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Suppression of tumor suppressor Tsc2 and DNA repair glycosylase Nth1 during spontaneous liver tumorigenesis in Long-Evans Cinnamon rats

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Abstract

Chronic inflammation and oxidative stress are arguably associated with an increased risk of cancer. Certain diseases that are characterized by oxyradical overload, such as Wilson's disease (WD), have also been associated with a higher risk of liver cancer. The Long-Evans Cinnamon (LEC) rat, an animal model for WD, is genetically predisposed to the spontaneous development of liver cancer and has been shown to be very useful for studying the mechanisms of inflammation-mediated spontaneous carcinogenesis. Endonuclease III (Nth1) plays a significant role in the removal of oxidative DNA damage. Nth1 and a tumor suppressor gene Tuberous sclerosis 2 (Tsc2) are bi-directionally regulated in humans, mice, and rats by a common minimal promoter containing two Ets-binding sites (EBSs). In this study, we examined the expression of Nth1 and Tsc2 genes during disease progression in the LEC rat liver. During the period of acute hepatitis (16–17 weeks), we observed decreased Nth1 and Tsc2 mRNA levels and a continued decrease of the Tsc2 gene in 24 weeks in LEC rats, while the effect was minimal in Long-Evans Agouti (LEA) rats. This reduction in the mRNA levels was due to the reduced binding of EBSs in the Nth1/Tsc2 promoter. Increase in protein oxidation (carbonyl content) during the same time period

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(16–24 weeks) may have an effect on the promoter binding of regulatory proteins and consequent decrease in Nth1 and Tsc2 gene expressions during tumorigenesis.

Keywords

Long-Evans Cinnamon rat; Long-Evans Agouti rat; Reactive oxygen species; Wilson's disease; Liver cancer; Hepatitis

Introduction

Considerable evidence has linked oxidative damage and cancer. Reactive oxygen species (ROS) are generated in the mitochondria of normal mammalian cells as a byproduct of normal respiration and are formed in other subcellular locations as a function of oxygen-mediated biochemical reactions. High ROS levels are toxic to the cell; however, low ROS levels are involved in physiological functions such as the activation and modulation of signal transduction pathways, modulation of the activities of redox-sensitive transcription factors, and regulation of mitochondrial enzyme activities. The ROS levels are reduced by antioxidant defenses, but are elevated by transition metals such as iron or copper and by exogenous agents such as ionizing radiation or ozone. Understanding the mechanisms by which specific genes are inactivated after oxidative damage is of great interest and importance. Specific gene loss may be explained on the basis of physical gene structure, chromosomal location, and/or physiological function of the gene [1].

Damage to membrane lipids by ROS such as peroxide, superoxide anion, hydroxyl radical, and singlet oxygen may ultimately lead to inflammation [2]. Although there is a strong association between chronic inflammation and cancer, investigators have not yet uncovered all the molecules, pathways, and mechanisms involved, and there are numerous questions regarding the mechanisms and targets of pro-inflammatory mediators of tumor development [3].

Certain diseases that are characterized by oxyradical overload, such as Wilson's disease (WD), have been associated with chronic inflammation and a higher risk of liver cancer [4]. The Long-Evans Cinnamon (LEC) rat is a model for WD and is characterized by a mutation in the *Atp7b* gene. This mutation leads to defective copper excretion and, therefore, copper accumulation in the liver. In LEC rats, the human disease is mimicked in terms of copper accumulation in the liver and impaired hepatic excretion of copper into the blood and bile. Aberrations in copper dynamics may create favorable conditions for superoxide-yielding redox cycling, thereby leading to increased ROS production. This may result in oxidative damage in susceptible regions such as the liver or brain. The LEC rat has been shown to be very useful for studying the mechanisms of inflammation-mediated DNA damage and spontaneous carcinogenesis. Recent investigations revealed considerable increases in DNA single-strand breaks and 8-oxoguanine levels—two indices of DNA oxidative damage—in the brain, liver, and kidney of affected animals [5–9]. In addition, other findings have suggested the involvement of lipid peroxidation in Cu-mediated toxicity in the LEC liver [10]. In order to eliminate the deleterious effects of oxidized bases, organisms have developed efficient repair mechanisms. Oxidized base lesions are removed by enzymes of the BER pathway. Endonuclease III (Nth1) and 8-oxoguanine DNA glycosylase (OGG1) play significant roles in the removal of oxidized bases such as thymine glycol, 5-hydroxy cytosine, and 8-oxoguanine [11–14].

