

Prevention of liver carcinogenesis by amarogentin through modulation of G₁/S cell cycle check point and induction of apoptosis

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Amarogentin, a secoiridoid glycoside, is an active component of the medicinal plant *Swertia chirata*. In this study, chemopreventive and chemotherapeutic actions of amarogentin were evaluated in a carbon tetrachloride (CCl₄)/N-nitrosodiethylamine (NDEA)-induced liver carcinogenesis mouse model system during continuous and posttreatment schedule. Better survival, no toxicity and increased body weight were noted in amarogentin-treated mice. Reduction in proliferation and increase in apoptosis frequency were evident in amarogentin-treated groups. In carcinogen control group moderate dysplasia, severe dysplasia and hepatocellular carcinoma were evident at 10th, 20th and 30th week, respectively. Amarogentin was found to prevent progression of liver carcinogenesis at mild dysplastic stage. Exposure to CCl₄/NDEA resulted in upregulation of ppRb807/811, cyclinD1 and cdc25A at 10th week and additional activation of cMyc and mdm2 along with downregulation of LIMD1, p53 and p21 at 20th week. This was followed by activation of ppRb567 and downregulation of RBSP3 at 30th week. Prevention of carcinogenesis by amarogentin in both groups might be due to cumulative upregulation of LIMD1, RBSP3, p16 and downregulation of cdc25A at 10th week along with activation of p53 and p21 and downregulation of ppRb807/811 and ppRb567 at 20th week, followed by downregulation of cyclinD1, cMyc and mdm2 at 30th week. During carcinogenesis reduction of apoptosis was evident since 20th week. However, amarogentin treatment could significantly induce apoptosis through upregulation of the Bax-Bcl2 ratio, activation of caspase-3 and poly ADP ribose polymerase cleavage. This is the first report of chemopreventive/therapeutic role of amarogentin during liver carcinogenesis through modulation of cell cycle and apoptosis.

Introduction

Epidemiological and experimental studies have identified many natural compounds with cancer prevention and therapeutic potential (1). *Swertia chirata*, a medicinal plant traditionally used in Indian ayurvedic medicine for treatment of liver disorders (2). Amarogentin, a bitter glycoside, is active component of this plant. The anticarcinogenic activity of this compound was first reported in a mouse skin carcinogenesis model. Amarogentin was shown to inhibit cellular proliferation and induce apoptosis (3). However, no study has yet been undertaken to elucidate chemopreventive or therapeutic effect of amarogentin during liver carcinogenesis. Liver cancer is one of the most lethal malignancies worldwide (4) and the prognosis of liver cancer after surgery is very poor along with various side effects of chemotherapeutic drugs (5). Hence chemopreventive intervention for

Abbreviations: AFP, α -fetoprotein; BrdU, 5-bromo-2-deoxyuridine; CCl₄, carbon tetrachloride; HCC, hepatocellular carcinoma; NDEA, N-nitrosodiethylamine; PARP, poly ADP ribose polymerase; Rb, retinoblastoma; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

prevention of liver carcinogenesis may provide a feasible alternative strategy for prevention of hepatocellular carcinoma.

In the normal liver, hepatocytes are mitotically quiescent but can rapidly proliferate in response to tissue injury (6). These cells are under tight control of cell division and programmed cell death. Aberrations in cell cycle and apoptosis have been reported in hepatocellular carcinoma (HCC) (7,8). It seems that perturbation of sequential retinoblastoma (Rb) phosphorylation due to deregulation of different cell cycle kinases like cdk2/4/6 might be associated with tumor development in liver. Overexpression of ppRb, cMyc, cyclinD1, cdc25A and mdm2 and inactivation of p16, p21 and p53 have been reported in several malignancies including HCC (9–19). In addition, there are certain cell cycle inhibitors like LIMD1 that facilitates Rb–E2F interaction (20), RBSP3 that dephosphorylates Rb at Ser 807/811 at early G₁ phase of cell cycle (21). However, association of these genes with developmental HCC is not known although their associations with other tumors like head and neck, breast and lungs have been reported (20–25). Apoptosis induction is an important event for a chemopreventive and chemotherapeutic drugs (26,27). The apoptosis occurs in a cell through either intrinsic or extrinsic pathway (28). In a mouse skin carcinogenesis model amarogentin was found to induce apoptosis through intrinsic pathway (29). In intrinsic pathway, increase in Bax:Bcl2 ratio releases cytochrome-C from mitochondria leading to activation of caspase-3 and inactivation of poly ADP ribose polymerase (PARP) (30). Thus, it is pertinent to analyze the regulatory genes in the cell cycle particularly at G₁/S phase and apoptosis to understand the chemopreventive mechanism of amarogentin.

In this study, chemopreventive efficacy of amarogentin was evaluated on a mouse liver carcinogenesis model. Cellular proliferation and apoptosis were analyzed along with expression analysis of several regulatory genes associated with G₁/S phase of cell cycle and apoptosis during the process of carcinogenesis. Our study clearly demonstrates that amarogentin prevents liver carcinogenesis at mild dysplastic stage through modulation of cellular proliferation and apoptosis.

Materials and methods

Experimental animals

Female Swiss albino mice (5 weeks, average weight 25 g) were obtained from animal house of Chittaranjan National Cancer Institute, Kolkata, India. Animals were maintained at 25 ± 5°C temperature under alternating 12 h light/dark cycle with 45–55% humid conditions. Drinking water and food pellets (Lipton India Ltd) were provided. Food pellets contain wheat flour 22.5%, roasted black gram flour 60%, skimmed milk powder 5%, casein 4%, refined ground nut oil 4%, salt mix with starch 4% and vitamin mix 0.5%. Animal handling and experimental protocol were approved by institutional ethical committee.

Chemicals

Extraction and purification of amarogentin (>99% pure in high-performance liquid chromatography analysis) (Supplementary document 1, available at *Carcinogenesis* Online) were done at the National Research Institute for Ayurvedic Drug Development, Kolkata, India. NDEA was purchased from Sigma Chemical Co. 5-Bromo-2-deoxyuridine (BrdU) Labeling and Detection Kit II, In situ Cell Death Detection Kit II and POD kit were procured from Roche Molecular Biochemicals, Mannheim, Germany. TRIzol reagent was product of Roche, Mannheim, Germany. All required primary antibody, IgG-horseradish peroxidase-conjugated secondary antibody and luminol reagent were purchased from Santa Cruz Biotechnology, Inc.

