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Role of *Atp7b* Gene in Spontaneous and *N*-Diethylnitrosamine-induced Carcinogenesis in a New Congenic Strain, WKAH.C-*Atp7b* Rats

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To examine whether Long-Evans Cinnamon (LEC) rats, a mutant rat model of Wilson's disease, have a susceptibility gene(s) to hepatocarcinogenesis in addition to the causative gene, *Atp7b*, we established a new congenic strain, WKAH.C-*Atp7b* rats, in which the *Atp7b* gene of the LEC rats is inserted into the normal Wistar-King Aptekman Hokkaido (WKAH) background. Hepatocellular tumors developed spontaneously in both sexes of WKAH.C-*Atp7b* rats, their incidence being slightly lower than that in LEC rats. Incidences of spontaneous liver tumors in LEC, WKAH.C-*Atp7b* and WKAH rats correlated with hepatic copper and iron concentrations. Medium-term liver bioassay showed that LEC rats were more susceptible to the induction of glutathione *S*-transferase placental form-positive preneoplastic foci than WKAH.C-*Atp7b* rats, and WKAH.C-*Atp7b* rats were more susceptible than WKAH rats. In an *N*-diethylnitrosamine (DEN)-induced long-term carcinogenicity study, 1) LEC rats were similarly or rather less susceptible to hepatocellular tumors than WKAH.C-*Atp7b* and WKAH rats, indicating that the progression of the preneoplastic foci to liver cancer in LEC rats was worse than that in WKAH.C-*Atp7b* and WKAH rats, 2) the incidences of kidney tumors in LEC and WKAH.C-*Atp7b* rats were higher than that in WKAH rats and high copper concentrations in the kidneys were observed in LEC and WKAH.C-*Atp7b* rats, 3) LEC rats were resistant to lung carcinogenesis. These data indicate that the susceptibility of LEC rats to liver and kidney carcinogenesis could be explained by *Atp7b* gene mutation and that the susceptibility to lung carcinogenesis is controlled by gene(s) other than *Atp7b*.

Key words: LEC rat — *Atp7b* — Congenic — *N*-Diethylnitrosamine — Copper

LEC rats were established as a mutant strain in which hepatitis (hepatocellular necrosis) with severe jaundice and liver tumors develop spontaneously.[1–3] About 10–20% of male, and 40–50% of female rats die of fulminant hepatitis.[2–4] Their hepatitis is controlled by a single recessive gene designated as *Wnd* (*Atp7b*), which is a copper-transporting ATPase gene homologous to the Wilson's disease gene, and LEC rats have a partial deletion of the *Atp7b* gene.[5] The *Atp7b* gene was originally named the *hts* gene, and mapped on chromosome 16.[6] These rats also show arrest of maturation from $CD4^+8^+$ to $CD4^+8^-$ cells in the thymus, which is not linked to hepatitis genetically and the T-helper immunodeficiency (*thid*) gene is mapped on chromosome 1.[7,8] In LEC rats, hepatocellular and renal cell tumors develop due to excess copper and/or hemolysis-induced iron accumulation in the organs.[9–11] An *in vivo* short-term assay study suggested that LEC rats are a DEN-susceptible strain[12] and the susceptibility of this strain to hepatocarcinogens is genetically independent of copper accumulation in the liver.[13] We have been employing genetic linkage analysis to determine hepatocarcinogen susceptibility loci using a total of 139 DEN-treated (F344×LEC)F2 and (LEC×F344)F2 rats, but we have not yet found susceptibility loci (unpublished data). To determine whether LEC rats have a susceptibility-to-hepatocarcinogenesis gene(s), we compared spontaneous and DEN-induced medium- and long-term carcinogenicity of *Atp7b* congenic rats with those of LEC and WKAH rats.

