

# Epigenetic Reprogramming of Liver Cells in Tamoxifen-Induced Rat Hepatocarcinogenesis

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Tamoxifen, a nonsteroidal anti-estrogen, is a potent genotoxic hepatocarcinogen in rats, with both tumor initiating and promoting properties. Recently it has been demonstrated that genotoxic carcinogens, in addition to exerting genotoxic effects, often cause epigenetic alterations and these induced epigenetic changes may play important mechanistic role in carcinogenesis. In the present study, we investigated the role of tamoxifen-induced epigenetic changes in hepatocarcinogenic process. The results of the study showed that exposure of female F344 rats to tamoxifen resulted in progressive loss of CpG methylation in regulatory sequences of long interspersed nucleotide elements (LINE-1) and prominent increase in expression of LINE-1 elements and *c-myc* proto-oncogene. The accumulation of tamoxifen-induced DNA lesions was accompanied by the decreased level of Rad51, Ku70, and DNA polymerase  $\beta$  (Pol $\beta$ ) proteins that play a crucial role in maintenance of genomic stability. Furthermore, feeding rats with tamoxifen-containing diet led to increased regenerative cell proliferation, as indicated by the increased level of Ki-67 and proliferating cell nuclear antigen (PCNA) proteins. These data indicate that exposure of animals to genotoxic hepatocarcinogen tamoxifen led to early phenotypical alterations in livers characterized by emergence of epigenetically reprogrammed cells with a specific cancer-related epigenetic phenotype prior to tumor formation.

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**Key words:** tamoxifen; rat; hepatocarcinogenesis; DNA hypomethylation; cell proliferation

## INTRODUCTION

Tamoxifen, a nonsteroidal anti-estrogen, has been the most important hormonal agent for treatment of breast cancer for more than two decades, and recently been approved as a long-term chemopreventive agent for breast cancer in healthy women at high risk for developing breast cancer [1–3]. However, a number of studies have demonstrated tamoxifen to be hepatocarcinogenic in rats [4,5] with both initiating [6] and promoting [7] properties. The mechanism of cancer initiation by tamoxifen in rats is due to genotoxic reactivity of tamoxifen metabolism products resulting in formation of tamoxifen-DNA adducts [6,8–12]. The promoting activity of tamoxifen is associated in part with its ability to induce cell proliferation in liver [8,13,14] and this action certainly contributes to carcinogenic potential of tamoxifen.

Recently it has been demonstrated that genotoxic carcinogens, in addition to exerting genotoxic effects, often cause epigenetic alterations [15]. Furthermore, it is becoming increasingly evident that these induced epigenetic changes may play a role in mechanisms of carcinogenesis [15–19].

However, it has not yet been established whether epigenetic changes induced by carcinogens and found in tumors play a causative role in carcinogenesis or are merely a consequence of the transformed state [15,20]. In our previous study using tamoxifen-induced hepatocarcinogenesis in rats, we demonstrated that long-term exposure of female Fisher 344 rats to tamoxifen-containing diet leads to the formation of tamoxifen-DNA adducts and to substantial global epigenetic changes characterized by decreased cytosine DNA methylation, decreased histone H4 lysine 20 trimethylation, and altered activity and expression of maintenance and de novo

Abbreviations: LINE-1; long interspersed nucleotide elements; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Pol $\beta$ , DNA polymerase  $\beta$ ; PCNA, proliferating cell nuclear antigen; ORFs, open reading frames; TUNEL, d-UTP nick end labeling; ANOVA, analysis of variance.

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DNA methyltransferases [21]. It has been hypothesized that alterations in epigenetic status induced by carcinogen exposure and sustained by continuing replication in the cellular genome result in the emergence of epigenetically reprogrammed cells that are indispensable preliminary events in carcinogenesis [19]. These epigenetically reprogrammed cells are characterized by global demethylation of the genome, activation of repetitive DNA sequences, hypomethylation of proto-oncogenes associated with persistent proliferative activity, hypermethylation of tumor-suppressor genes involved in the cell-cycle arrest, and inactivation of DNA repair genes [19]. These epigenetic changes are similar to those observed in cancer cells; as such they may have the potential to be used as early biomarkers for carcinogenicity. In light of these considerations, the present study was undertaken to determine whether or not exposure of rats to genotoxic hepatocarcinogen tamoxifen would result in emergence of epigenetically reprogrammed cells with a specific cancer-related epigenetic phenotype prior to tumor formation.

## MATERIALS AND METHODS

### Animals, Treatments, and Tissue Preparations

Female weanling F344 rats were obtained from the National Center for Toxicological Research breeding facility, housed two per cage in a temperature-controlled (24°C) room with a 12-h light-dark cycle, and given ad libitum access to water and NIH-31 laboratory diet. At 6 wk of age, the rats (body weight 150 g) were allocated randomly to receive either NIH-31 diet containing 420 ppm tamoxifen (Dyets, Inc., Bethlechem, PA) or control NIH-31 diet. Diets were stored at 4°C and given ad libitum with biweekly replacement. Five rats per diet group and five rats per control group were sacrificed at 12 and 24wk after diet initiation. The livers were excised, frozen immediately in liquid nitrogen, and stored at -80°C for subsequent analyses.

