

Supplementary Information

Metronidazole enhances steatosis-related early-stage hepatocarcinogenesis in high fat diet-fed rats through

DNA double-strand breaks and modulation of autophagy

Environmental Science and Pollution Research

Ayumi Eguchi^a, Sayaka Mizukami^{a,b}, Misato Nakamura^a, Sousuke Masuda^a, Hirotsada Murayama^a, Masashi

Kawashima^a, Mari Inohana^a, Rei Nagahara^a, Mio Kobayashi^a, Risako Yamashita^a, Suzuka Uomoto^a, Emi Makino^c,

Ryoichi Ohtsuka^c, Naofumi Takahashi^c, Shim-Mo Hayashi^d, Robert R. Maronpot^c, Makoto Shibutani^a,

Toshinori Yoshida^{a,*}

^a Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan

^b Pathogenetic Veterinary Science, United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu-shi, Gifu 501-1193, Japan

^c The Institute of Environmental Toxicology, 4321, Uchimoriya-machi, Joso-shi, Ibaraki 303-0043, Japan

^d Global Scientific and Regulatory Affairs, San-Ei Gen F. F. I., Inc., 1-1-11 Sanwa-cho, Toyonaka, Osaka 561-8588, Japan

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*Correspondence: Toshinori Yoshida, D.V.M., Ph.D., Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan. E-mail: yoshida7@cc.tuat.ac.jp;

Tel +81-42-367-5874; Fax: +81-42-367-5771

Supplementary table 1. Sequence of primers used for real-time RT-PCR

Accession no.	Gene	Forward primer (5' →3')	Reverse primer (5' →3')
NM_178095	<i>Abca1</i>	CCAGGAGCGT GTGAGCAAAG	ACCAGTGTAG CAGGGACCAC ATAA
NM_053502	<i>Abcg1</i>	GCCTGGCCAT TGCCTAGAA C	GGACACCACT TGGAAGCAAG A
NM_017340	<i>Acox1</i>	GCGCAAGGAG CGGGCCTCC	CTCGACGGCG CCGGGTATTC
NM_001012097.1	<i>Atg7</i>	TTCTTAGAAGATTTGACTGGTCTTACA	TCACTCATGTCCCAGATCTCA
NM_012520	<i>Catalase</i>	ATTGCCGTCC GATTCTCC	CCAGTTACCA TCTTCAGTGT AG
NM_053437	<i>Dgat1</i>	CACGAATCAT TGAGCGTCTC TTA	GCCAATAGAA GAAGATGAGC CATATC
NM_017332	<i>Fasn</i>	GCGGGCGTGG TAATGCT	CTGTTTCGAA ATACGCTCCA T
NM_030826	<i>Gpx1</i>	GCTGCTCATT GAGAATGTGC	GAATCTCTTCA TTCTTGCCAT T
NM_183403	<i>Gpx2</i>	GTGTGATGTC AATGGCAGA AT	AGGGCAGCTT GTCTTTCAGG TA
NM_017051	<i>Mn-SOD</i>	GACCTGCCTT ACGACTATG	TACTTCTCCT CGGTGACG
NM_031789	<i>Nfe2l2</i>	TGCCCTGGA AGTGTCAAA	GGCTGTACTG TATCCCCAGA AGA
NM_024160	<i>Cyba</i>	TGTTGCAGGA GTGCTCATCT GTCT	AGGACAGCCC GGACGTAGTA ATTT
NM_012857	<i>Lamp1</i>	GCAAGGCGCTCGCCCTCAAT	GCCCCGCTGACTCCTCTTCC
NM_017068	<i>Lamp2</i>	AGCAGGTGGTTTCCGTGTCTCG	AGGGCTGTCCCACCGCTAT
NM_022867	<i>LC3</i>	CGGGTTGAGGAGACACACAA	TCTTTGTTTGAAGCTCCGGC
NM_024160.1	<i>P22phox</i>	TGTTGCAGGAGTGCTCATCTGTCT	AGGACAGCCCCGGACGTAGTAATTT
NM_001100984	<i>P67phox</i>	GAAAGCATGAAGGATGCCTGG	ATAGACCAAGATCACATCTCC
NM_001007144	<i>Plin2</i>	CCGAGCGTGG TGACGAGGG	GAGGTCACGG TCCTCACTCC C
NM_001134637	<i>Plin5</i>	GGATGTCCGG TGATCAGAC	GTGCACGTGG CCCTGACCAG
NM_001108509	<i>Pnpla2</i>	CGGTGGATGA AGGAGCAGAC A	TGGCACAGAC GGCAGAGACT
NM_013196	<i>Ppara</i>	CCCCACTTGA AGCAGATGAC C	CCCTAAGTAC TGGTAGTCCG C
NM_001145366	<i>Pparg</i>	GACCACTCCC ATTCCTTTGA	CATTGGGTCA GCTTGTGA
NM_001276708	<i>Srebf1</i>	GGAGCCATGG ATTGCACATT	GCTTCCAGAG AGGAGCCCAG
NM_001033694	<i>Srebf2</i>	TTCAGCACCG CTCCACAGA	GCACCTGCTG CTGGATGGTA
NM_031144	<i>Actb</i>	CCCTGGCTCC TAGCACCAT	AGAGCCACCA ATCCACACAG A