Experimental design

Following experimental groups were selected for this study:

- Group I:** Normal control without any treatment.
- Group II:** Mice in this group received intraperitoneal injection (i.p.) of CCl₄ (50 μ l/kg body weight) in liquid paraffin successively for

4 days followed by NDEA (75 mg/kg body weight) injection i.p. weekly for three successive weeks and 100 mg/kg body weight for another three successive weeks. This protocol was adapted from our pilot study based on previous reports (31,32).

Group III: Mice of this group received oral administration of amarogentin (0.2 mg/kg body weight) twice weekly, 15 days prior to carcinogen application. Then, amarogentin was administered once a week during carcinogen application and continued during the experimental period.

Group IV: This group of mice received amarogentin orally once a week (0.2 mg/kg body weight) starting 1 week after completion of carcinogen application and continued till the end of the experiment.

Number of animals were 12 for each group. Mice were under observation for their well being, body weight, toxicity and survival. Mice from different experimental groups were killed at 10th, 20th and 30th week of first carcinogen application. At each time point three animals were killed from each group.

Determination of amarogentin dose

To select the specific dose of amarogentin for oral administration, subacute toxicity study was conducted in mice for 15 days (twice weekly) at different doses (0.1, 0.2, 0.4 and 0.8 mg/kg body weight) of amarogentin. There were six mice in each group including control mice. Blood was collected from orbital sinus of the mice of each group after scheduled time. Biochemical parameters related to liver toxicity and nephrotoxicity were studied with respect to control mice.

Sample collection

After killing, liver was dissected out from each mouse. The affected lobe of liver was washed in phosphate buffer saline and divided for following analysis: BrdU incorporation for cell proliferation assay, formalin fixation (10%) for histopathological analysis and *in situ* cell death assay [terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay] and preparation of RNA and protein for expression analysis.

Histopathological evaluation

The formalin-fixed tissue samples were processed conventionally to prepare paraffin blocks followed by tissue sectioning at 3–4 μ m and hematoxylin-eosin staining. Stained slides were observed under light microscope and photographed.

In situ cell proliferation assay

Percentages of proliferative cells in liver sections were determined using BrdU labeling and detection kit II. Liver tissue was placed into pre-warmed (37°C) cell culture medium containing BrdU and incubated for 1 h followed by fixation of tissues. After paraffin embedding and sectioning, tissue sections were processed for BrdU assay according to manufacturers' protocol. For each sample BrdU positive cells were counted at six randomly chosen fields at 40 \times magnification in blinded manner. Percentage of proliferating cells was determined from the average BrdU positive cells.

In situ cell death detection using TUNEL assay

In situ apoptosis analysis was done in the paraffin-embedded tissue sections (3–4 μ m) by the TUNEL method using *in situ* cell death detection and POD kit according to manufacturers' protocol. For each sample, TUNEL positive cells were counted at six randomly chosen fields at 40 \times magnification in blinded manner. Then percentage of apoptotic cells was determined from the average TUNEL positive cells.

Quantitative real-time-PCR analysis

Total tissue RNA was extracted from affected liver tissue parts by TRIzol reagent (Invitrogen) according to the manufacturers' protocol. The mRNA expression was analyzed by real-time PCR (ABI Prism 7500) using primers mentioned in (Supplementary document 2, available at *Carcinogenesis* Online) and Power SYBR Green PCR Master Mix (Applied Biosystems). Mouse β 2-microglobulin gene was used as control. Each sample was loaded in triplicate. Relative level of gene expression was determined by comparative threshold cycle (ddCt) method (33) after normalization against β 2-microglobulin gene. Relative expression was graphically represented.

Protein extraction and western blot analysis

Total protein was extracted from liver lesion by sonication with RIPA buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.1% Triton-X 100, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF and 1 mM sodium orthovanadate]. After quantification protein gel was electrophoresed in 10–15% sodium dodecyl sulfate-polyacrylamide gel. The electrophoretically resolved proteins were then transferred onto Immobilon P-polyvinylidene difluoride membrane (Millipore,

MA). Membrane was then blocked with 5% bovine serum albumin for 1 h at room temperature and then proteins were detected with appropriate primary antibodies (enlisted in Supplementary document 2, available at *Carcinogenesis* Online) followed by horseradish peroxidase-conjugated specific secondary antibodies (Supplementary document 2, available at *Carcinogenesis* Online). The target protein bands were then visualized using luminol reagent and autoradiographed on X-ray film (Kodak). The band intensities were quantified using densitometric scanner (Bio-Rad GS-800). Peak densities of the proteins of interest were normalized using peak density of loading control tubulin and graphically represented after normalization with Group I.

Immunohistochemical analysis

The expression of Rb, pRB (SER 807/811), pRB (SER 567), LIMD1, RBSP3 and α -fetoprotein (AFP) proteins was determined by immunohistochemistry. About 3–5 μ m paraffin sections were dewaxed rehydrated (antigen retrieval was performed using citrate buffer pH 6 at 90°C for 30–45 min as requirement) and incubated overnight with primary antibodies with specific dilution at 4°C. Horseradish peroxidase-conjugated secondary antibodies were added followed by color substrate reaction using diaminobenzidine chromogen and counterstaining with hematoxylin. The staining intensity (1 = weak, 2 = moderate and 3 = strong) and the percentage of immunopositive cells (<1 = 0, 1–20 = 1, 20–50 = 2, 50–80 = 3 and >80 = 4) were determined by two observers independently and by combining the two scores, final evaluation of expression was done (0–2 = low, 3–4 = intermediate, 5–6 = normal and 7 = high) (34). The expression pattern of a gene in the experimental group was compared with its expression in normal control (Group I).