Tuberous sclerosis (TSC) is an autosomal dominant systemic disorder associated with renal cell carcinoma (RCC). Germline mutations in the Tsc1 or Tsc2 gene have been associated

with TSC [15, 16]. Loss of heterozygosity (LOH) at the Tsc1 or Tsc2 loci has been detected in TSC-associated RCC and in hamartoma [17–20], suggesting their roles as tumor suppressors. Some LOH-negative RCCs may have suppression of Tsc2 expression by promoter disregulation [21–23]. However, the latter's role in liver cancer is not known yet. Interestingly, Nth1 and Tsc2 are bi-directionally regulated in humans, mice, and rats by a common core promoter [24–26]. In our earlier study, we observed a marked decrease during acute hepatitis in the LEC rat liver in the activity and expression of Nth1 protein and mRNA, and to a certain extent in OGG1 [9]. In this study, we examined the expression of Nth1 and Tsc2 genes during the course of inflammation-mediated liver cancer in the LEC rat liver. Our findings revealed that protein oxidation and its consequent effect on the binding of regulatory proteins to Nth1/Tsc2 promoter may have a role in Nth1 and Tsc2 gene expressions in the LEC rat liver during liver carcinogenesis.

Materials and methods

Liver tissues

Liver tissues were procured from LEC and Long-Evans Agouti (LEA) rats of various age groups, as described previously, and the same animals were used for all the experiments [9].

Protein isolation

Liver extracts were prepared from LEA and LEC rats based on a modified published procedure. Briefly, liver tissues (12 g) from LEC and LEA rats of various ages were minced, washed thoroughly with PBS, and homogenized. After centrifugation of the homogenate, the pellet was resuspended in chilled buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitor mixture (Roche Diagnostics, IN)] and allowed to swell on ice for 15 min. The swollen pellet was mixed with 0.6% NP40 and 0.4 M NaCl and left on a rocking shaker for 15 min at 4°C. The lysate was centrifuged, and the supernatant (whole liver extract; 4–8 mg/ml) was stored at −80°C in small aliquots and thawed only once for the gel shift assay and protein oxidation (carbonyl content) analysis; this was done to avoid inactivation resulting from repeated freeze–thaw cycles [9].

RNA extraction and cDNA synthesis

Total RNA was extracted from the homogenized samples by using the SV Total RNA Isolation System (Promega, Madison, WI, USA), according to the manufacturer's instructions. The cDNA pool was synthesized using the QuantiTect Rev. Transcription Kit (Qiagen, GmbH, Hilden).

Gel shift assay

The reaction mixture contained the following components in a total volume of 12.5 μl: 1 ng 5′ end-labeled double-stranded 47-bp oligonucleotide containing both Ets-binding sites (EBSs); 10 μg nuclear extract from the LEA/LEC rat liver; 1 μg double-stranded poly deoxyinosinate-deoxycytidylate [poly(dIdC)]; and binding buffer [20 mM HEPES (pH 7.9), 0.1 mM EDTA, 1 mM $MgCl_2$, 40 mM KCl, and 0.5 mM dithiothreitol]. Incubation was carried out at room temperature for 30 min, and the reaction products were separated on 4% non-denaturing polyacrylamide gels, which were subsequently dried and autoradiographed. The binding specificities of the probes were evaluated by incubating the extracts with an excess of non-labeled wild type and mutant EBS oligonucleotides [26].

Evaluation of Nth1 and Tsc2 gene expressions by real-time PCR

Primers for real-time PCR were designed using the Primer3 (v. 0.4.0) software [27]. The primer sequences are listed in Table 1. The specificity of the primer pairs was assessed by

regular PCR (50 cycles) and by running the products on an agarose gel to confirm the amplicon size.