Abbreviations: *Abca1*, ATP binding cassette subfamily A member 1; *Abcg1*, ATP binding cassette subfamily G member 1; *Acox1*, acyl-CoA oxidase 1; *Actb*, actin, beta; *Atg7*, Autophagy related 7; *Dgat1*, diacylglycerol O-acyltransferase 1; *Fasn*, Fatty acid synthase; *Gpx1*, Glutathione peroxidase 1; *Gpx2*, Glutathione peroxidase 2; *Lamp1*, Lysosomal Associated Membrane Protein 1; *Lamp2*, Lysosomal Associated Membrane Protein 2; *LC3*, Microtubule Associated Protein 1 Light Chain 3 Alpha; *Mn-SOD*, Manganese superoxide dismutase; *Nfe2l2*, nuclear factor, erythroid 2-like 2; *Ncf2*, neutrophil cytosolic factor 2; *Cyba*, cytochrome b-245 alpha chain; *Plin2*, Perilipin2; *Plin5*, Perilipin 5; *Pnpla2*, Patatin-Like Phospholipase Domain Containing 2; *Ppara*, Peroxisome proliferator activated receptor alpha; *Pparg*, peroxisome proliferator activated receptor alpha; Perilipin2; RT-PCR, reverse transcription polymerase chain reaction; *Scd*, Stearoyl-CoA desaturase; *Srebf1*, sterol regulatory element binding transcription factor 1; *Srebf2*, sterol regulatory element binding transcription factor 2.

Supplementary table 2. Antibodies for immunohistochemistry

Antigen	Host species	Clonality (Clone)	Dilution	Antigen retrieval	Manufacture
ADFP	Mouse	Monoclonal (AP125)	1:400	Microwaving at 90°C for 10 min in citrate buffer, pH6.0	Abcam (Cambridge, UK)
Cleaved Caspase-3	Rabbit	Polyclonal	1:500	Autoclaving at 121°C for 10 min in antigen retrieval solution (Dako), pH9.0	Cell Signaling Technology, Inc. (Massachusetts, USA)
GST-P	Rabbit	Polyclonal	1:1000	none	Medical & Biological
Histone H2A.X	Rabbit	Monoclonal (EP854(2)Y)	1:3000	Autoclaving at 121°C for 10 min in citrate buffer, pH6.0	Abcam (Cambridge, UK)
Ki-67	Mouse	Monoclonal (MIB-5)	1:50	Autoclaving at 121°C for 10 min in citrate buffer, pH6.0	Dako (Glostrup, Denmark)
PCNA	Mouse	Monoclonal (PC10)	1:200	Microwaving at 90°C for 10 min in citrate buffer, pH6.0	Dako (Glostrup, Denmark)
PPAR γ	Rabbit	Monoclonal (C26H12)	1:800	Microwaving at 90°C for 10 min in citrate buffer, pH9.0	Cell Signaling Technology, Inc. (Massachusetts, USA)
p22phox	Rabbit	Polyclonal	1:200	Autoclaving at 121°C for 10 min in antigen retrieval solution (Dako), pH9.0	Bioss Inc. (Massachusetts, USA)
p62;SQSTM1	Rabbit	Polyclonal	1:400	Autoclaving at 121°C for 10 min in citrate buffer, pH6.0	Proteintech Group, Inc (IL, USA)
LC3	Rabbit	Polyclonal	1:200	Autoclaving at 121°C for 10 min in antigen retrieval solution (Dako), pH9.0	Bioss Inc. (Massachusetts, USA)

Supplemental table 3. Blood biochemistry in rats[§]

Parameters	CTL	HFD	HFD+MNZ‡
No. of animals	13	13	13
AST (U/L)	100.4±29.8	91.1±7.5	83.8±37.1
ALT (U/L)	52.8±5.5	49.2±3.1	37.5±3.8 ^{ab}
ALP (U/L)	604.9±65.2	524.5±41.0 ^a	506.8±42.6 ^a
T.BIL (mg/dL)	0.04±0.01	0.06±0.01 ^a	0.07±0.01 ^a
LBIL (mg/dL)	0.03±0.01	0.05±0.01 ^a	0.05±0.01 ^a
D.BIL (mg/dL)	0.01±0.00	0.02±0.00 ^a	0.01±0.01
T.CHOL (mg/dL)	57.2±5.6	54.1±8.4	61.2±8.6
TG (mg/dL)	56.2±14.3	37.3±14.5 ^a	51.4±23.0

Abbreviations: AST, aspartate aminotransaminase; ALP, alkaline phosphatase; ALT, alanine aminotransaminase; CTL, control; D.Bil, direct bilirubin; LBIL, indirect bilirubin; HFD, high fat diet; MNZ, metronidazole; T.BIL, total bilirubin; T.CHOL, total cholesterol; TG, triglyceride.