Statistical analysis

Data obtained from Group II were compared with Group I and data obtained from Group III and IV were compared with Group II. Student's paired *t*-test was used for pair-wise group comparisons, as needed. Fisher's exact test was used to determine the association between carcinoma formation after exposure of CCl₄/NDEA with or without amarogentin. Survival curve was plotted according to Kaplan–Meier method by using statistical program SPSS (SPSS Inc, Chicago, IL). All statistical tests were two-sided, and $P < 0.05$ and $P < 0.001$ were considered statistically significant. Data are expressed as mean with standard deviation.

Results

Selection of amarogentin dose

It was evident that amarogentin at 0.2 mg/kg body weight has better protective effect on liver and kidney than higher doses (Supplementary Figure 1A, available at *Carcinogenesis* Online). For this reason, amarogentin at a dose of 0.2 mg/kg body weight was selected for this study.

Effect of amarogentin on changes in body weight and survival during liver carcinogenesis

Group I mice showed gradual increase in body weight. In Group II body weight of mice gradually decreased upto 10th week, then it became comparable in the following weeks. Overall, mice body weight in this group significantly decreased ($P = 0.00009$). Amarogentin treatment significantly increased the body weight (Group III: $P = 0.00007$; Group IV: $P = 0.004$). Body weight of mice in Group III initially decreased up to sixth week of carcinogen treatment and then it became comparable till end of the experiment. In Group IV decrease in mice body weight was noted upto 10th week, and then body weight gradually increased in the following weeks (Supplementary Figure 1B, available at *Carcinogenesis* Online).

Survival of mice was analyzed during liver carcinogenesis by Kaplan–Meier survival analysis on a different set of experiment without sacrificing any animal upto 30th week of carcinogen treatment. Group II mice showed 67% survival, which is significantly ($P = 0.0318$) lower than Group I. Amarogentin treatment increased the survival percentage in Group III (83%) and Group IV (75%) (Supplementary Figure 1C, available at *Carcinogenesis* Online).

Analysis of macroscopic liver images and histopathological changes during liver carcinogenesis

Effects of both carcinogen and amarogentin were very prominent on liver, visualized macroscopically. In case of Group II, normal

smoothness of liver was lost and it became very rough in its texture and appearance at 10th week; small foci were observed at 20th week; large foci all over the liver were evident at 30th week. In contrast, no marked changes were visible in mice of Group III and Group IV at different time points, except for few foci evident at 30th week in few Group IV mice liver (Supplementary Figure 2A, available at *Carcinogenesis* Online).

Histopathological analysis revealed larger cell size, nuclear size and derangement in tissue architecture indicating moderate dysplasia in Group II mice at 10th week; severe dysplastic changes and HCC were evident at 20th and 30th week, respectively (Table I; Supplementary Figures 1D and 2B, available at *Carcinogenesis* Online). Amarogentin treatment significantly prevented ($P = 0.008$) carcinogenesis. In Group III, mild dysplastic liver lesions were evident in 10th to 30th week except 2/6 mice with moderate dysplastic liver lesions at 30th week (Table I; Supplementary Figures 1D and 2B, available at *Carcinogenesis* Online). Few lymphocytic infiltrations at 10th week were noted in Group III (image not shown). In Group IV moderate dysplastic liver lesions were evident at 10th week, whereas mild dysplastic liver lesions were predominantly evident at 20th week (4/5) and 30th week (3/5) (Table I; Supplementary Figures 1D and 2B, available at *Carcinogenesis* Online).

Analysis of cellular proliferation and apoptosis during liver carcinogenesis

In Group I mice frequency of proliferating liver cells (BrdU-incorporated cells) was about 20%, which were mainly localized surrounding portal vein area (Supplementary Figure 2C, available at *Carcinogenesis* Online; Figure 1A). In Group II mice liver lesions, frequency of proliferating cells significantly increased at all time points. Amarogentin treatment resulted in significant decrease in frequency of proliferating cells at all time points in Group III. In Group IV mice liver lesion and low frequency of proliferating cells were noted at 10th week, with significant decrease at 20th and 30th weeks (Figure 1A).

On the other hand, Group I mice showed ~12% apoptotic cells around portal vein area (Supplementary Figure 2D, available at *Carcinogenesis* Online; Figure 1B). The frequency of apoptotic cells was slightly higher at 10th week in Group II, which however became lower at 20th and 30th week. In Group III, the frequency of apoptotic cells was high at 10th week, which continued to increase at 20th and 30th week. Similarly frequency of apoptotic cells was significantly increased in Group IV at 20th and 30th week than Group II (Figure 1B).

Expression of AFP in liver lesions

AFP is a major plasma protein produced by the yolk sac and liver during fetal development that is thought to be the fetal form of serum albumin. AFP is a marker of liver progenitor cells and its level increases in hepatocellular carcinoma. AFP positive liver tumor showed poor prognosis than AFP negative (35). High cytoplasmic expression of AFP in liver section of Group II was observed at the 30th week indicating hepatocellular carcinoma (Supplementary Figure 2E, available at *Carcinogenesis* Online). In other groups notable expression was not observed (Supplementary Figure 2E, available at *Carcinogenesis* Online).

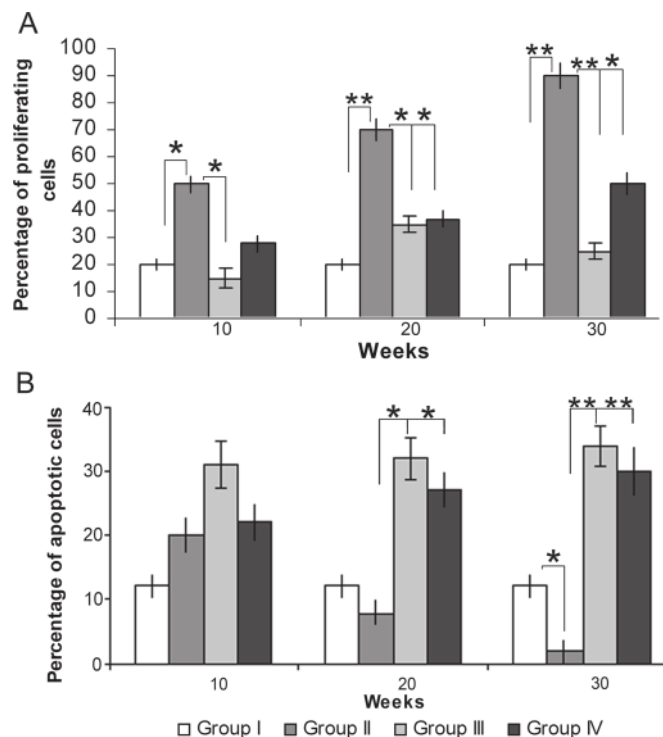


Fig. 1. Graphical representation of percentage of proliferating (A) and apoptotic (B) cells of different experimental groups at different time points. * $P < 0.05$; ** $P < 0.001$. White bars represent Group I, dark gray bars represent Group II, light gray bars represent Group III and black bars represent Group IV.