### Determination of Methylation Status of Long Interspersed Nucleotide Elements (LINE-1) by COBRA Assay

Genomic DNA was isolated from frozen livers by digestion with proteinase K, followed by standard phenol/chloroform extraction and ethanol precipitation [22]. The methylation status of LINE-1 was determined by COBRA assay, which consists of a standard bisulfite modification of genomic DNA, subsequent PCR amplification and digestion of PCR product with appropriate restriction endonuclease [23–25]. The combination of sodium bisulfite treatment and PCR amplification results in methylation-dependent creation of new restriction endonuclease sites, such as RsaI (GTAC), or methylation-

dependent retention of preexisting sites, such as BstUI (CGCG). Briefly, 2 µg of genomic DNA was treated with sodium bisulfite as previously described [23,25]. The bisulfite-modified DNA was PCR amplified with primers corresponding to the regulatory region sequence of rat LINE-1 [24]. The sense primer was 5'-TTTGGTGAGTTTGGGATA-3', and the antisense primer was 5'-CTCAAAAATACCCACCTAAC-3'. The PCR amplification consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 60 s, and extension at 72°C for 60 s. The PCR products were digested with 20 units of RsaI or BstUI restriction endonuclease (New England Biolabs, Beverly, MA) for 16 h, respectively. The digested PCR products were separated on 3% high-resolution agarose gels (Sigma, St. Louis, MO), stained with ethidium bromide, photographed, and the band intensity was analyzed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

### Determination of Methylation Status of the *p16<sup>INK4A</sup>* Promoter by Methylation-Specific PCR and Methylation-Sensitive PCR Assays

The methylation status of *p16<sup>INK4A</sup>* promoter was determined by methylation-specific PCR as previously described [26,27]. Recently, we determined that the CpG island in rat *p16<sup>INK4A</sup>* gene begins ~180 bp upstream from transcription initiation site and expands into first exon of the gene (GenBank accession number: AY145882). To determine the methylation status of the first CpG sites at the beginning of CpG island, we used methylation-sensitive PCR. Briefly, genomic DNA was treated with SmaI and HinP1I methylation-sensitive restriction endonucleases followed by PCR amplifications of a 448 bp fragment of the *p16<sup>INK4A</sup>* 5'-region. By using primers that flank the SmaI and HinP1I cleavage sites within the 5'-region of the *p16<sup>INK4A</sup>* gene, quantitative recovery of PCR product will vary directly with the extent of SmaI- or HinP1I-induced DNA breaks at unmethylated CCCGGG and GCGC sites. One microgram of genomic DNA was treated with 20 units of SmaI or HinP1I methylation-sensitive restriction endonuclease (New England Biolabs) for 16 h according to the manufacturer's instructions. The primer sequences which corresponded to the *p16<sup>INK4A</sup>* upstream region are: the sense primer 5'-AGGGTGTTCAGCGTGGGTAGCAGG TG-3' and the antisense 5'-ACGACCGAAAGTGTTCCGGGCGTTTGGTGAA-3'. The PCR amplification consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 60 s, and extension at 72°C for 60 s. The PCR products were separated on 2% high-resolution agarose gels, stained with ethidium bromide, and photographed.

#### Determination of Tamoxifen-DNA Adduct Levels

The DNA samples were hydrolyzed to nucleosides and analyzed for (*E*)- $\alpha$ -(deoxyguanosin- $N^2$ -yl)-tamoxifen (dG-Tam) and (*E*)- $\alpha$ -(deoxyguanosin- $N^2$ -yl)-*N*-desmethyltamoxifen (dG-DesMeTam) by electrospray ionization tandem mass spectrometry (ES-MS/MS) coupled with on-line sample preparation and high-performance liquid chromatography (HPLC) [28] as described by Schild et al. [29].

#### Determination of S-Adenosylmethionine (SAM) and S-Adenosylhomocysteine (SAH) Concentrations

The determination of SAM and SAH content in liver tissue extracts was performed by a HPLC method with coulometric electrochemical detection as previously described [30].

#### Analysis of Rad51, Ku70, and DNA Polymerase $\beta$ (Pol $\beta$ ) DNA Repair Proteins

The level of RAD51, Ku70 DNA, Pol $\beta$ , and proliferating cell nuclear antigen (PCNA) was determined by Western immunoblotting analysis. Briefly, tissue samples were homogenized, sonicated in 500  $\mu$ L of ice-chilled 1% sodium dodecyl sulphate (SDS), and boiled for 10 min. Protein concentrations were determined by the Bradford assay (Pierce, Rockford, IL). Equal amounts of proteins (20  $\mu$ g) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using slab gels of 8% or 12% polyacrylamide, made in duplicate, and transferred onto PVDF membranes (GE Healthcare Bio-Sciences, Piscataway, NJ). Membranes were incubated with primary antibodies against RAD 51 (1:1000, BD Biosciences, Mountain View, CA), Ku70 (1:1000, BD Biosciences), Pol $\beta$  (1: 1000, Biomeda, Foster City, CA), PCNA (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with a horseradish peroxidase-conjugated secondary antibody, antibody binding was detected with the ECL Plus Immunoblotting Detection System (GE Healthcare Bio-Sciences). Chemiluminescence was detected by Biomax MR Films (Eastman Kodak, New Haven, CT). To confirm equal protein loading, unaltered PVDF membranes were stained with Coomassie Blue. Images are representative of three independent immunoblots. Signals were quantified using NIH ImageJ 1.63 Software and normalized relative to  $\beta$ -actin.

