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Biochemical and Biophysical Research Communications 362 (2007) 183–187
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Different mutation patterns of mitochondrial DNA displacement-loop in hepatocellular carcinomas induced by *N*-nitrosodiethylamine and a choline-deficient L-amino acid-defined diet in rats

Mariko Onishi [a,b], Yui Sokuza [a], Tomoki Nishikawa [a], Chiharu Mori [a], Kimiko Uwataki [a], Kanya Honoki [c], Toshifumi Tsujiuchi [a,*]

[a] *Laboratory of Cancer Biology and Bioinformatics, Department of Life Science, Faculty of Science and Engineering, Kinki University, 3-4-1, Kowakae, Higashiosaka, Osaka 577-8502, Japan*
[b] *Department of Pathology, Osaka City University Medical School, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan*
[c] *Department of Orthopedic Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan*

Received 28 July 2007
Available online 8 August 2007

Abstract

Mutations of the mitochondria DNA (mtDNA) displacement loop (D-loop) were investigated to clarify different changes of exogenous and endogenous liver carcinogenesis in rats. We induced hepatocellular carcinomas (HCCs) in rats with *N*-nitrosodiethylamine (DEN) and a choline-deficient L-amino acid-defined (CDAA) diet. DNAs were extracted from 10 HCCs induced by DEN and 10 HCCs induced by the CDAA diet. To identify mutations in mtDNA D-loop, polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis, followed by nucleotide sequencing, was performed. Mutations were detected in 5 out of 10 HCCs (50%) induced by DEN. Four out of 5 mutations were G/C to A/T transitions at positions 15707, 15717, 15930, and 16087, and one T/A to C/G transition at position 15559. By contrast, no mutations were found in 10 HCCs induced by the CDAA diet. These results demonstrated that mutations in mtDNA D-loop occur in rat HCCs induced by DEN but not by the CDAA diet, suggesting that mtDNA D-loop is a target of exogenous liver carcinogenesis in rats.

Keywords: Mitochondria DNA; D-loop; Hepatocellular carcinoma; Mutation; Rat

Liver carcinogenesis can be divided into two categories: carcinogenesis induced by exogenous carcinogens changes and that induced by endogenous changes that occur without the need for exposure to any chemical carcinogens. *N*-Nitrosodiethylamine (DEN) is one of the most well-known liver carcinogens in rats. It have been reported that a cell cycle disturbance induced in DEN-initiated hepatocytes by colchicine gives a growth advantage to putative preneoplastic lesions under conditions of partial hepatectomy and selection pressure, so that a high incidence of HCCs can be obtained within a short latent period [1,2]. It has also been reported that unequivocal liver tumors associated with cirrhosis can be induced by prolonged feeding of rats with a choline-deficient L-amino acid-defined (CDAA) diet that does not contain any known carcinogens [3]. Because our previous studies revealed differential effects of chemopreventive agents and different genetic alterations in our two liver models, there is a possibility that different mechanisms underlie exogenous and endogenous hepatocarcinogenesis in rats [4,5].

Human mitochondrial DNA (mtDNA) consists of 16,569 nucleotide pair double-stranded, comprising a circular molecule [6,7]. The mtDNA encodes 2 rRNAs, 22

Abbreviations: mtDNA, mitochondrial DNA; D-loop, displacement-loop; HCC, hepatocellular carcinoma; DEN, *N*-nitrosodiethylamine; CDAA diet, choline-deficient L-amino acid defined diet; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism.

* Corresponding author. Fax: +81 6 6723 2721.
E-mail address: ttujiuch@life.kindai.ac.jp (T. Tsujiuchi).

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doi:10.1016/j.bbrc.2007.07.175

tRNAs, and 13 polypeptide genes which involve in respiration and oxidative phosphorylation [6,7]. There are hundreds mtDNA copies per human cell, and the vast majority of these copies are identical after birth [7]. The mutation rate of mammalian mtDNA is 10-fold higher than that of nucleic DNA, since the mitochondria lack highly efficient DNA repair system or protective histones [7–9]. A displacement loop (D-loop), which is a non-coding region in mtDNA, contains the heavy-strand and light-strand promoters [10]. The D-loop region is considered as a hot spot for mtDNA alterations [11–15]. Indeed, it has been reported to be alterations of the D-loop region in human several malignancies, including HCCs [11–15].