Effect of amarogentin on Rb phosphorylation during carcinogenesis

The expression of Rb mRNA/protein in liver lesions in different groups was comparable except slight decrease at 30th week in Group II (Figure 2A and 2B). Interestingly, in Group II ppRb (Ser 807/811) protein expression significantly increased at 10th week and became comparable in the following weeks (Figure 2B). However, its expression in Groups III and IV gradually decreased with time and became significantly lower at 30th week (Figure 2B). On the other hand, the expression of ppRb (Ser 567) protein was comparable among groups at 10th week. But its expression was increased in Group II at 20th week and became significantly higher at 30th week (Figure 2B). However, its decreased expression was evident in Groups III and IV at 20th and 30th week, with significant decrease in Group III at 30th week.

In immunohistochemical analysis, moderate nuclear Rb expression along with diffused cytoplasmic expression were observed in Group I (Figure 2C). Similar expression pattern was evident in Groups III and IV at all time points (Supplementary Figure 3, available at *Carcinogenesis* Online; Figure 2C), whereas, in Group II its lower expression was seen. In Group I, moderate nuclear and cytoplasmic expression of ppRb (807/811) and ppRb (Ser 567) were seen (Figure 2C). Whereas, strong nuclear/cytoplasmic expression of ppRb 807/811 was seen at all time points in Group II (Supplementary Figure 3, available at *Carcinogenesis* Online; Figure 2C). Gradual

Table I. Effect of amarogentin on liver lesion histopathology at different time points during the process of carcinogenesis

| | Experimental groups | 10th Week | 20th Week | 30th Week |
|--------------------------|---------------------|---|---|---|
| Normal | Group I | No change in liver histology [100% (6/6)] | No change in liver histology [100% (6/6)] | No change in liver histology [100% (6/6)] |
| Carcinogen | Group II | Moderate dysplasia [100% (6/6)] | Severe dysplasia [66.67% (4/6)] | Hepatocellular carcinoma [100% (5/5)] |
| Amarogentin + Carcinogen | Group III | Mild dysplasia [100% (6/6)] | Mild dysplasia [100% (6/6)] | Mild dysplasia [66.67% (4/6)] |
| | Group IV | Moderate dysplasia [100% (6/6)] | Mild dysplasia [80% (4/5)] | Mild dysplasia [60% (3/5)] |