#### mRNA Isolation and Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

Total mRNA was isolated from frozen livers by using QuikPrep Mikro mRNA Purification Kit (GE Healthcare Bio-Sciences), and first-strand cDNA template was synthesized using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Bio-Sciences) according to the manufacturer's instructions. PCR was carried out in 25  $\mu$ L of AmpliTaq Gold Master Mix (Applied Biosystems, Foster City, CA)

containing 1  $\mu$ L of the synthesized cDNA, 25 pmol each primer. The primer sets used for amplification of two open reading frames (ORFs) fragments of LINE-1, *Aurora-A*, *c-myc*, and *p16<sup>INK4A</sup>* genes, annealing temperatures, PCR conditions, and sizes of PCR products are summarized in Table 1. The PCR conditions were optimized to ensure that the reactions were on the linear phase of amplification. As an internal control for RT-PCR, equivalent amounts of cDNA were amplified by PCR using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers. As a negative control for each sample, we used PCR without addition of reverse transcriptase. Following PCR amplification, 10  $\mu$ L of PCR products was electrophoresed on 2% agarose gels, stained with ethidium bromide and photographed. The band intensity was analyzed by ImageQuant software (Molecular Dynamics).

#### Immunohistochemistry

The extent of cell proliferation and apoptosis was determined in liver sections after 24 wk of feeding the rats with tamoxifen-containing diet. Formalin-fixed paraffin-embedded liver sections were deparaffinized and rehydrated. For evaluation of proliferative activity the liver sections were stained for Ki-67 protein. Endogenous peroxidases were inhibited by incubation with freshly prepared 3% hydrogen peroxide with 0.1% sodium azide for 10 min at room temperature. The tissue sections were then placed in an antigen retrieval solution (0.01 M citrate buffer, pH 6.0) for 15 min in a microwave oven at 100°C at 600 W. After incubation with normal rat 10% serum, mouse monoclonal anti-rat Ki-67 (clone MIB-5, DAKO, Carpinteria, CA) was applied to the sections at the dilution of 1:50 (3.8  $\mu$ g/mL) for 1 h at room temperature. After incubation with primary antibody, tissue sections were incubated with biotinylated rat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:400 for 30 min at room temperature and later with streptavidin-conjugated horseradish peroxidase (ExtrAvidin Kit, Sigma) at a dilution of 1:30 for 30 min at room temperature. Staining was developed with 3,3-diaminobenzidine; slides were counterstained with hematoxylin, dehydrated, and mounted. For a negative control 3.8  $\mu$ g/mL mouse IgG (Jackson ImmunoResearch) or phosphate-buffered saline replaced the primary antibody.

Apoptotic bodies in liver sections were detected by terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP nick end labeling (TUNEL) of DNA fragments using an ApopTag Peroxidase in situ Apoptosis Detection Kit obtained from Serologicals Corporation (Norcross, GA). The TUNEL assay was performed according to the manufacturer's suggestions. Briefly, endogenous peroxidases were quenched as described above and the liver sections were incubated with proteinase K (20  $\mu$ g/mL) for

Table 1. Primer Sequences and Expected Size of RT-PCR Products

Target gene	Primer sequences		Product size (bp)	Annealing T (°C)	Cycles No.
	Forward	Reverse			
Line 1 (ORF1)	5'-AAGAAACACCTCCCGTCACA-3'	5'-CCTCCTTATGTTGGG CTTTACC-3'	350	60	27
Line 1 (ORF2)	5'-CCCACTCTCCCTACTT A-3'	5'-TATAGAGGAAGGCAACTGAT-3'	260	55	28
Aurora-A	5'-GGCGAATGCTTTGCTACT-3'	5'-CCGTACACAAAGTCAGGGAAT-3'	350	55	32
c-myc	5'-GGAACATATGACCTCGACTACGACTC-3'	5'-GCAGCGGCCGAGAAAGCCGCTCCACAT-3'	406	60	35
P16 <sup>INK4A</sup>	5'-CTTCACCAACGCCCCGAACAC-3'	5'-CGGAGAGGGTGGTGGGTC-3'	130	68	35
GAPDH	5'-GGTGCTGAGTATGCTGTGGA-3'	5'-GCCATGCCAGTGAGCTTCCC-3'	425	60	35

15 min at room temperature. The permeabilized tissue sections were enzymatically labeled with digoxigenin-nucleotide via TdT and subsequently exposed to horseradish peroxidase-conjugated anti-digoxigenin antibody. Staining was developed with 3,3-diaminobenzidine, and sections were counterstained with methyl green, dehydrated, and mounted. Sections were examined by light microscopy (BX40, Olympus, Tokyo, Japan). Ki-67-positive hepatocytes and TUNEL-labeled apoptotic bodies were counted in 14 random microscopic fields at 40× magnification (~10 000 cells) and labeling indices were expressed as the percentage of labeled hepatocytes or apoptotic bodies per 10 000 cells.

#### Statistical Analysis

Results are presented as mean ± SD and were assessed by two-way analysis of variance (ANOVA), using treatment and weeks as fixed factors, or one-way ANOVA, using treatment as the fixed factor. When necessary to maintain an equal variance or a normal data distribution, the results were ln transformed before conducting the ANOVA. *P*-Values < 0.05 were considered significant.