Real-time PCR was carried out using the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Each PCR reaction contained 2.5 μl master mix (Power SYBR Green PCR Master Mix; Warrington, UK), 0.5 μM forward and reverse primers, and 100 ng first-strand cDNA in deionized water. The PCR protocol used was as follows: each sample was heated to 95°C for 10 min, followed by 50 cycles at 94°C for 10 s, 55°C for 30 s, and 72°C for 30 s. All samples were run in triplicates, and the PCRs for the housekeeping gene β-actin and the target genes were run in parallel for each sample. The PCR reactions were carried out on a 384-well plate (ABgene House, Surrey, UK) in 5 μl reaction volumes. The $2^{-\Delta\Delta Ct}$ method was used for calculating the fold relationships in gene expression between the time points.

Measurement of protein oxidation

The protein oxidation was measured by the assessment of reactive carbonyl content in protein, which was measured by the widely applied procedure using a commercial 2,4-dinitrophenylhydrazine (DNPH)-based colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI). Assays were carried out with 5–8 mg/ml protein following manufacturer's procedure. The carbonyl contents in oxidized proteins were calculated as nmol per mg of protein by using a molar extinction coefficient of 22,000 M^{-1} cm^{-1} for DNPH at 370 nm. Values were expressed as means ± SD of at least three independent measurements from two rats of each strain.

Results

Downregulation of Nth1 and Tsc2 in the LEC rat liver during acute hepatitis

The expression profiles of Nth1 and Tsc2 in the livers of LEC and LEA rats of various ages and disease stages (Fig. 1a) were obtained by real-time PCR analysis and are shown in Fig. 1b. It was observed that Nth1 and Tsc2 mRNA expressions were severely affected during the period of acute hepatitis (16–17 weeks), with the continuation of reduction of Tsc2 expression in 24 weeks in LEC rats. The expression levels of Nth1 were reduced by 47 and 62% at 16 and 17 weeks, respectively. This led to the reduced Nth1 protein expression and activity, as reported earlier [9]. Similarly, the expression levels of Tsc2 were decreased by 50–60% during 16–24 weeks. The expressions of both genes beyond 17 weeks were higher than at 8 weeks, probably to compensate for the lost/reduced expression during acute hepatitis. However, in LEA rats, β-actin normalized expression was not greatly affected at the time points tested. Overall, the expression patterns of Nth1 and Tsc2 in both LEC and LEA rats indicate the known so far bi-directional regulatory mechanisms of Nth1/Tsc2 promoter [26]. In LEC rats, the reduced mRNA expression prompted us to investigate the mechanism of regulation of Nth1 and Tsc2 expressions at the transcriptional level.

Reduced binding of NTH1/Tsc2 common core promoter during acute hepatitis

In an earlier study, Honda et al. [9] characterized the Nth1/Tsc2 promoter and found a 108-bp (Fig. 2a) active core promoter which contained two EBSs. In order to determine whether the ETS family proteins directly bind to EBSs in the core promoter and thereby contribute to Nth1 and Tsc2 regulations in LEC rats, we performed a gel shift assay using a portion of the promoter (a 47-bp oligonucleotide) that spans the two EBSs along with the nuclear extract from the liver cells of LEA and LEC rats. The β-actin expression is shown as loading control. The specificity of the generated band was determined by competitive EMSA. The results revealed that the band was completely out competed by a 40-fold molar excess of unlabeled wild-type (W) oligonucleotide, but was unaffected by the mutant—this provided

proof of the specificity of the band. As shown in Fig. 2b, the oligonucleotide-protein binding pattern was greatly affected in LEC rats during acute hepatitis, with the maximum effect at 16 and 17 weeks. Obvious changes in the binding pattern were not observed at the other time points (8, 14, 24, and 40 weeks) tested. These results suggest that ETS family proteins are involved in the binding of EBSs in LEA and LEC rats. Reduced binding of ETS protein(s) to the promoter during acute hepatitis could be a possible reason for decreased Nth1 and Tsc2 expressions in LEC rats.