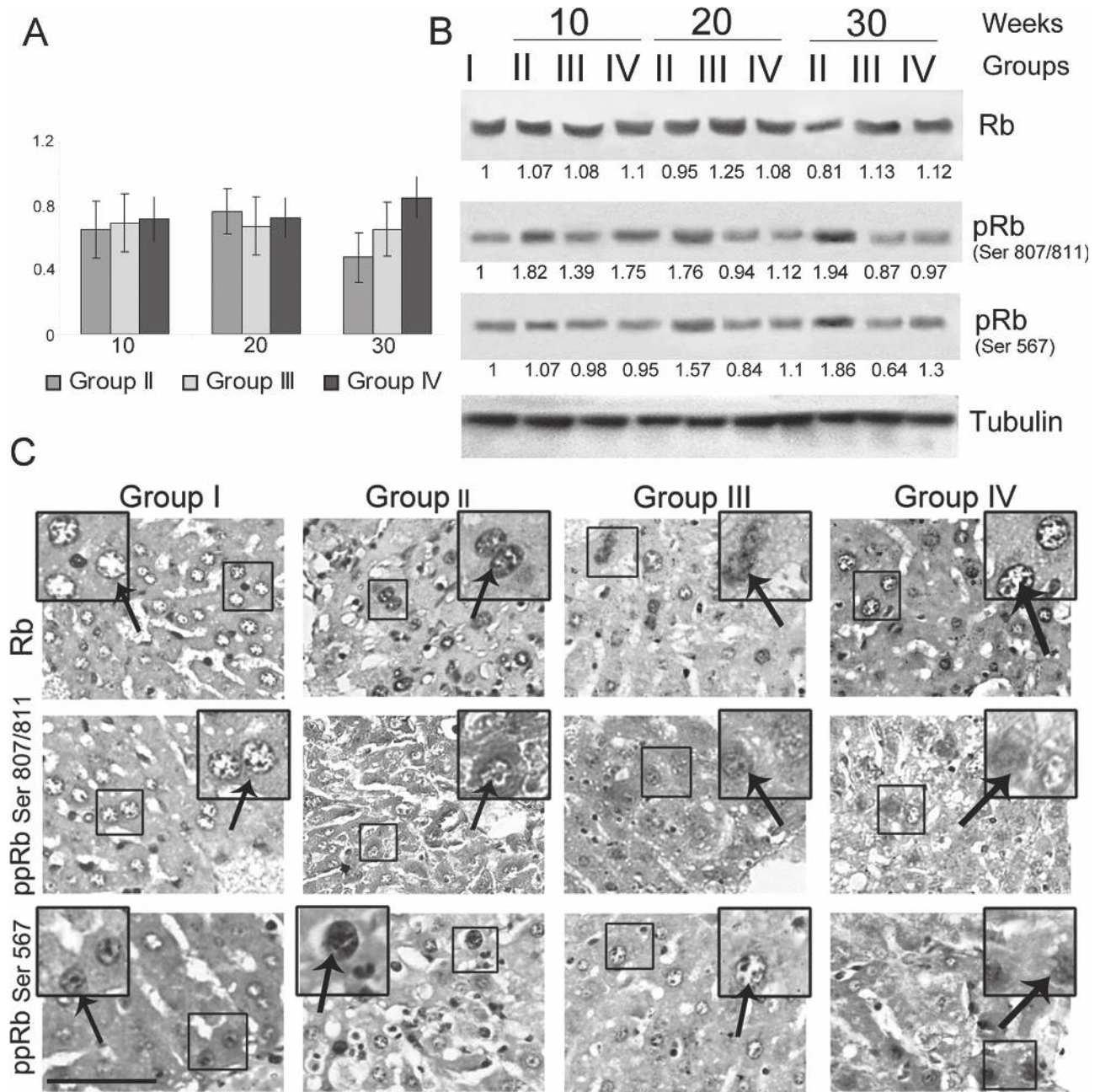


Fig. 2. (A) Quantitative RT-PCR showing mRNA expression of Rb. The x-axis showed weeks and y-axis showed relative expression. Data presented as mean \pm SD. (B) Western blot analysis of Rb, ppRb (Ser 807/811) and ppRb (Ser 567) proteins in liver lesions of all experimental groups (Groups I-IV) of 10th, 20th and 30th weeks. Tubulin was used as loading control. Dark gray bars represent Group II, light gray bars represent Group III and black bars represent Group IV. (C) Immunohistochemical analysis of above-mentioned proteins in liver lesion of Group I and other groups at 30th weeks. Magnification of tissue samples is 20 \times and for inset magnification is 40 \times . Scale bars represent 50 μ m. Arrows indicate respective nuclear/cytoplasmic expression.

increase in nuclear expression of ppRb (Ser 567) was evident in this group (Supplementary Figure 3, available at *Carcinogenesis Online*; Figure 2C). In Groups III and IV low nuclear and cytoplasmic expression were observed at all time points (Supplementary Figure 3, available at *Carcinogenesis Online*; Figure 2C).

Effect of amarogentin on expression of cell cycle inhibitors during liver carcinogenesis

The mRNA expression of LIMD1 and RBSP3 was gradually decreased in Group II during progression of carcinogenesis. Interestingly, their expression highly increased at 20th and 30th week in Groups III and IV. The protein expression pattern of these genes showed concordance with mRNA expression (Figure 3I a, b). In

addition, significantly higher protein expression of LIMD1 was seen at 20th and 30th week in Group III/IV and of RBSP3 at 30th week in Group III (Figure 3I a, b). In immunohistochemistry analysis high nuclear/cytoplasmic expression LIMD1 and nuclear expression of RBSP3 were seen in Group I (Figure 3I c; Supplementary Figure 3, available at *Carcinogenesis Online*). However, reduced expression of these proteins was evident in Group II. Interestingly, amarogentin could enhance both nuclear/cytoplasmic expression of these proteins in Group III/IV (Figure 3I c; Supplementary Figure 3, available at *Carcinogenesis Online*).

The mRNA expression of p16 in Group II was comparable or slightly higher at all time points. The p21 mRNA expression gradually decreased in Group II with progression. Their expression was