MATERIALS AND METHODS

Animals LEC/Tj and WKAH/Tj inbred rats were bred in the Institute for Animal Experimentation, the University of Tokushima School of Medicine, under specific pathogen-free conditions. To establish a congenic strain for the *Atp7b* gene, we crossed a LEC male rat with a normal WKAH female rat. The N1 rats were further backcrossed with WKAH rats. At the N2 generation, all N2 rats were crossed with LEC rats first to distinguish the *Atp7b*/+ heterozygotes and +/+ homozygotes in each N2 rat by determination of serum ceruloplasmin activity.[14] Then *Atp7b*/+

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Abbreviations: LEC, Long-Evans Cinnamon; DEN, *N*-diethylnitrosamine; GST-P, glutathione *S*-transferase placental form; WKAH, Wistar-King Aptekman Hokkaido; BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine.

heterozygotes among the N2 rats were further backcrossed with WKAH rats. This process was repeated until the N8 generation, when both *Atp7b*/+ female and male rats were crossed to get *Atp7b*/*Atp7b* homozygotes. Finally, we obtained *Atp7b*/*Atp7b* homozygotes at the N8F4 generation, and designated them as the WKAH.C-*Atp7b*/Tj congenic strain. WKAH.C-*Atp7b* rats have been bred by sister-brother matings. The allele distribution for 21 biochemical loci of the WKAH.C-*Atp7b* strain is completely concordant with that of the WKAH strain (data not shown), indicating that the *Atp7b* gene of LEC rats is fixed in the WKAH background.

Animals were housed three to a plastic cage with sterilized woodchips for bedding in an air-conditioned room at 23±2°C and 55±10% humidity with a 12 h light/dark cycle, and given pellet diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*.

Spontaneous tumors Totals of 192 male and 37 female LEC rats, 18 male and 45 female WKAH.C-*Atp7b* rats and 54 male and 42 female WKAH rats were used to examine spontaneous tumors. The number of male WKAH.C-*Atp7b* rats was limited because of their early death. Animals were killed at 51–120 weeks old under ether anesthesia. The sizes of liver tumors of more than 5 mm in diameter were recorded. Grossly apparent lesions and tissues from major organs were fixed in 10% phosphate-buffered formalin. Paraffin-embedded tissues were sectioned, stained with hematoxylin and eosin, and examined histologically. Histological types of liver tumors were classified as reported.[15] Hepatocellular carcinomas were subclassified into three grades.

Medium-term liver bioassay Six-week-old male LEC, WKAH.C.-*Atp7b* and WKAH rats (n=15) were treated with i.p. injection of 200 mg/kg DEN. All rats were subjected to two-thirds partial hepatectomy in week 3 and killed in week 8. The liver slices were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned and immunostained with rabbit anti-GST-P serum (MBL Co., Nagoya) by the avidin-biotin peroxidase method (LSAB kit, DAKO, Carpinteria, CA). The numbers and areas of GST-P-positive hepatic foci (≥0.1 mm in diameter) were measured with a color image analyzer (SP-500, Olympus Optical, Tokyo). This procedure corresponds to the control group of the medium-term bioassay for hepatocarcinogenesis established by Ito *et al*.[16]

Carcinogenicity study Six-week-old male LEC, WKAH.C-*Atp7b*, and WKAH rats (n=21) were given s.c. injections of 100 mg/kg DEN (Tokyo Chemical Industry Co., Tokyo) in weeks 0, 2 and 4, and controls were treated with 0.9% NaCl. All surviving animals were killed in week 50, and their liver, kidneys, lung, thymus, pancreas, spleen and cecum were examined histologically.

Analyses of metals Tissues of the liver and kidneys of untreated rats of 22 and 56 weeks old (n=4 to 6) were ashed with nitric acid, and their copper and iron concentrations were measured in an atomic absorption spectrophotometer (AA-782, Nippon Jarrel Ash, Co., Kyoto).

Statistical analyses Data were analyzed statistically by means of Fisher's exact probability test and Student's t test.

RESULTS

General features of WKAH.C-*Atp7b* rats Jaundice of WKAH.C-*Atp7b* rats developed later and was more mild than that of LEC rats. Liver cells of untreated WKAH.C-*Atp7b* rats showed megalocytosis, being similar to those of LEC rats (Fig. 1). Karyomegaly of proximal renal tubular cells was observed in both LEC[17] and WKAH.C-*Atp7b* rats. The thymic medulla of LEC rats showed hypoplasia as we have reported,[7] but the thymus of WKAH.C-*Atp7b* rats was histologically similar to that of WKAH rats.