§: All animals were subjected to a medium-term liver carcinogenesis bioassay.

‡: Rats were given HFD mixed with MNZ during weeks 2 and 11.

Data are shown as the mean ± standard deviation.

a: p<0.05 vs CTL (Tukey's or Steel-Dwass test).

b: p<0.05 vs HFD (Tukey's or Steel-Dwass test).

Supplemental table 4. Immunoexpression of p22phox, Ki-67 and cleaved caspase-3 in the preneoplastic liver foci and surrounding hepatocytes in rats§

Parameter	Distribution	Unit	CTL	HFD	HFD+MNZ‡
p22phox	inside	(%)	2.4±1.4	5.6±4.0 ^a	4.2±2.2
	outside	(No/10HPF)	11.3±4.8	15.8±5.2 ^a	12.2±3.7
Ki-67	inside	(%)	3.0±2.1	3.3±2.7	2.6±1.6
	outside	(No/10HPF)	71.1±15.4	91.6±28.9	86.9±29.2
Cleaved caspase-3	inside	(%)	0.1±0.1	0.2±0.3	0.1±0.1
	outside	(No/10HPF)	1.7±1.3	2.6±1.6	2.0±1.2

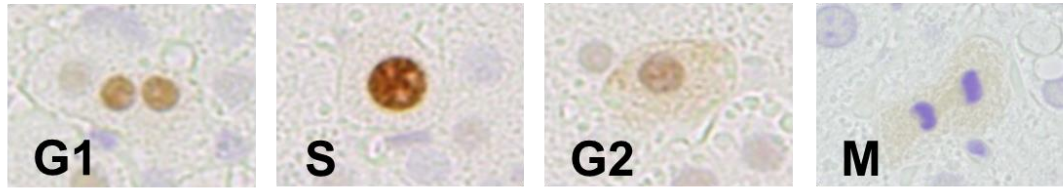
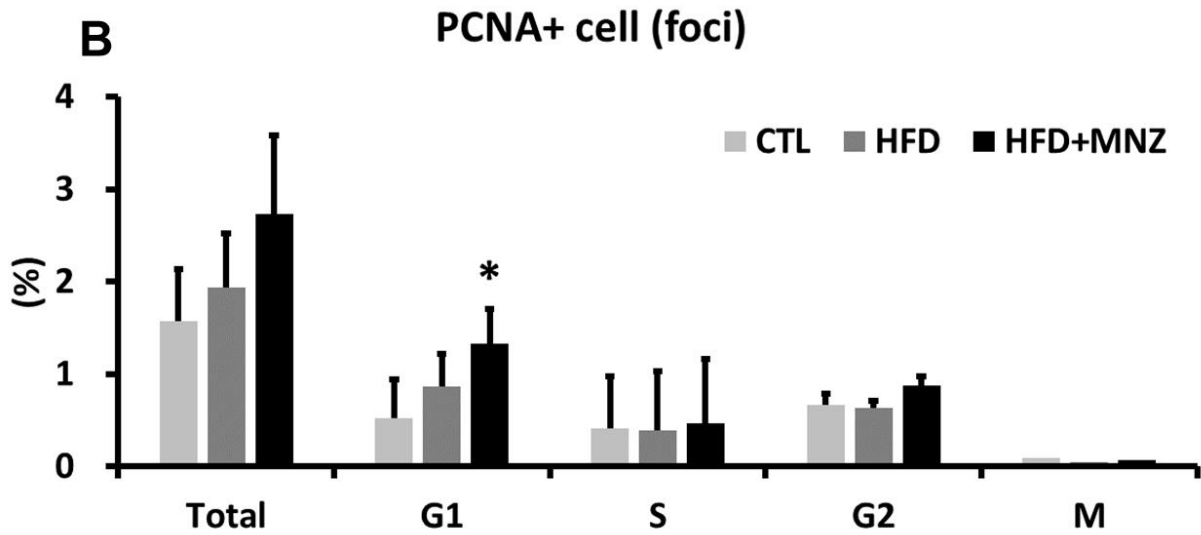
Abbreviations: CTL, control; HFD, high fat diet; HPF, high power field; MNZ, metronidazole.

‡: Rats were given HFD mixed with MNZ during the study.

Three parameters were examined in the preneoplastic liver foci (inside of foci) and surrounding hepatocytes (outside of foci).

Data are shown as the mean ± standard deviation.

a: p<0.05 vs CTL (Tukey's or Steel-Dwass test).

A**B**

Supplemental Figure 1. PCNA-positive cells in rat liver. (A) Representative images of hepatocytes in G1, S, G2, and M phases. (B) Quantification of PCNA-positive cells in total, and in each phase (G1, S, G2, M) is presented as (%) of total cell number counted. Data are presented as mean \pm standard deviation. * $p < 0.05$ compared with the CTL group (multiple comparison tests: Tukey or Steel-Dwass test).