In the present study, to clarify whether different mutation pattern of mtDNA may exist in exogenous and endogenous liver carcinogenesis in rats, we conducted the search for mutation in mtDNA D-loop region in rat HCCs induced by DEN and the CDAA diet.

Materials and methods

Animals and treatment. The method for the production of HCCs using exogenous carcinogens was as described previously [1,2]. A total of 10 male F344 rats, at 6 weeks of age (Japan SLC Inc.), received DEN (Wako Pure Chemical Co., Ltd., Kyoto, Japan) i.p. at a dose of 10 mg/kg body weight, and underwent a partial hepatectomy 4 h later [16]. Colchicine (Sigma Chemical Co., St. Louis, MO, USA) was injected i.p. 1 and 3 days after DEN treatment at a dose of 0.5 mg/kg body weight. After an 11-day recovery period, the rats were placed on the selection regimen comprising a diet including 0.02% 2-acetylaminofluorene (Nakalai Tesque Co. Ltd.) for 2 weeks and a single intragastric administration of carbon tetrachloride (Nakalai Tesque Co. Ltd.) at 1 mg/kg body weight, followed by the procedure described by Cayama et al. [17]; rats were killed under ether anesthesia 42 weeks after the beginning of the experiment.

In order to produce HCCs by means of endogenous carcinogens, 10 male F344 rats, at 6 weeks of age (Japan SLC Inc.), were continuously given a CDAA diet until 75 weeks after the beginning of experiments [3].

Tissue preparation. Upon sacrifice, livers were immediately removed, fixes in formalin at 4 °C, and routinely processed for paraffin embedding. Two serial thin sections were made. One cut at 3-μm thickness were stained with hematoxylin and eosin staining for histological examination. The other section at 5-μm thickness was used for DNA extraction. Liver lesions were classified according to the diagnostic criteria described previously [1–3].

Polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis. DNA extraction from paraffin-embedded sections of 10 HCCs induced by DEN and 10 HCCs induced by the CDAA diet, and normal liver tissues adjacent to all HCCs was performed as described previously [18]. Two normal liver tissues form non-treated rats were used as controls.

PCR-SSCP analysis was then conducted to seek for mutations in the mtDNA D-loop. Four sets of primers were designed against the rat mtDNA D-loop sequence (GenBank Accession No. NC_001665) (Table 1). Briefly, PCR for SSCP analysis was performed in 10 μl of reaction mixture containing 1 μM of each primer, 200 μM of each dNTP, 1× PCR buffer (Applied Biosystems Japan Ltd., Tokyo, Japan), 2.5 U of AmpliTaq (Applied Biosystems Japan) and 0.5 μl of extracted DNA under the following reaction conditions; primary denaturation for 2 min at 96 °C followed by 36 cycles of 15 s of denaturation at 96 °C, 15 s of annealing at 53–58 °C and 1 min of extension at 72 °C, and a final extension period of 5 min at 72 °C. PCR products were diluted with 10 μl of loading solution containing 90% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue. Aliquots containing 6 μl of diluted products were electrophoresed on polyacrylamide gels using a GeneGel Excel 12.5/24 kit (Amersham Pharmacia Biotech Co. Ltd., Tokyo, Japan) at 8, 15, 18, and 20 °C for 90 min at 15 W, using a GenePhor Electrophoresis Unit (Amersham Pharmacia Biotech). After electrophoresis, the gels were stained with a DNA Silver Staining kit (Amersham Pharmacia Biotech).

DNA nucleotide sequencing. Following PCR-SSCP analysis, DNA fragments in abnormally shifted bands in the gel were extracted and reamplified. The obtained PCR products were directly sequenced using a BigDye terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems Japan) and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan). To confirm the results, PCR amplification was repeated on the same samples and each PCR product was sequenced with forward and reverse primers at least twice.

The products obtained from PCR amplification with each primer set were also directly sequenced.

