231.
- [15] X. Sheng, Y. Sun, Y. Yin, T. Chen, Q. Xu, Cirsilineol inhibits proliferation of cancer cells by inducing apoptosis via mitochondrial pathway, *J. Pharm. Pharmacol.* 60 (2008) 1523–1529.
- [16] J. Brognard, A.S. Clark, Y. Ni, P.A. Dennis, Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation, *Cancer Res.* 61 (2001) 3986–3997.
- [17] M.E. St-Germain, V. Gagnon, S. Parent, E. Asselin, Regulation of COX-2 protein expression by Akt in endometrial cancer cells is mediated through NF- κ B/I κ B pathway, *Mol. Cancer* 3 (2004) 7–13.
- [18] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, *Cell* 144 (2011) 646–674.
- [19] A.S. Tsao, T. McDonnell, S. Lam, J.B. Putnam, N. Bekele, W.K. Hong, J.M. Kurie, Increased phospho-AKT (Ser473) expression in bronchial dysplasia implications for lung cancer prevention studies, *Cancer Epidemiol. Biomark. Prev.* 12 (2003) 660–664.
- [20] S. Fatrai, L. Elghazi, N. Balcazar, C. Cras-Méneur, I. Krits, H. Kiyokawa, E. Bernal-Mizrachi, Akt induces β -cell proliferation by regulating cyclin D1, cyclin D2, and p21 levels and cyclin-dependent kinase-4 activity, *Diabetes* 55 (2006) 318–325.
- [21] J.P. Alao, The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic intervention, *Mol. Cancer* 6 (2007) 24–40.
- [22] D.P. Bartel, MicroRNAs: target recognition and regulatory functions, *Cell* 136 (2009) 215–233.
- [23] X. Wu, M.G. Piper-Hunter, M. Crawford, G.J. Nuovo, C.B. Marsh, G.A. Otterson, S.P. Nana-Sinkam, MicroRNAs in the pathogenesis of lung cancer, *J. Thorac. Oncol.* 4 (2009) 1028–1034.

- [24] C.M. Zhang, J. Zhao, H.Y. Deng, miR-155 promotes proliferation of human breast cancer MCF-7 cells through targeting tumor protein 53-induced nuclear protein 1, *J. Biomed. Sci.* 20 (2013) 79–89.
- [25] T. Donnem, K. Eklo, T. Berg, S.W. Sorbye, K. Lonvik, S. Al-Saad, L.T. Busund, Prognostic impact of MiR-155 in non-small cell lung cancer evaluated by in situ hybridization, *J. Transl. Med.* 9 (2011) 1–9.
- [26] Y. Wang, J. Li, L. Tong, J. Zhang, A. Zhai, K. Xu, M. Chu, The prognostic value of miR-21 and miR-155 in non-small-cell lung cancer: a meta-analysis, *Jpn. J. Clin. Oncol.* 43 (2013) 813–820.
- [27] S. Li, T. Chen, Z. Zhong, Y. Wang, Y. Li, X. Zhao, microRNA-155 silencing inhibits proliferation and migration and induces apoptosis by upregulating BACH1 in renal cancer cells, *Mol. Med. Rep.* 5 (2012) 949–954.
- [28] Y.J. Surh, Cancer chemoprevention with dietary phytochemicals, *Nat. Rev. Cancer* 3 (2003) 768–780.
- [29] G.J. Kelloff, J.A. Crowell, V.E. Steele, R.A. Lubet, W.A. Malone, C.W. Boone, C.C. Sigman, Progress in cancer chemoprevention: development of diet-derived chemopreventive agents, *J. Nutr.* 130 (2000) 467S–471S.
- [30] M.H. Pan, C.S. Lai, J.C. Wu, C.T. Ho, Molecular mechanisms for chemoprevention of colorectal cancer by natural dietary compounds, *Mol. Nutr. Food Res.* 55 (2011) 32–45.
- [31] M. Gu, S. Roy, K. Raina, C. Agarwal, R. Agarwal, Inositol hexaphosphate suppresses growth and induces apoptosis in prostate carcinoma cells in culture and nude mouse xenograft: PI3K–Akt pathway as potential target, *Cancer Res.* 69 (2009) 9465–9472.
- [32] N.H. Shafie, N.M. Esa, H. Ithnin, N. Saad, A.K. Pandurangan, Pro-apoptotic effect of rice bran inositol hexaphosphate (IP6) on HT-29 colorectal cancer cells, *Int. J. Mol. Sci.* 14 (2013) 23545–23558.
- [33] M. Pandey, K.P. Gupta, Epigenetics, an early event in the modulation of gene expression by inositol hexaphosphate in ethylnitrosourea exposed mouse lungs, *Nutr. Cancer* 63 (2011) 89–99.
- [34] K.P. Gupta, N.K. Mehrotra, Status of ornithine decarboxylase activity and DNA synthesis in mancozed-exposed mouse skin, *Carcinogenesis* 13 (1992) 131–133.
- [35] M. Pandey, S. Sahay, P. Tiwari, D.S. Upadhyay, S. Sultana, K.P. Gupta, Involvement of EZH2, SUV39H1, G9a and associated molecules in pathogenesis of urethane induced mouse lung tumors: potential targets for cancer control, *Toxicol. Appl. Pharmacol.* 280 (2014) 296–304.
- [36] E. Graf, J.W. Eaton, Suppression of colonic cancer by dietary phytic acid, *Nutr. Cancer* 19 (1993) 11–19.
- [37] K. Toshiya, T. Testuya, H. Akira, T. Takuji, Cancer chemoprevention through the induction of apoptosis by natural compounds, *J. Biophys. Chem.* 3 (2012) 156–173.
- [38] D. Wang, R.N. DuBois, The role of COX-2 in intestinal inflammation and colorectal cancer, *Oncogene* 29 (2009) 781–788.
- [39] A.K. Bauer, L.D. Dwyer-Nield, A.M. Malkinson, High cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) contents in mouse lung tumors, *Carcinogenesis* 21 (2000) 543–550.