## RESULTS

### Effect of Tamoxifen on LINE-1 Methylation in Liver

In a previous study, using a sensitive HpaII-based cytosine extension assay that measures the proportion of unmethylated CCGG sites, we showed that feeding female rats with a tamoxifen-containing diet leads to a rapid decrease of cytosine DNA methylation in liver [21]. The majority of cytosine methylation in mammals resides in transposons and exons other than the first exon [31,32]. Transposons are interspersed repetitive DNA sequences that constitute almost 40% of rat genome [33]. DNA methylation plays a key role in maintaining stability of the genome by silencing the expression of these repetitive DNA sequences [31–35]. It has been suggested that genome-wide DNA hypomethylation in cancer cells largely affects transposons [31,34,35]. In view of this, we measured the effect of tamoxifen on the methylation status of LINE-1, a repetitive DNA sequence that constitutes almost 23% of rat genome [33].

Figure 1 shows the extent of LINE-1 regulatory region methylation, as determined by COBRA assay, in livers of control rats and rats fed tamoxifen. After 12 wk of feeding tamoxifen the ratio of undigested and BstUI-digested PCR products did not differ between tamoxifen-fed and control rats. When assessed after 24 wk, the undigested/BstUI-digested PCR product ratio in control rats did not change whereas the ratio was 1.9 times greater in the tamoxifen-fed rats, a difference that was significant. These results indicate a decrease in LINE-1 methylation after 24 wk of feeding because

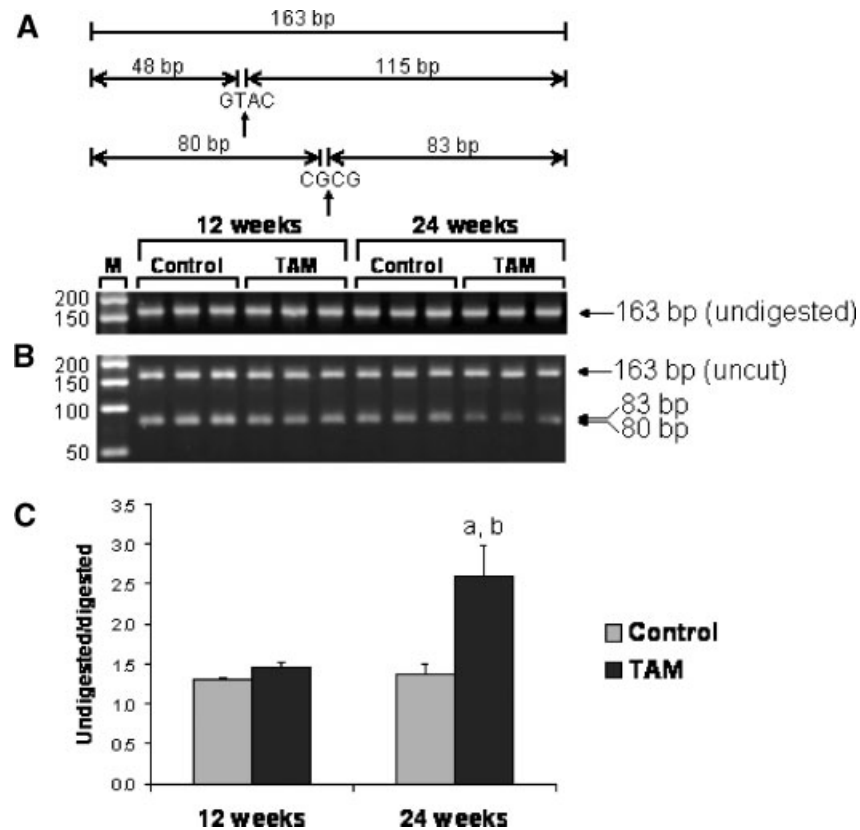


Figure 1. Methylation status of LINE-1 in the liver of control and tamoxifen-exposed rats as detected by COBRA assay. (A) Diagram of the PCR product of rat LINE-1 regulatory region. The location of RsaI and BstUI sites analyzed for methylation status by COBRA is shown. (B) Representative photographs show a COBRA assay with the BstUI restriction endonuclease. The upper panel shows representative photograph of ethidium bromide stained gel with undigested PCR product of rat LINE-1 regulatory region. The lower panel shows ethidium bromide stained gel after digestion of PCR product of rat LINE-1 regulatory region with BstUI restriction endonuclease. (C)

Ratio between uncut (163 bp) and BstUI-cut (80–83 bp) PCR products in liver of tamoxifen-fed rats and the age-matched control rats ( $n = 5$ , mean  $\pm$  SD). The increased ratio between undigested and BstUI-digested PCR products in liver of tamoxifen-fed rats indicates the loss of LINE-1 methylation because cleavage of PCR products would occur only if the CpG dinucleotides within BstUI recognition sequence (CGCG) are methylated. a, significantly different from control rats at the same time point; b, significantly different 12-wk time point.

cleavage of PCR products would occur only if the CpG dinucleotides within BstUI recognition sequence (CGCG) are methylated. Rats fed tamoxifen also had a decrease in cytosine methylation at RsaI site in LINE-1 regulatory region, although the degree of hypomethylation at this site was less pronounced (data not shown).