Spontaneous tumors Table I shows the incidences of spontaneous hepatocellular tumors (adenomas plus carcinomas) in LEC, WKAH.C-*Atp7b* and WKAH rats. Hepatocellular tumors developed spontaneously in both LEC and WKAH.C-*Atp7b* rats, but the incidence of tumors in WKAH.C-*Atp7b* rats was lower, and their onset was thought to be later than in LEC rats, although the number of WKAH.C-*Atp7b* rats was small. The first hepatocellular carcinomas in LEC rats developed in week 63 in a male and in week 64 in a female. On the other hand, in WKAH.C-*Atp7b* rats the first carcinomas developed in week 77 in a male and in week 96 in a female.

Medium-term liver bioassay As shown in Fig. 2, the numbers and areas of GST-P-positive hepatic foci in LEC and WKAH.C-*Atp7b* rats were significantly higher than those of WKAH rats. The numbers of GST-P-positive foci in LEC rats were 1.8 times those of WKAH.C-*Atp7b* rats (P<0.001), but the areas of the foci were not different in these two strains.

Carcinogenicity study Seventeen (81%) of the control WKAH.C-*Atp7b* rats died of hepatic or renal injury by 26 weeks, although jaundice was not severe. The kidneys of the dead WKAH.C-*Atp7b* rats showed acute tubular necrosis histologically. In contrast, one (5%) of the control LEC rats died of hepatic injury in 9 weeks. The first tumor developed in a LEC rat at week 27, but most of the rats in each group were killed after week 42. Rats which were found dead with advanced postmortem change were excluded. Hepatocellular tumors developed in 9 (50%) of the control LEC rats, but in none of the control WKAH.C-*Atp7b* rats (Table II). Liver/body weights in DEN-treated WKAH.C-*Atp7b* and WKAH rats were higher than those in LEC rats, and numbers of liver tumors in WKAH.C-*Atp7b* and WKAH rats were not countable. The incidences of hepatocellular tumors in DEN-treated LEC, WKAH.C-*Atp7b* and WKAH rats were 94%, 100% and 100%,