Oxidation of proteins during hepatitis

We then tested whether the protein oxidation is the cause of loss of binding of EBS in Nth1/Tsc2 promoter sequences, using a commercial 2,4-dinitrophenylhydrazine (DNPH)-based colorimetric assay kit for protein carbonyls. The most general indicator and by far the most commonly used marker of protein oxidation is protein carbonyl content. Copper or iron often binds to cation binding locations on proteins and with the help of further attack by H_2O_2 or O_2, transforms side chain amino groups on multiple amino acid residues, such as lysine, arginine, proline, and histidine into carbonyls. Aldehydes independent of metals also can oxidize proteins. DNPH reacts with protein carbonyls, forming a Schiff base to produce the corresponding hydrazone, which is analyzed spectrophotometrically at an absorbance between 360 and 385 nm. Here, protein carbonyl content increased significantly in 16–24 weeks (Fig. 3), suggesting that global protein oxidation may be a cause of decreased Nth1/Tsc2 promoter binding of regulatory proteins and a consequent decrease in Nth1 and Tsc2 gene expressions during acute hepatitis.

Discussion

Accumulation of copper and iron in the liver leads to oxidative DNA damage that may result in mutations and the development of fulminant hepatitis, hepatic fibrosis, and subsequent hepatocarcinogenesis in LEC rats. Hence, Nth1, which is an enzyme responsible for the removal of oxidative damage, is important in terms of protection against oxidative DNA damage. In this context, it is important to understand the mechanism of Nth1 regulation in an LEC animal model during the initial phase of disease progression. Interestingly, *Nth1* and *Tsc2*, a tumor suppressor gene, are bi-directionally regulated in human, mouse, and rat by a common core promoter [24–26]. The Tsc2 gene is known to suppress renal cancer; however, its role in liver cancer is not known. In the present study, we investigated the mechanisms of expression of Nth1 and Tsc2 genes during the course of inflammation-mediated liver cancer in the LEC rat liver mechanism of Nth1 regulation.

Real-time PCR studies in the LEC rat liver revealed that both Nth1 and Tsc2 expressions were severely affected during acute hepatitis. This finding is in agreement with the decreased repair activity of Nth1 observed in our earlier study [9].

In order to understand the mechanism of Nth1 and Tsc2 regulations, we used EMSA to examine Nth1/Tsc2 promoter binding with the nuclear extracts from the livers of LEA and LEC rats of various ages. The core promoter of Nth1 contains two EBSs. The presence of ETS family proteins in the nuclear extracts was observed by the formation of DNA–protein complexes, which were observed as specific bands. However, this binding pattern was severely affected during the period of acute hepatitis (16–17 weeks) in LEC rats but not in LEA rats. This could be due to the elevated oxidative stress experienced by LEC animals. Nair et al. observed a marked increase in lipid peroxidation, Mn-SOD, 8-oxo-dG, and etheno adducts during acute hepatitis in LEC animals [28].

In addition to DNA oxidation, protein oxidation also has profound biological effect during oxidative stress. Increased production of the hydroxyl radical during inflammation (such as

during acute hepatitis) leads to the formation of lipid hydroperoxides, which ultimately results in the formation of α, β, and unsaturated aldehydes. Notably, the production of such aldehydes results in extensive protein modification, termed protein carbonylation, which leads to the loss- or gain-of-function of various cellular proteins, including many gene regulatory factors [29]. In fact, our results show that protein carbonyl content was significantly increased during the same time period (acute hepatitis), when the Nth1 promoter binding by ETS family proteins was significantly reduced. Thus, it is tempting to speculate that those regulatory proteins could also undergo modifications that lead to the loss of their Nth1/Tsc2 promoter binding activity and hence the reduced expression of Nth1 and Tsc2 during oxidative stress. It is important to note that total carbonyl content measurement showed an overall trend of protein oxidation, which had probably affected the binding of target proteins in early time points; however, at 24 and 40 week time points although the protein oxidation was still high, the cells apparently adopted to the acute environment of severe oxidative stress, which probably allowed the promoter binding proteins becoming more stable than before. The situation is obviously more complex than a simple one-to-one cause and effect relationship.

In addition, the roles of other regulatory pathways, such as those involving miRNA at the post-transcriptional level or the epigenetic regulatory mechanism via DNA methylation and/or histone modification, may not be ruled out for Nth1 and Tsc2 regulations during oxidative stress and will be investigated in future studies.