#### Effect of Tamoxifen on $p16^{INK4A}$ Promoter Methylation in Liver

The changes in DNA methylation are not limited to repetitive DNA sequences, but also affect important genes involved in DNA repair, cell cycle, and genome maintenance control [36]. The  $p16^{INK4A}$  tumor suppressor gene is one of the most frequent targets for DNA methylation-related inactivation [37]. The methylation status of  $p16^{INK4A}$  gene was assessed with a sensitive methylation-specific PCR and methylation-sensitive PCR assays. Treatment with tamoxifen did not lead to the appearance of de novo methylation in promoter of  $p16^{INK4A}$  gene, as

determined by methylation-specific PCR or by methylation-sensitive PCR assay (data not shown). This finding is in good agreement with evidence that de novo methylation of promoter region of tumor suppressor genes occurred at later stages of carcinogenic process [35].

#### Effect of Tamoxifen on SAM and SAH Content in Liver

Because global loss of cytosine DNA methylation could be linked to altered intracellular SAM and SAH content, the concentration of hepatic SAM and SAH was analyzed in control and tamoxifen-fed rats (Figure 2). The level of SAM did not differ between tamoxifen-fed and control rats at either 12 or 24 wk (Figure 2A). SAH levels increased significantly between 12 and 24 wk for both control and tamoxifen-fed rats (Figure 2B); furthermore, compared to the control rats, the level of SAH was significantly decreased in the rats fed tamoxifen at both 12 and 24 wk. As a reflection of the nearly

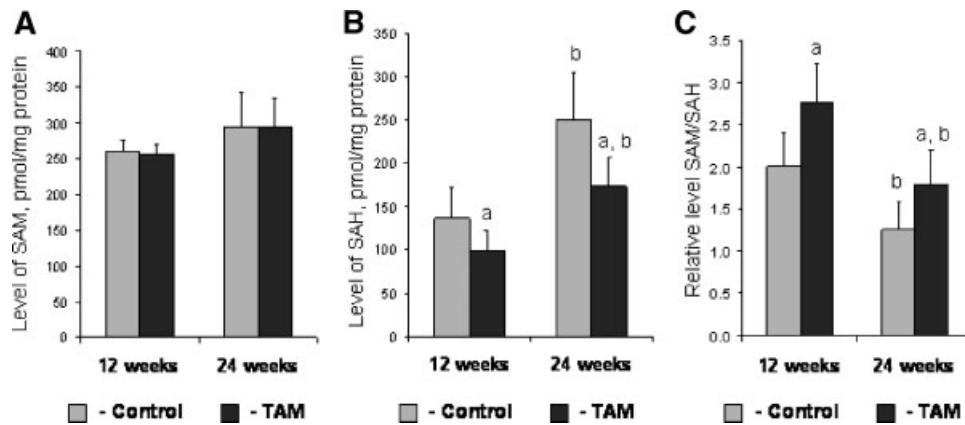


Figure 2. Level of SAM (A), SAH (B), and SAM/SAH ratio (C) in the liver of control rats and rats fed tamoxifen-containing diet as detected by HPLC assay. a, significantly different from control rats at the same time point; b, significantly different 12-wk time point.

constant level of SAM, the SAM/SAH ratio was significantly decreased at 24 wk compared to 12 wk for both groups, and in control rats compared to tamoxifen-fed rats at both times (Figure 2C).

#### Level of Rad51, Ku70, and Pol $\beta$ Proteins in Liver of Rats Fed Tamoxifen-Containing Diet

Considering the fact that global DNA hypomethylation, especially hypomethylation associated with repetitive DNA sequences, could predispose to genomic instability via homologous recombination between repetitive elements [35], we measured the level of Rad51, Ku70, and Pol $\beta$ , proteins that play a crucial role in DNA repair and in the maintenance of genomic stability [38–41]. Figure 3 shows that feeding rats with tamoxifen-containing diet for 24 wk resulted in significant decrease in level of Rad51, Ku70, and Pol $\beta$  proteins. Levels of Rad51, Ku70, and Pol $\beta$  proteins in liver of animals after 24 wk of expose to tamoxifen were 25%, 27%, and 36% lower than in control group, respectively. In contrast, the level of PCNA in liver of tamoxifen-fed rats was significantly higher at both 12 and 24 wk.

#### Effect of Tamoxifen on *LINE-1* (*ORF1* and *ORF2*), *c-myc*, *Aurora-A*, *p16* mRNA Expression

Figure 4 shows the effect of feeding rats with tamoxifen-containing diet upon *LINE-1* (*ORF1* and *ORF2*), *Aurora-A*, *c-myc*, and *16<sup>INK4A</sup>* expression as detected by semiquantitative RT-PCR. In rats fed tamoxifen, the expression of *LINE-1* (*ORF1*), *LINE-1* (*ORF2*), and *c-myc* was increased at both 12 and 24 wk; furthermore, the expression of *LINE-1* (*ORF2*) was increased at 24 wk compared to 12 wk. In contrast, level *Aurora-A* and *16<sup>INK4A</sup>* mRNA (data not shown) did not differ between the tamoxifen-exposed and age-matched control rats after 12 wk of feeding, and was decreased in tamoxifen-fed rats after 24 wk of feeding.