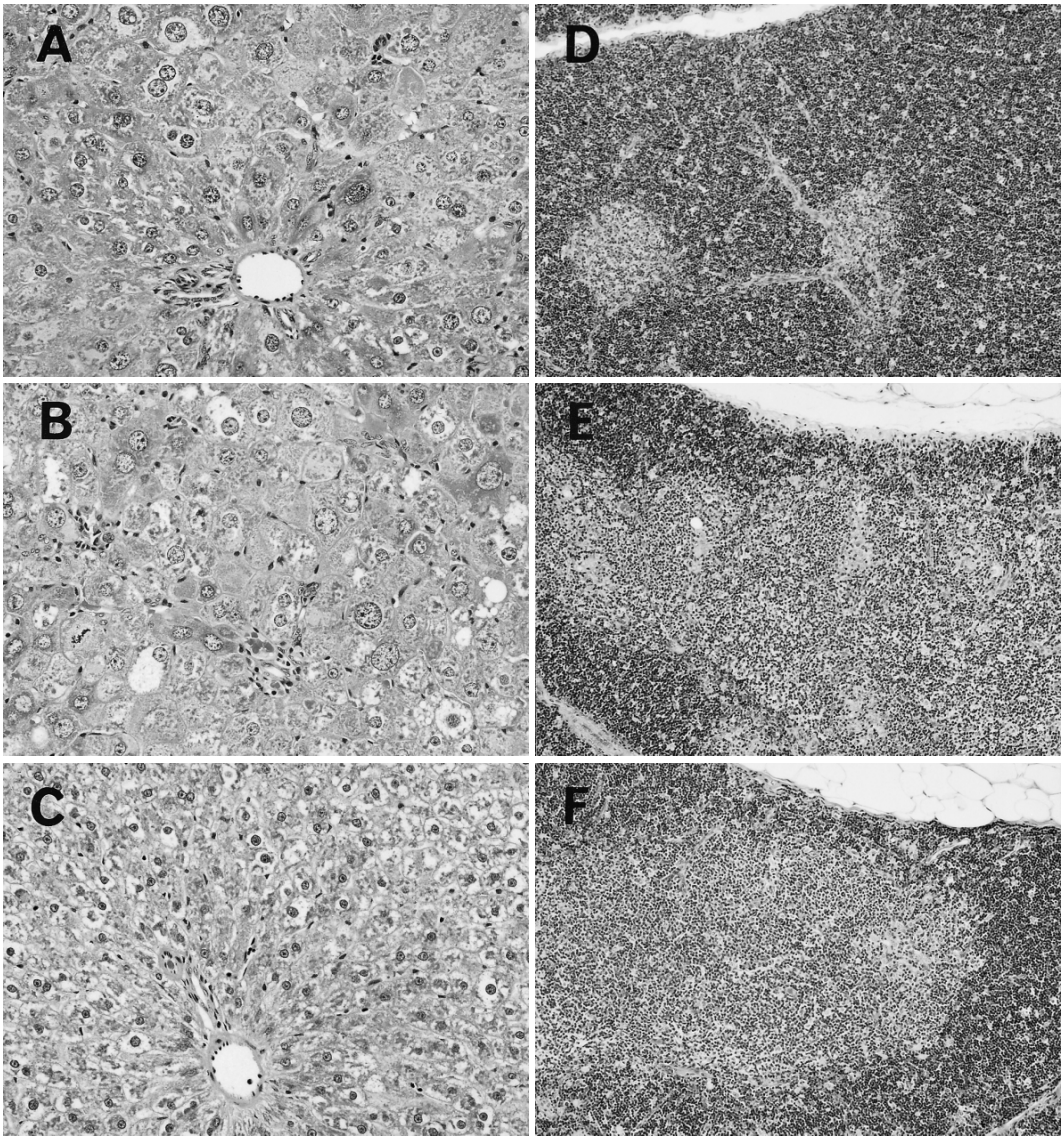

Fig. 1. Histological appearances of the liver and thymus of 56-week-old untreated male LEC, WKAH.C-*Atp7b* and WKAH rats. A–C, livers of (A) LEC, (B) WKAH.C-*Atp7b* and (C) WKAH rats (×150); D–F, thymuses of (D) LEC, (E) WKAH.C-*Atp7b* and (F) WKAH rats (×75). The livers of LEC and WKAH.C-*Atp7b* rats contained megalocytic hepatocytes. The appearance of thymic medulla of WKAH.C-*Atp7b* rats was similar to that of WKAH rats.

respectively, and all DEN-treated WKAH.C-*Atp7b* rats had hepatocellular carcinomas. The histological grades of hepatocellular tumors in WKAH.C-*Atp7b* rats were higher than those in LEC and WKAH rats, and the incidences of poorly differentiated hepatocellular carcinomas in LEC, WKAH.C-*Atp7b* and WKAH rats were 14%, 37% and 6%, respectively. Consequently WKAH.C-*Atp7b* rats showed a higher incidence of metastasis to the lung than LEC rats. WKAH.C-*Atp7b* and LEC rats showed similar susceptibilities to DEN-induced renal carcinogenesis (Table III). LEC rats were the most resistant to DEN-induced lung carcinogenesis, and WKAH.C-*Atp7b* rats showed the same susceptibility as WKAH rats.

Metal concentration The hepatic copper and iron concentrations of untreated WKAH.C-*Atp7b* rats in week 56 were lower than those of LEC rats (Fig. 3). In contrast, the renal copper concentration of WKAH.C-*Atp7b* rats was higher than that of LEC rats, and was maintained until week 56. The hepatic iron concentration of WKAH.C-*Atp7b* rats in week 22 and 56 was lower than that of LEC

Table I. Incidences of Spontaneous Hepatocellular Tumors (≥5 mm) in LEC, WKAH.C-*Atp7b*, and WKAH Rats