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Abbreviations

LEC	Long-Evans Cinnamon rat
LEA	Long-Evans Agouti rat
Nth1	Endonuclease III
ROS	Reactive oxygen species
WD	Wilson's disease
OGG1	8-Oxoguanine DNA glycosylase

References

1. Oberley TD. Oxidative damage and cancer. Am J Pathol. 2002; 160:403–408. [PubMed: 11839558]
2. Symons AM, King LJ. Inflammation, reactive oxygen species and cytochrome P450. Inflammopharmacol. 2003; 11:75–86.
3. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002; 420:860–867. [PubMed: 12490959]
4. Hussain SP, Raja K, Amstad PA, Sawyer M, et al. Increased p53 mutation load in nontumorous human liver of Wilson disease and hemochromatosis: oxyradical overload diseases. Proc Natl Acad Sci USA. 2000; 97:12770–12775. [PubMed: 11050162]
5. Klein D, Lichtmannegger J, Finckh M, et al. Gene expression in the liver of Long-Evans Cinnamon rats during the development of hepatitis. Arch Toxicol. 2003; 77:568–575. [PubMed: 14574444]
6. Samuele A, Mangiagalli A, Armentero MT, et al. Oxidative stress and pro-apoptotic conditions in a rodent model of Wilson's disease. Biochim Biophys Acta. 2005; 1741:325–330. [PubMed: 16081251]

7. Kato J, Kobune M, Kohgo Y, Sugawara N, et al. Hepatic iron deprivation prevents spontaneous development of fulminant hepatitis and liver cancer in Long-Evans Cinnamon rats. J Clin Invest. 1996; 98:923–929. [PubMed: 8770863]
8. Sternlieb I, Quintana N, Volenberg I, et al. An array of mitochondrial alterations in the hepatocytes of Long-Evans Cinnamon rats. Hepatology. 1995; 22:1782–1787. [PubMed: 7489989]
9. Choudhury S, Zhang Z, Frenkel K, et al. Evidence of alterations in base excision repair of oxidative DNA damage during spontaneous hepatocarcinogenesis in Long Evans cinnamon rats. Cancer Res. 2003; 63:7704–7707. [PubMed: 14633694]
10. DiDonato M, Sarkar B. Copper transport and its alterations in Menkes and Wilson diseases. Biochim Biophys Acta. 1997; 1360:3–16. [PubMed: 9061035]
11. Ikeda S, Biswas T, Roy R, et al. Purification and characterization of human Nth1, a homologue of *Escherichia coli* endonuclease III. J Biol Chem. 1998; 273:21585–21593. [PubMed: 9705289]
12. Liu X, Roy R. Mutation at active site lysine 212 to arginine uncouples the glycosylase activity from the lyase activity of human endonuclease III (hNth1). Biochemistry. 2001; 40:13617–13622. [PubMed: 11695910]
13. Liu X, Roy R. Truncation of amino-terminal tail stimulates activity of human endonuclease III. J Mol Biol. 2000; 321:265–276. [PubMed: 12144783]