#### Morphological Changes in Liver of Rats Fed Tamoxifen-Containing Diet

The extent of cell death, as indicated by the presence of apoptotic bodies, and cell proliferation, as indicated by the expression of Ki-67 protein, was measured in liver sections after 24 wk of feeding rats. Representative examples of TUNEL stain and Ki-67 protein expression in liver from tamoxifen-treated rats are shown in Figure 5A and B, respectively. Feeding the tamoxifen-containing diet for 24 wk resulted in increase of apoptotic cell death and cell proliferation in the liver (Figure 5C). The increased rate of cell death in liver of tamoxifen-treated animals was accompanied by regenerative cell proliferation, as indicated by the expression of Ki-67 protein, with the number of proliferating hepatocytes in the liver of tamoxifen-fed rats being seven times higher than in the control group, whereas number of apoptotic bodies was increased 2.6-fold only (Figure 5C).

#### DISCUSSION

In this report we describe that in addition to the genotoxic components, tamoxifen-induced hepatocarcinogenesis in rat is characterized by the substantial phenotypic changes. The results of the study showed that exposure of female F344 rats to tamoxifen resulted in progressive loss of CpG methylation in *LINE-1* regulatory region (Figure 1). In contrast, methylation status of promoter of *p16<sup>INK4A</sup>* tumor-suppressor gene, which plays a central role in the cell-cycle arrest [37], did not change and remained in unmethylated state in liver of tamoxifen-exposed rats.

Hypomethylation of *LINE-1* sequences has been reported in many human cancers, including urothelial and renal cell carcinomas [42], chronic myeloid leukemia [43], prostate [44] and liver [45] cancers. This has led to the suggestion that hypomethylation

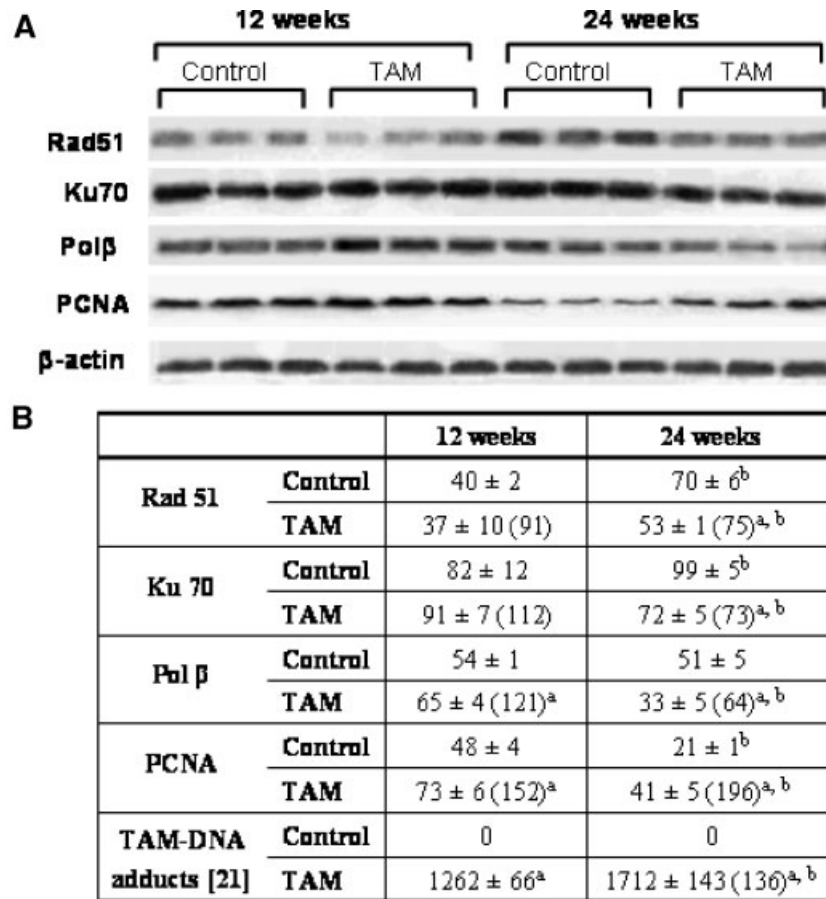


Figure 3. Western blot analysis of Rad51, Ku70, and DNA polymerase  $\beta$  proteins in the liver of control and tamoxifen-exposed rats. (A) Liver tissue lysates were separated by SDS-PAGE and subjected to the immunoblotting using specific antibodies against Rad51, Ku70, and DNA polymerase  $\beta$  proteins. Equal sample loading was confirmed by the immunostaining against  $\beta$ -actin. These results were reproduced in two independent experiments. Representative

western immunoblot images of three independent immunoblots are shown. (B) Quantitative evaluation of the Rad51, Ku70, and DNA polymerase  $\beta$  proteins in liver of control and tamoxifen-fed rats. Protein levels are presented as relative to age-matched control rats after normalization to  $\beta$ -actin ( $n=5$ , mean  $\pm$  SD). a, significantly different from control rats at the same time point; b, significantly different 12-wk time point.