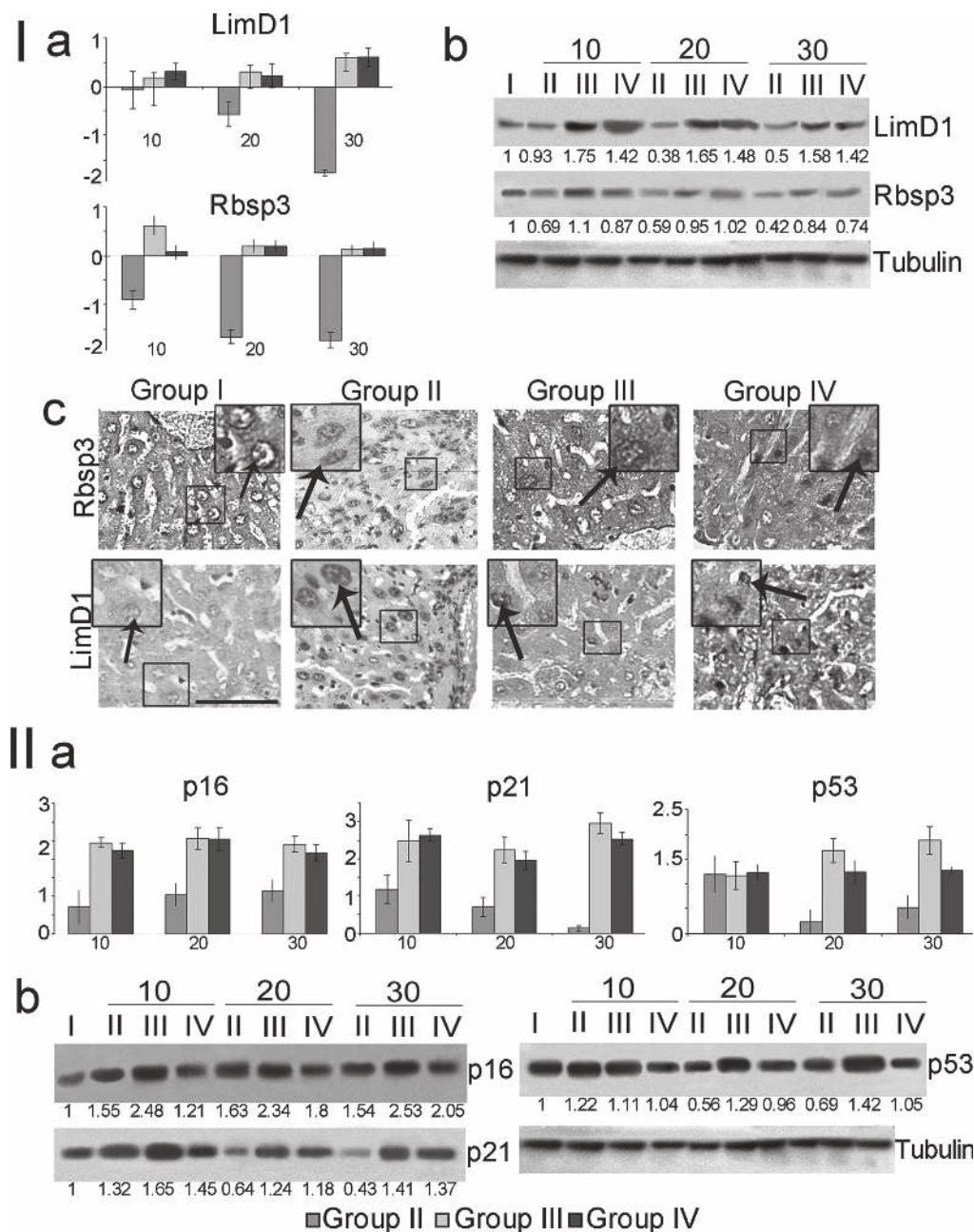


Fig. 3. [I] Expression analysis of LIMD1 and RBSP3. (a) Quantitative RT-PCR showing mRNA expression; (b) western blot analysis of corresponding proteins; (c) immunohistochemical analysis in liver lesion of Group I and other groups at 30th weeks. Magnification of tissue samples is 20x and for inset magnification is 40x. Scale bar represents 50 μm. Arrows indicate corresponding protein expression. Arrows in Group II indicate no expression. [II] Expression analysis of p16, p21 and p53. (a) Quantitative RT-PCR showing mRNA expression; (b) western blot analysis of corresponding proteins. In case of mRNA expression x-axis showed weeks and y-axis showed relative expression. Data presented as mean ± SD. **P* < 0.05; ***P* < 0.001. Dark gray bars represent Group II, light gray bars represent Group III and black bars represent Group IV. Western blot analysis was done in liver lesions of all experimental groups (Groups I-IV) of 10th, 20th and 30th weeks. Tubulin was used as loading control.

comparatively higher in Groups III and IV at all time points. The protein expression of p16 and p21 showed concordance with the mRNA expression (Figure 3II a, b). In addition, significant increase in protein expression of p16 and p21 was seen at 30th week in Group III/IV (Figure 3II a, b). On the other hand, mRNA expression of p53 was comparable in all groups at 10th week. However, its decreased expression was evident in Group II at 20th and 30th week. In Groups III and IV significantly increased expression was evident at 20th and 30th week. The protein expression showed concordance with mRNA expression with significant increase at 20th and 30th week in Group III/IV (Figure 3II a, b).

Effect of amarogentin on expression of cell cycle activators during liver carcinogenesis

The mRNA expression of cMyc and cyclinD1 gradually increased with progression in Group II (Figure 4A). However, differential expression of these genes was evident in Groups III and IV. In Group III their expression was gradually decreased with progression of the lesion, whereas in Group IV their reduced expression was mainly evident in 30th week. Interestingly, high expression of cdc25A was evident in Group II at 10th week and became comparable in the following weeks (Figure 4A). But its expression was gradually decreased in Groups III and IV with progression. The mdm2

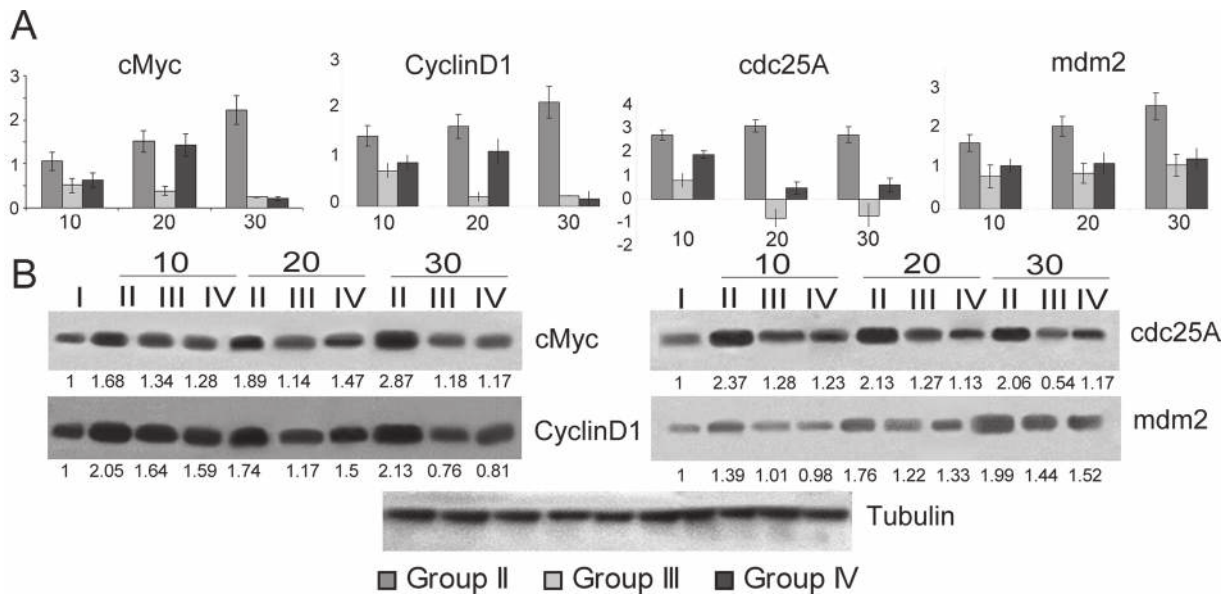


Fig. 4. (A) Quantitative RT-PCR showing mRNA expression of cMyc, cyclinD1, cdc25A and Mdm2. The x-axis showed weeks and y-axis showed relative expression. Data presented as mean \pm SD. Dark gray bars represent Group II, light gray bars represent Group III and black bars represent Group IV. (B) Western blot analysis of corresponding proteins in liver lesions of all experimental groups (Groups I-IV) of 10th, 20th and 30th weeks. Tubulin was used as loading control.

expression gradually increased with progression in Group II (Figure 4A). However, in Groups III and IV its expression was low at 10th week and became comparable in the following weeks. The protein expression of these genes showed concordance with mRNA expression in different weeks (Figure 4B). The expression of cMyc and cyclinD1 proteins decreased significantly at 30th week in Group III/IV, whereas significant decrease in cdc25A protein expression was seen in Group III at 30th week (Figure 4B).