14. Hill J, Hazra TK, Izumi T, et al. Stimulation of human 8-oxoguanine-DNA glycosylase by AP-endonuclease: potential coordination of the initial steps in base excision repair. Nucleic Acids Res. 2001; 29:430–438. [PubMed: 11139613]
15. van Slegtenhorst M, de Hoogt R, Hermans C, et al. Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. Science. 1997; 277:805–808. [PubMed: 9242607]
16. The European Chromosome 16 Tuberous Sclerosis Consortium. Identification and characterization of the tuberous sclerosis gene on chromosome 16. Cell. 1993; 75:1305–1315. [PubMed: 8269512]
17. Bjornsson J, Short MP, Kwiatkowski DJ, Henske EP. Tuberous sclerosis-associated renal cell carcinoma. Clinical, pathological, and genetic features. Am J Pathol. 1996; 149:1201–1208. [PubMed: 8863669]
18. Green AJ, Johnson PH, Yates JR. The tuberous sclerosis gene on chromosome 9q34 acts as a growth suppressor. Hum Mol Genet. 1994; 3:1833–1834. [PubMed: 7849709]
19. Henske EP, Neumann HP, Scheithauer BW, Herbst EW, Short MP, Kwiatkowski DJ. Loss of heterozygosity in the tuberous sclerosis (TSC2) region of chromosome band 16p13 occurs in sporadic as well as TSC-associated renal angiomyolipomas. Genes Chromosom Cancer. 1995; 13:295–298. [PubMed: 7547639]
20. Carbonara C, Longa L, Grosso E, et al. Apparent preferential loss of heterozygosity at TSC2 over TSC1 chromosomal region in tuberous sclerosis hamartomas. Genes Chromosom Cancer. 1996; 15:18–25. [PubMed: 8824721]
21. Kobayashi T, Urakami S, Hirayama Y, et al. Intragenic Tsc2 somatic mutations as Knudson's second hit in spontaneous and chemically induced renal carcinomas in the Eker rat model. Jpn J Cancer Res. 1997; 88:254–261. [PubMed: 9140109]
22. Kubo Y, Mitani H, Hino O. Allelic loss at the predisposing gene locus in spontaneous and chemically induced renal cell carcinomas in the Eker rat. Cancer Res. 1994; 54:2633–2635. [PubMed: 8168090]
23. Kobayashi T, Mitani H, Takahashi R, et al. Transgenic rescue from embryonic lethality and renal carcinogenesis in the Eker rat model by introduction of a wild-type Tsc2 gene. Proc Natl Acad Sci USA. 1997; 94:3990–3993. [PubMed: 9108092]
24. Imai K, Sarker AH, Akiyama K, Ikeda S, Yao M, Tsutsui K, Shohmori T, Seki S. Genomic structure and sequence of a human homologue (NTHL1/Nth1) of Escherichia coli endonuclease III with those of the adjacent parts of TSC2 and SLC9A3R2 genes. Gene. 1998; 222:287–295. [PubMed: 9831664]
25. Ikeda S, Mochizuki A, Sarker AH, Seki S. Identification of functional elements in the bidirectional promoter of the mouse Nthl1 and Tsc2 genes. Biochem Biophys Res Commun. 2000; 273(3): 1063–1068. [PubMed: 10891372]