of LINE-1 sequences may promote genomic instability and facilitate tumor progression [34,46]. However, it has not yet been established whether epigenetic changes, including hypomethylation of LINE-1 elements, which are often found in tumors play a causative role in carcinogenesis or are merely a consequence of the transformed state [15,20]. Recently, a progressive hypomethylation of LINE-1 regulatory region in liver was found at early stages of hepatocarcinogenesis induced by chronic feeding of folate/methyl-deficient [25] and by choline-deficient diets to rats [24]. The result of our study showing the loss of LINE-1 CpG methylation provides additional evidence of the importance of LINE-1 hypomethylation in carcinogenic process. Several possible mechanisms, including alteration of methyl group metabolism, inhibition of DNA methyltransferases, formation of DNA lesions, and alterations in DNA repair could contribute to loss of cytosine methylation during carcinogenesis. Because in the

present study we did not detect differences in SAM content, and the direction of changes in SAH level and SAM/SAH ratio in livers of tamoxifen-exposed rats would not result in DNA hypomethylation, we conclude that loss of LINE-1 methylation induced by tamoxifen may not be associated with dysregulation of cellular one-carbon metabolism. In our previous study, we showed that long-term exposure to tamoxifen resulted in diminished expression of the maintenance and de novo DNA methyltransferases [21]. This could contribute to loss of LINE-1 cytosine methylation. Other important factors that may contribute to LINE-1 hypomethylation are formation of tamoxifen-DNA adducts and DNA repair processes associated with DNA adduct formation. Considering that major DNA adducts induced by tamoxifen, (*E*)- $\alpha$ -(deoxyguanosin- $N^2$ -yl)-tamoxifen and (*E*)- $\alpha$ -(deoxyguanosin- $N^2$ -yl)-*N*-desmethyltamoxifen, occur at GC base pairs, and the fact that LINE-1 sequences constitute almost 23% of rat

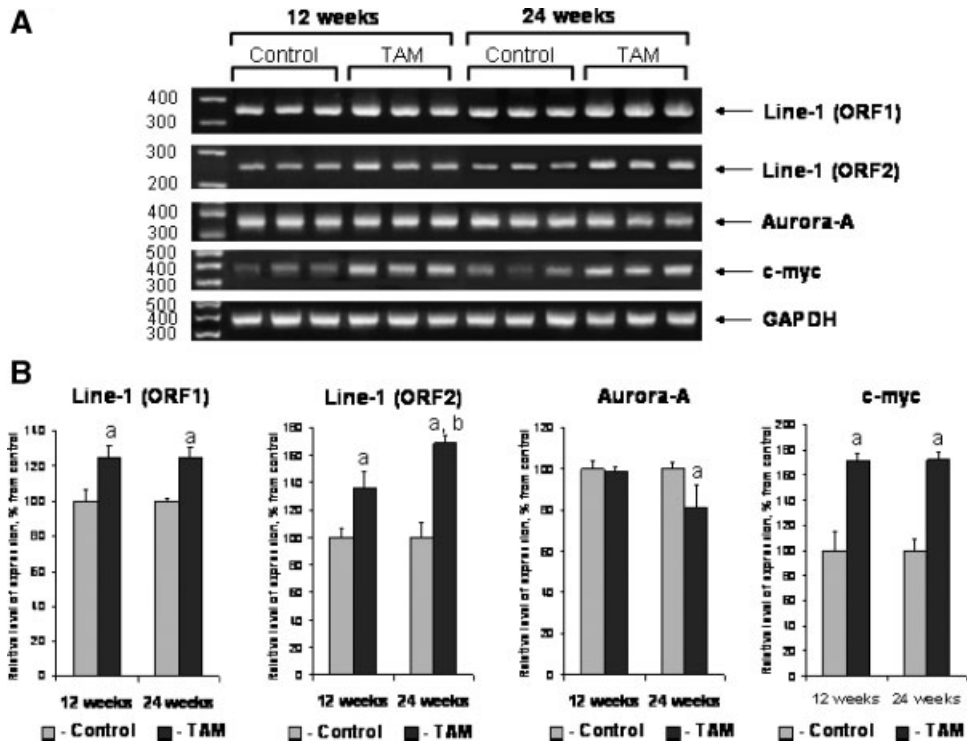


Figure 4. Expression of LINE-1, *Aurora-A*, and *c-myc* gene in liver of control rats and rats fed tamoxifen-containing diet. (A) Expression of LINE-1, *Aurora-A*, and *c-myc* gene in liver of control rats and rats fed tamoxifen-containing diet, as revealed by semiquantitative RT-PCR. GAPDH was used as control for loading. These results were reproduced in two independent experiments. (B) Quantitative

analysis of LINE-1, *Aurora-A*, and *c-myc* mRNAs in liver of control and tamoxifen-fed rats. Expression presented as relative to age-matched control animals after normalization to GAPDH ( $n=5$ , mean  $\pm$  SD). a, significantly different from control rats at the same time; b, significantly different from same treatment at 12 wk.

genome [33], we hypothesize that formation of tamoxifen-DNA adducts at methylated CpG sites within LINE-1 sequences may be a pivotal factor leading to LINE-1 hypomethylation.