Effect of amarogentin on expression of apoptosis-associated genes during liver carcinogenesis

In Group II, Bcl2 mRNA expression increased gradually during progression of carcinogenesis (Figure 5A). In Groups III and IV its decreased expression was evident at all time points. Protein expression showed concordance with mRNA expression with significant increase at 30th week in Group II (Figure 5B). In Groups III and IV significant decrease in its protein expression was evident at 30th week. On the other hand, Bax mRNA expression was gradually decreased with tumor progression in Group II (Figure 5A). Increased expression was evident in Group III/IV at 20th and 30th week. Similar expression pattern was evident in case of protein expression with significant decrease at 30th week in Group II (Figure 5B). In Group III/IV its expression significantly increased at 20th and 30th week (Figure 5B). Interestingly, the Bax-Bcl2 ratio gradually decreased during progression in Group II (Figure 5C). Whereas, in Groups III and IV increase in this ratio was evident at 10th week. The significant difference in this ratio was seen in 20th and 30th week in Group III/IV (Figure 5C).

Procaspase-3 cleavage is an indicator of apoptosis. Procaspase-3 protein expression did not change significantly among any time points and any experimental groups (Figure 5B). Active caspase-3 (20 kDa) expression gradually decreased with progression in Group II. In Groups III and IV also gradual increase in caspase-3 (20 kDa) expression was observed with significant increase at 20th and 30th week. Comparable expression of PARP (116 kDa) protein was found at all time points and in all experimental groups (Figure 5B). Cleaved PARP (24 kDa) expression gradually decreased in Group II during progression of carcinogenesis. Further increase in its expression was observed in Groups III and IV (Figure 5B).

Discussion

The chemopreventive and chemotherapeutic efficacy of amarogentin were demonstrated in CCl₄/NDEA-induced mouse liver carcinogenesis model. Continuous treatment (Group III) and posttreatment (Group IV) with amarogentin resulted in increased body weight, no alteration in kidney and liver function and better survival compared with carcinogen control group (Group II). Toxicity of amarogentin was reported previously in a hamster model (36), which used a much higher dose of this compound. Moreover, the extraction procedure was also different from that used for the present experiment. Amarogentin solution used in this study contains 10% ethanol. A separate experimental group was taken using 10% EtOH as vehicle control group. No changes in liver histology and toxicity were evident in this group. This suggests that the vehicle control group and normal control group (Group I) are comparable in all respect (Supplementary Figure 4, available at *Carcinogenesis* Online). Histopathological analysis revealed, moderate dysplasia, severe dysplasia and hepatocellular carcinoma at 10th, 20th and 30th week of carcinogenic exposure in Group II. Interestingly, amarogentin could prevent liver carcinogenesis at mild dysplasia upto 30th week in both Groups III (incidence 67%) and IV (incidence 60%). If amarogentin administration was stopped midway (20th week) liver foci formation started again (data not shown). Administration of carcinogens resulted in increased proliferation of liver cells that was observed since 10th week. Amarogentin treatment was found to significantly inhibit cellular proliferation. To understand the mechanism regulating the inhibition of proliferation, Rb phosphorylation status along with expression analysis of different genes associated with G₁/S phase of cell cycle check point were analyzed.

The mRNA/protein expression of Rb showed no significant change among different time points and experimental groups except slight decrease in Group II at 30th week. However, in Group II, ppRb (Ser 807/811) expression was found to be significantly increased since moderate dysplastic stage (10th week) indicating role of early G₁/S check point in carcinogenesis (Supplementary Figure 5, available at *Carcinogenesis* Online). These two sites of Rb were reported to be important for E2F binding (37). Significant increase in expression (RNA/protein) of cyclinD1 and cdc25A since 10th week suggests their role in phosphorylation of Rb at Ser 807/811 at early G₁ phase of cell cycle for selective growth advantage of the initiating clones.