26. Honda S, Kobayashi T, Kajino K, Urakami S, Igawa M, Hino O. Ets protein Elf-1 bidirectionally suppresses transcriptional activities of the tumor suppressor Tsc2 gene and the repair-related Nth1 gene. Mol Carcinog. 2003; 37:122–129. [PubMed: 12884363]

27. Rozen, S.; Skaletsky, HJ. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S.; Misener, S., editors. Bioinformatics methods and protocols: methods in molecular biology. Humana Press; Totowa, NJ: 2000. p. 365-386.

28. Nair J, Sone H, Nagao M, et al. Copper-dependent formation of miscoding ethano-DNA adducts in the liver of Long Evans cinnamon (LEC) rats developing hereditary hepatitis and hepatocellular carcinoma. Cancer Res. 1996; 56:1267–1271. [PubMed: 8640812]

29. Levonen AL, Landar A, Ramachandran A, et al. Cellular mechanisms of redox cell signalling: role of cysteine modification in controlling antioxidant defences in response to electrophilic lipid oxidation products. Biochem J. 2004; 378:373–382. [PubMed: 14616092]

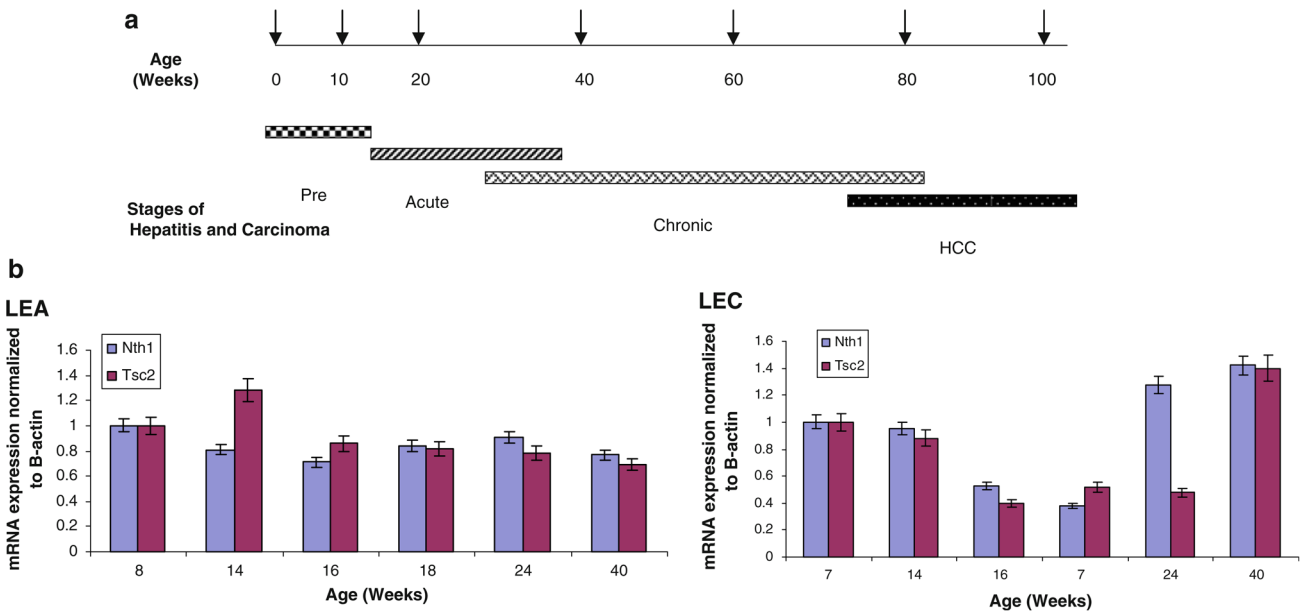

Fig. 1.

a Stages of hepatitis and hepatocellular carcinoma (HCC) in LEC rat. **b** mRNA expression profile of Nth1 and Tsc2 in livers from LEA and LEC. The data presented are the mean ± SD values of three to five independent measurements from two rats of each strain. The *arrows* denote the important time points for development and progression of hepatitis and HCC in LEC rats