Strain	Sex	No. of rats	Age (weeks)						
			51–60	61–70	71–80	81–90	91–100	101–110	111–120
LEC	M	192	7/46 (15)	33/72 (46)	22/28 (79)[b, c]	6/7 (86)[e]	12/12 (100)[e]	13/13 (100)[d]	14/14 (100)[a]
	F	37	—	13/30 (43)	2/2 (100)[c]	1/1 (100)	2/2 (100)[d]	—	2/2 (100)
WKAH.C-*Atp7b*	M	18	0/4	0/2	1/8 (13)	1/1 (100)	2/3 (67)[c]	—	—
	F	45	—	0/4	1/9 (11)	3/5 (60)[d]	10/16 (63)[e]	8/11 (73)	—
WKAH	M	54	—	—	0/3	0/12	1/37 (3)	0/2	—
	F	42	—	—	0/5	0/18	0/14	1/4 (25)	0/1

a) Two rats with hepatocellular carcinomas had metastases to the lung or adrenal gland.
b) Significantly different from WKAH.C-*Atp7b* rats at $P<0.01$ by Fisher's exact probability test.
c, *d*, *e*) Significantly different from WKAH rats by Fisher's exact probability test: *c*) $P<0.05$, *d*) $P<0.01$, *e*) $P<0.001$.
Values in parentheses are percentages.

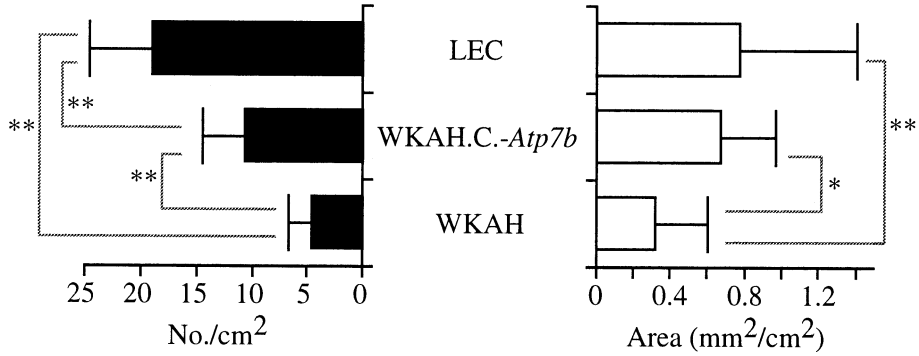

Fig. 2. Numbers and areas of GST-P-positive foci in male LEC, WKAH.C-*Atp7b* and WKAH rats. Differences are significant at * $P<0.01$ and ** $P<0.001$ by Student's *t* test. Bars, SD.

Table II. Incidences of Liver Tumors in LEC, WKAH.C-*Atp7b* and WKAH Rats Treated with DEN ($n=21$)

Treatment	Strain	Effective no. of rats (≥27 weeks)	Body weight (g)	Liver/body weight (%)	Liver tumor			
					Hepatocellular tumor	Hepatocellular adenoma	Hepatocellular carcinoma	Carcinoma metastasized to the lung
0.9% NaCl	LEC	18	338±16[a]	3.6±1.4	9 (50)	8 (44)	1 (6)[c]	0
	WKAH.C-*Atp7b*	4	527±84	3.2±0.2	0	0	0	0
	WKAH	21	508±29	2.9±0.1	0	0	0	0
DEN	LEC	17	315±21	7.1±3.8[b]	16 (94)	16 (94)	14 (82)[d]	4 (24)[g]
	WKAH.C-*Atp7b*	19	408±47	15.3±4.5	19 (100)	19 (100)	19 (100)[e]	11 (58)
	WKAH	18	394±71	18.3±8.8	18 (100)	18 (100)	16 (89)[f]	8 (44)

a) Mean±SD.
b) Significantly different from WKAH.C-*Atp7b* rats ($P<0.001$) and WKAH rats ($P<0.01$, Student's *t* test).
c) Well differentiated, 1 (100%).
d) Well differentiated, 2 (14%); moderately differentiated, 10 (71%); poorly differentiated, 2 (14%).
e) Well differentiated, 1 (5%); moderately differentiated, 11 (58%); poorly differentiated, 7 (37%).
f) Well differentiated, 4 (25%); moderately differentiated, 11 (69%); poorly differentiated, 1 (6%).
g) Significantly different from WKAH.C-*Atp7b* rats ($P<0.05$, Fisher's exact probability test).
Values in parentheses are percentages.