Results and discussion

The 10 HCCs induced by DEN in 10 rats and 10 HCCs induced by the CDAA diet in 10 rats used in this study were all histologically well-differentiated. There were no differences in inflammation in the livers and histological grade of HCCs.

Representative results of PCR-SSCP and DNA sequencing analyses are shown in Fig. 1. Five out of 10 HCCs (50%) induced by DEN showed bandshifts in nucleotides 15617–15878 and 15839–16125 in mtDNA D-loop. Four out of 5 mutations were G/C to A/T transitions at positions 15707, 15717, 15930, and 16087, and one mutation T/A to C/G transition at position 15559. One (the case of HCC8) out of five mutations showed to harbor a homoplasmic mutation, because of loss of normal bands which include normal mtDNA sequences. No mutations were

Table 1
The primers used for PCR-SSCP analysis

Nucleotide position[a]	Primer sequence	Product size (bp)	Annealing temperature (°C)
15396–15660	IF: 5′-TCTATTTAAACTACTTCTTGTC-3′ 1R: 5′-CAGGGATAGTCATATGGAAG-3′	265	56
15617–15878	2F: 5′-CACCATTAAGTCATAAACCT-3′ 2R: 5′-GATCATGGGCTGATTAGACC-3′	263	53
15839–16125	3F: 5′-AAGACATCTCGATGGTAACG-3′ 3R: 5′-GGGTTTGGCATTGAAGTTTC-3′	287	58
16068–16313	4F: 5′-GAAAAATCTGTCAACAAACC-3′ 4R: 5′-TTTGGTGTATGTGGAATTTTC-3′	246	56

[a] Base numbering from GenBank NC_001665.

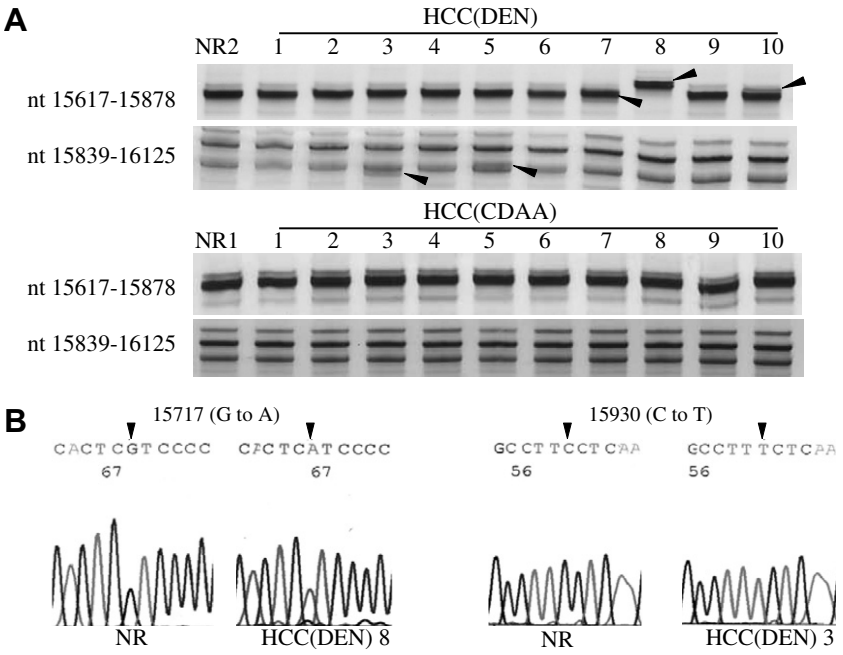

Fig. 1. (A) Representative results of PCR-SSCP analysis for mtDNA D-loop in HCCs induced by DEN and the CDAA diet in rats. Arrowhead show abnormal band shifts. NR, normal liver tissue. (B) Representative results of nucleotide sequencing analysis. These results and the corresponding HCC number are summarized in Table 2. NR, normal liver tissue.

found in normal adjacent tissues to 10 HCCs (data not shown). By contrast, 10 HCCs induced by the CDAA diet showed no abnormal bandshifts, and we also confirmed by direct nucleotide sequencing that the PCR products amplified contained the normal mtDNA D-loop sequence (data not shown). These results are summarized in Table 2.