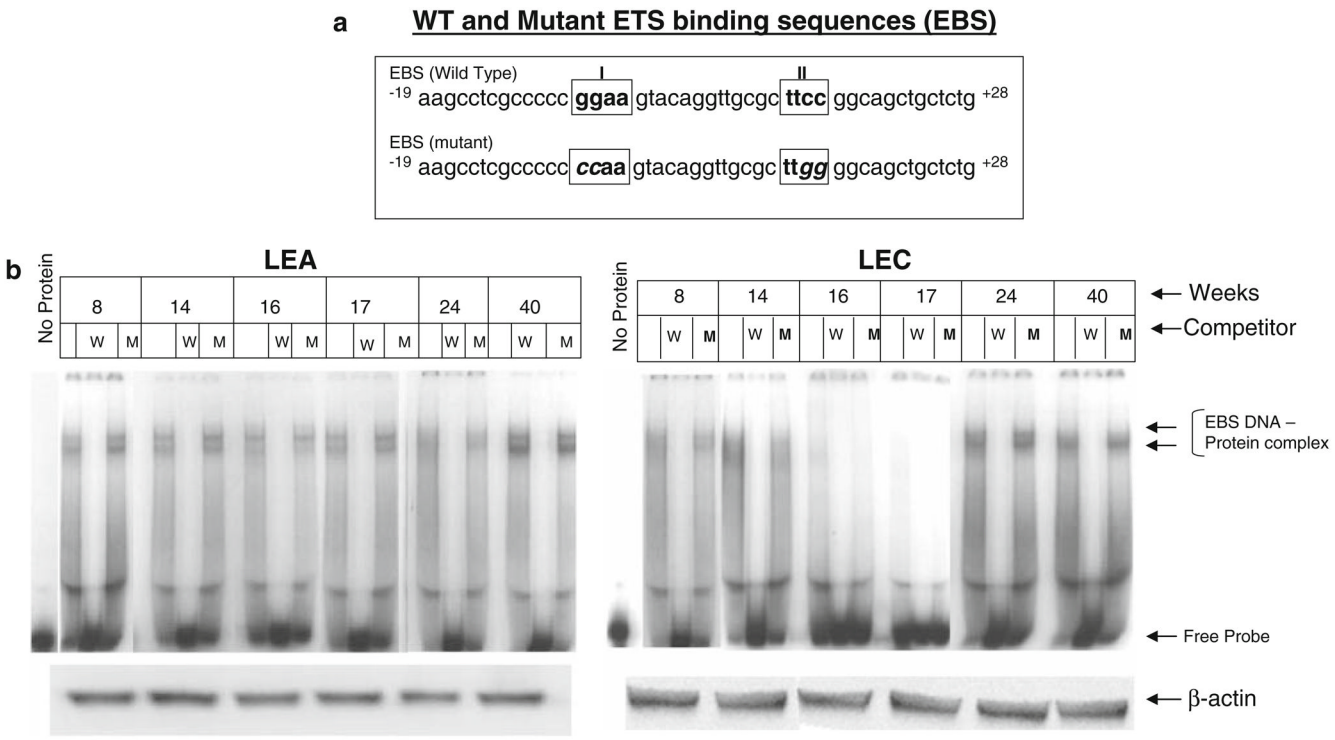

Fig. 2.

a Sequence of oligonucleotides used for gel shift assay. **b** Gel shift analysis of protein binding to the Nth1 promoter. All lanes contained 10 μg of nuclear extracts from livers of LEC and LEA rats and 1 ng of radiolabeled oligonucleotide containing two ETS-binding sites (EBS) denoted by I and II. *Arrowheads* indicate DNA bound with ETS family protein. Competition experiments were performed using non-radiolabeled 40-fold molar excess of oligonucleotide containing wild-type EBSs (lanes indicated by "W") or the mutant (lanes indicated by "M") EBSs. The *β*-actin expression is shown as loading control

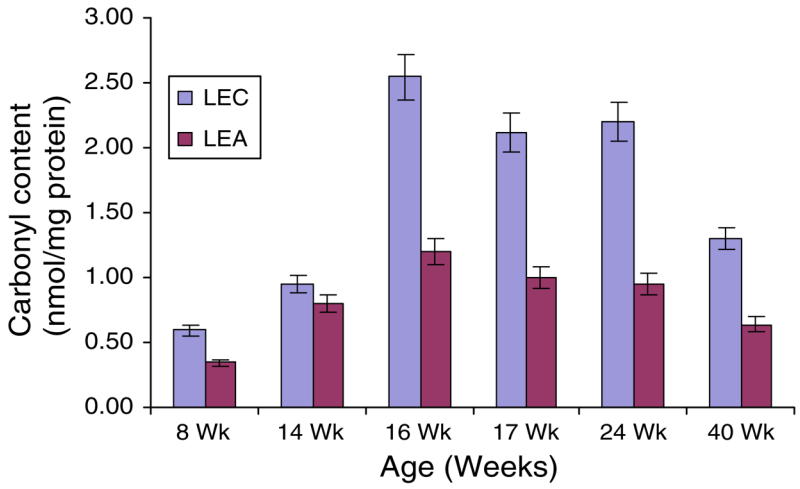

Fig. 3.

Protein oxidation profile in LEC and LEA liver tissues. Carbonyl content as an indicator of protein oxidation was measured by DNPH-based colorimetric analysis. The details are described in “Materials and methods” section. The data presented are the mean ± SD values of three to five independent measurements from two rats of each strain

Table 1

List of primers used for real-time PCR

Gene	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)	Amplicon size (bp)
β-actin	CAAGCCTCTCTGCACGTGTGTC	AGCTGCTCGCTGTCTTGGTTAAGT	100
Nth1	TGTGGCCTATGAGGTTGCTAACG	GCCAGTTGCTGCTGCCAGTT	95
Tsc2	GGAGAAGTTCAAGATACTATTAGGACTGGG	CGCTGTGATGATAAACTCCGTCTG	91