One of the primary functions of DNA methylation in mammalian somatic cells is silencing of repetitive DNA sequences [31]. Loss of cytosine methylation causes the reactivation of retrotransposons, which

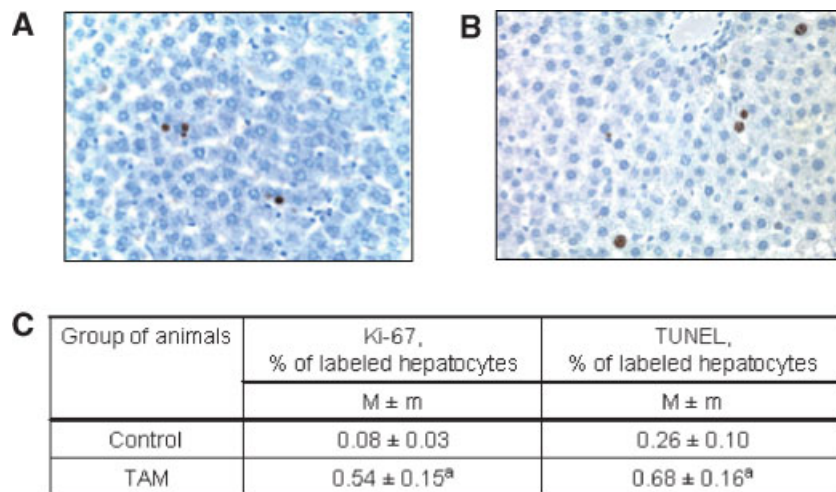


Figure 5. Apoptotic cell death and cell proliferation in the liver of control rats and rats fed tamoxifen-containing diet. (A) TUNEL-stained apoptotic bodies counterstained with methyl green; (B) Ki-67-stained hepatocytes counterstained with hematoxylin. (C) Ki-67 and TUNEL labeling indices in liver sections of control rats and rats fed tamoxifen-containing diet for 24 wk. Ki-67-positive hepatocytes

and TUNEL-labeled apoptotic bodies were counted in 14 random microscopic fields at 40 $\times$  magnification ( $\sim$ 10 000 cells) and labeling indices were expressed as the percentage of labeled hepatocytes or apoptotic bodies per 10 000 cells ( $n=5$ , mean  $\pm$  SD). a, significantly different from control rats.

affects the stability of the genome [34]. In present study, the loss of LINE-1 regulatory region methylation induced by long-term exposure to tamoxifen was associated with the increased LINE-1 expression. Given the number and distribution of LINE-1 sequences, the activation of LINE-1 elements may predispose to genome instability via homologous recombination between unmasked repeats [35], affecting stability of the genome through insertional mutagenesis, DNA breakage, retrotransposition of repetitive sequences, generation of pseudogenes and chimeric retrotranscripts, DNA breakage, and dysregulated gene expression mediated by the activation of transposon promoters within and around genes [34]. In light of these considerations, we examined the level of Rad51, Ku70, and Pol $\beta$  proteins in liver of rats exposed to tamoxifen and found that feeding the tamoxifen-containing diet resulted in the decreased expression of these proteins in liver (Figure 3).

Rad51, Ku70, and Pol $\beta$  proteins play key roles in the repair of DNA damage in somatic mammalian cells [38–41]. Ku70 and Rad51 proteins are crucial in homologous recombination repair [38,39], and Pol $\beta$  is the rate-determining enzyme in the base excision repair [41,47]. A common feature of these proteins is that loss of their activity results in extensive genomic instability [38–41]. Decreased expression of Rad51, Ku70, and Pol $\beta$  proteins in liver of tamoxifen-exposed rats may lead to accumulation of unrepaired DNA lesions, which further predispose cells to genomic instability. This suggestion is supported further by observation of an increased accumulation of tamoxifen-DNA adducts in liver between 12 and 24 wk, which indicates that tamoxifen-induced adducts are not repaired proficiently (Figure 3). Additionally, the loss of histone H4 lysine 20 trimethylation that occurs in the liver of tamoxifen-fed rats [21] may compromise the ability of cells to maintain cell-cycle arrest, which could result in genomic and chromosomal instabilities and in increased tumor risk [48,49].

The accumulation of tamoxifen-induced DNA lesions and the decreased expression of DNA repair proteins were accompanied by increased regenerative cell proliferation, as indicated by the expression of Ki-67 and PCNA proteins. An increase in cell turnover, regardless of the initiating cause, has been linked experimentally and conceptually to carcinogenesis and is considered one of the components contributing to hepatocarcinogenesis [50–53]. The continuing cell replication results in a stable activation of biological processes related to maintenance and promotion of replication in these proliferating cells. In contrast, processes related to inhibition of proliferation, activation of cell-cycle arrest and DNA repair pathways are suppressed in these cells. Indeed, the early and prolonged tamoxifen-induced increase in expression of *c-myc* gene, whose enhanced

expression contributes to almost every aspect of tumor cell biology, especially the ability to drive unrestricted cell proliferation [54] corresponds to this suggestion.

Considering the fact that DNA hypomethylation has been associated with chromosomal instability, also occurs early in tumorigenic process, we measured expression of centrosome kinase Aurora-A, the overexpression of which leads to neoplastic transformation of mammalian cells [55], and is frequently observed in a large number of human cancers, including liver cancer [56]. Feeding tamoxifen-containing diet did not result in increased expression of *Aurora-A* gene. Considering this finding and data presented above, we may suggest that exposure to tamoxifen induces early appearance of epigenetic changes in liver, which consequently may predispose cells to genomic instability.

In conclusion, we have demonstrated that exposure of rats to genotoxic hepatocarcinogen tamoxifen resulted in early phenotypical alterations in livers, characterized by demethylation and increased expression of LINE-1 sequences, diminished level of DNA repair proteins, increased expression of *c-myc* proto-oncogene, and increased cell proliferation. The analogous changes are often found in liver tumors [25]. The similarity between these changes provides experimental evidence of the early emergence of tumor-related epigenetically altered cells and the importance of epigenetic changes in carcinogenesis.

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