It has been considered that G/C to A/T transition is a common mutation induced by nitorosocompounds [19]. Previously, we indicated that Ki-ras mutations were all G/C to A/T transitions at codon 12 in rat lung and hamster pancreatic tumors induced by nitorosocompounds [20,21]. In this study, 4 out of 5 mutations in HCCs induced by DEN were G/C to A/T transitions. Therefore, it seems that these mutations were also caused by DEN per se. By contrast, no mutations in HCCs induced by the CDAA diet were detected. It has been reported that 8-hydroxyguanine, a representative feature of oxidative DNA damage, is generated by the CDAA diet [4], and it is well-established that 8-hydroxyguanine induces G/C to T/A or A/T to C/G transversions in *Escherichia coli* [22]. Therefore, it is suggested that mtDNA D-loop region is a target of nitrosocompounds in rat HCCs, but not 8-hydroxyguanine generated by the CDAA diet. Although the biological significance for alterations of D-loop region in rat liver carcinogenesis induced by DEN is not clarified, this region contains replication and transcription elements essential for mitochondria [11,23].

In human HCCs, it has been reported that 13 of 19 cases (68%) harbored mtDNA D-loop mutations, demonstrating mtDNA mutations within this region are a frequent event in HCCs [14]. Mitochondria is major targets during liver injury from several factors, such as metal overload, certain drugs and toxins, alcoholic liver injury, and conditions of oxidative stress, and DNA damage may be accumulated during liver carcinogenesis in mitochondria [14,24,25]. In the present study, we indicated oxidative stress may not contribute to mtDNA D-loop mutations in HCCs induced by the CDAA diet.

There are several reports of gene mutations in HCCs induced in exogenous and endogenous model. Ki-ras and

Table 2
Summary of mutations of mtDNA D-loop in rat HCCs induced by DEN and the CDAA diet

Samples	Incidence and patterns of mutations
DEN	5/10 (50%)
1	ND
2	ND
3	15930: C → T
4	ND
5	16087: C → T
6	ND
7	15707: C → T
8	15717: G → A
9	ND
10	15559: T → C
CDAA	0/10 (0%)
1	ND
2	ND
3	ND
4	ND
5	ND
6	ND
7	ND
8	ND
9	ND
10	ND

p53 mutations were found to be absent or low frequent in rat HCCs induced by DEN or the CDAA diet [26]. A high rate of β-catenin mutations was detected in rat HCCs induced by DEN, while HCCs induced by the CDAA diet indicated a low frequent mutation [5]. Therefore, together with the present study different genetic pathway may be involved in exogenous and endogenous liver carcinogenesis. Because of a multiple methyl group donor-deficiency, DNA hypomethylation is well known as one of the mechanisms underlying rat liver carcinogenesis resulting from the choline-deficient diet [27–29]. In fact, hypomethylation of growth-related genes, c-fos, c-myc, and c-Ha-ras was detected in livers of rats after short-term feeding with the choline-deficient diet [29]. By contrast, hypermethylation of CpG sites in the 5′upstream region of tumor suppressor genes, such as E-cadherin, connexin26 and Tslc1, has been also reported in rat HCCs induced by the CDAA diet [30].

In conclusion, we demonstrated different mutation patterns of mtDNA D-loop region in rat HCCs induced in exogenous and endogenous liver carcinogenesis models. It is unclear the differences in the rate of mutations between two models. In human colorectal tumors, it has been reported that mtDNA mutations may be related to the high level of reactive oxygen species in mitochondria [31]. Therefore, it is possible that this discrepancy may be due to a species or organ difference. Recent our investigations have shown that no alterations of mtDNA D-loop were detected in hamster pancreatic duct adenocarcinomas induced by nitrosocompound (unpublished data). To better understand the involvement of mtDNA during exogenous and endogenous liver carcinogenesis, further studies investigating alterations in other regions of the mtDNA are required.

Acknowledgments

This study was supported in part by the Foundation for Promotion of Cancer Research in Japan, and by a Grant (RK17-027) from the Faculty of Science and Engineering, Kinki University.

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