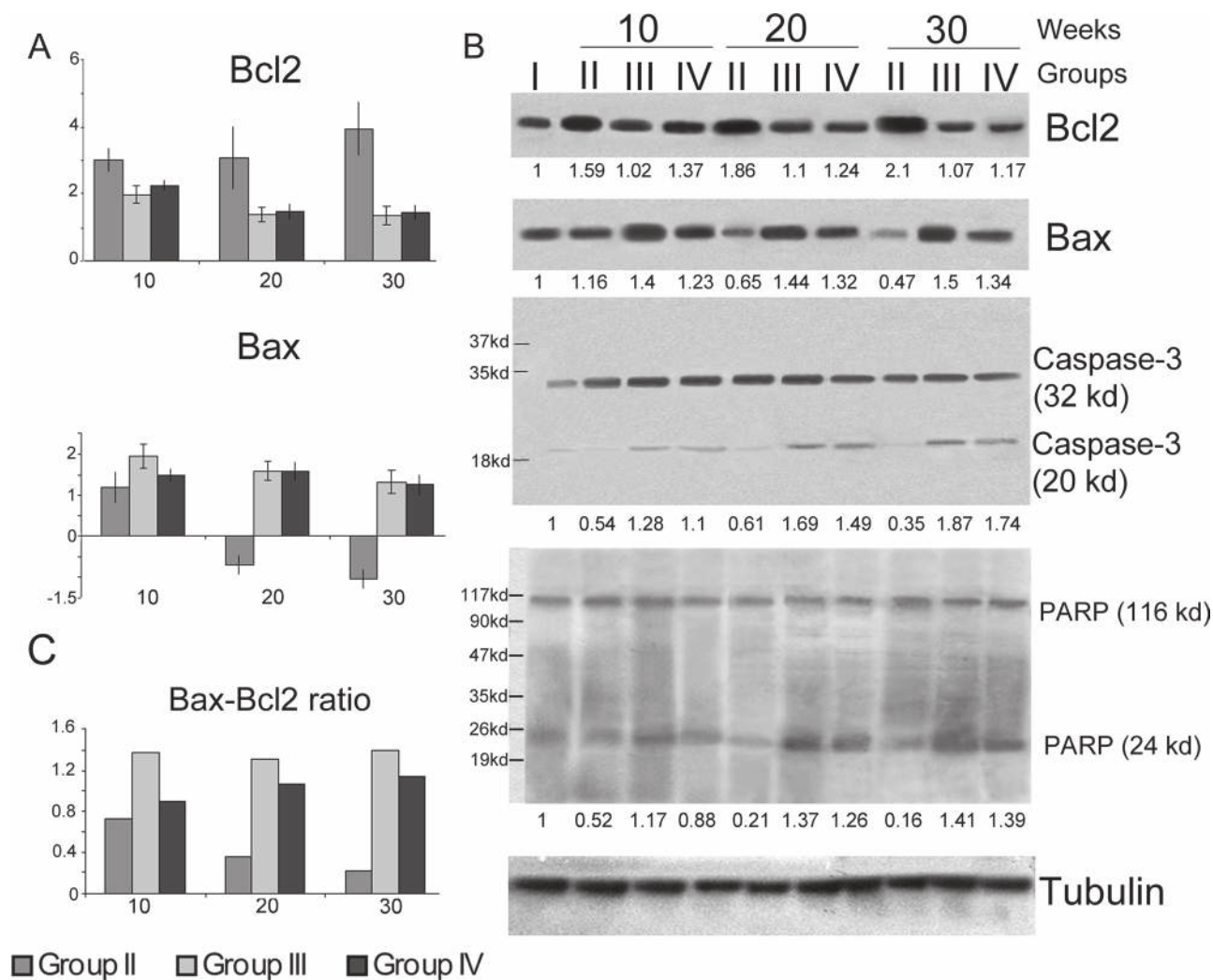


Fig. 5. (A) Quantitative RT-PCR showing mRNA expression of Bcl2 and Bax. The x-axis showed weeks and y-axis showed relative expression. Data presented as mean \pm SD. * $P < 0.05$; ** $P < 0.001$. Dark gray bars represent Group II, light gray bars represent Group III and black bars represent Group IV. (B) Western blot analysis of Bcl2, Bax, caspase-3 and PARP in liver lesions of all experimental groups (Groups I-IV) of 10th, 20th and 30th weeks. Tubulin was used as loading control. Molecular weight markers are marked in the left side of caspase-3 and PARP membrane. Band intensities in these two proteins are showing intensities of cleaved caspase-3 and PARP. (C) Graphical representation of Bax/Bcl2 ratio in liver lesions of all experimental groups of 10th, 20th and 30th weeks. The x-axis showed weeks and y-axis showed Bax/Bcl2 ratio. Dark gray bars represent Group II, light gray bars represent Group III and black bars represent Group IV.

Additional alterations of genes, like activation of cMyc and mdm2 and downregulation of LIMD1, p53 and p21 since 20th week, are needed for progression to severe dysplastic lesions. Interestingly, downregulation of RBSP3 along with phosphorylation of Rb at Ser 567 at 30th week are required for development of HCC. To the best of our knowledge differential expression of ppRb has not yet been reported during development of HCC. Overexpression of cMyc, cyclinD1, cdc25A and mdm2 has already been reported in HCC (15,16,38). However, there is ambiguity in the expression pattern of p53 and p21 in HCC. It was reported that mRNA expression of p21 is dependent on p53 expression and reduced expression of p21 is associated with HCC (18). Moreover, immunohistochemical expression of p21 is also associated with p53 and better survival in HCC (16). On the contrary, immunohistochemical overexpression of p53 was reported to be correlated with larger tumor size and poor survival in HCC (39). The role of LIMD1 and RBSP3 in development of HCC was not reported earlier. Hypermethylation of p16 was reported in HCC (40), although we have not seen promoter methylation of p16 in any groups (data not shown).

It was evident that amarogentin differentially modulates cell cycle regulatory genes in Groups III and IV (Supplementary Figure 5, available at *Carcinogenesis* Online). Significant increase in LIMD1,

RBSP3 and p16 expression along with downregulation of cdc25A in Group III were observed since 10th week. The cumulative alterations of these proteins may be responsible for the downregulated activity of CDK2/4/6 resulting in reduced expression of ppRb 807/811 and ppRb 567, seen in the 20th week. Cell cycle progression was thereby restricted to limit the lesion at mild dysplasia. In addition p53 and p21 at 20th week and downregulation of cMyc, cyclinD1 and mdm2 at 30th week might have synergistic effect in prevention of carcinogenesis probably through modulation of cell cycle. In Group IV, moderate dysplasia was observed at 10th week, but unlike Group II, in this group, increased expression of LIMD1 and p16 and downregulation of cdc25A were observed at this phase. This might be due to decreased proliferation than Group II at 10th week. Like Group III, prolonged treatment of amarogentin showed similar effect in Group IV at 20th and 30th week. Interestingly, from 10th to 30th weeks onward additional alterations of different genes are needed to check the process of carcinogenesis by amarogentin treatment in Group III/IV. It seems that the additional alterations of genes might produce a synergistic action to prevent carcinogenesis. This synergistic action seems to be lost by withdrawal of amarogentin when liver foci formation resurfaces (data not shown).

It has been noted that there is an inverse correlation of cellular proliferation and apoptosis during liver carcinogenesis. Amarogentin administration could reduce rate of proliferation and simultaneously induce apoptosis in the liver lesions particularly from 20th week onwards thereby preventing progress of carcinogenesis. The induction of apoptosis might be due to the increased Bax/Bcl2 ratio resulting in activation of caspase-3 and PARP cleavage.

From the present observation it may be concluded that cumulative alteration of both cellular proliferation and apoptosis, by amarogentin treatment, can prevent the progress of carcinogenesis in liver. The preventive action of amarogentin during carcinogenesis seems to be more effective than its therapeutic action. Continued work in this experimental model, to throw more light on the mechanism of action of amarogentin, would elucidate the best possible timing for and extent of intervention with this compound for prevention of liver carcinogenesis.

Supplementary material

Supplementary Documents 1 and 2 and Figures 1–5 can be found at <http://carcin.oxfordjournals.org/>

Funding

Council of Scientific and Industrial Research, New Delhi, India (27(0205)/09/EMR-II) to Dr C.K.P.

Acknowledgements

Authors are grateful to the Director, Chittaranjan National Cancer Institute, Kolkata, for his kind support during this study. Authors also thank Dr Sharmistha Banerjee for her encouragement about this study.

Conflict of Interest Statement: None declared.

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Received March 1, 2012; revised July 26, 2012; accepted August 28, 2012