Table III. Incidences of Kidney and Lung Tumors in LEC, WKAH.C-*Atp7b* and WKAH Rats Treated with DEN (n=21)

Treatment	Strain	Effective no. of rats (≥27 weeks)	Kidney tumor				Lung tumor		
			Renal cell tumor	Renal cell adenoma	Renal cell carcinoma	Nephro-blastoma	Lung tumor	Adenoma	Adeno-carcinoma
0.9% NaCl	LEC	18	0	0	0	0	0	0	0
	WKAH.C-*Atp7b*	4	0	0	0	0	0	0	0
	WKAH	21	0	0	0	0	0	0	0
DEN	LEC	17	8 (47)[a)]	7 (41)[a)]	1 (6)	1 (6)	0[c)]	0[c)]	0
	WKAH.C-*Atp7b*	19	9 (47)[a)]	9 (47)[b)]	2 (11)	0	10 (53)	10 (53)	3 (16)
	WKAH	18	2 (11)	1 (6)	1 (6)	0	4 (22)	3 (17)	1 (6)

a, *b*) Significantly different from WKAH rats by Fisher's exact probability test: *a*) $P<0.05$, *b*) $P<0.01$.
c) Significantly different from WKAH.C-*Atp7b* rats at $P<0.001$.
Values in parentheses are percentages.

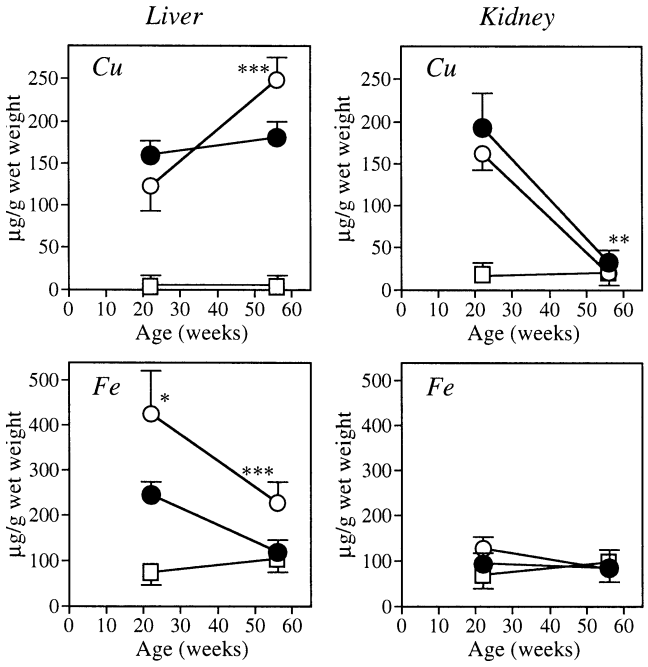

Fig. 3. Copper and iron concentrations in the liver and kidney of untreated LEC, WKAH.C-*Atp7b* and WKAH rats in weeks 22 and 56. Differences between LEC and WKAH.C-*Atp7b* rats are significant at * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ by Student's t test. ○ LEC, ● WKAH.C-*Atp7b*, □ WKAH. Bars, SD.

rats, but the renal iron concentrations in the two strains were not different.

DISCUSSION

A study using a modified Solt-Farber model assaying the number of glutathione *S*-transferase placental form-positive hepatic foci demonstrated that the susceptibility of LEC rats to hepatocarcinogens is genetically independent of hepatic copper accumulation.[13)] In our study using an F2 intercross, we could not find hepatocarcinogenesis susceptibility loci and there is no report on the cancer susceptibility or resistance gene(s) of LEC rats. Recently, genetic loci controlling rat hepatocarcinogenesis have been identified on several chromosomes.[18, 19)] Further experiments are needed to elucidate the susceptibility/resistance gene(s) of LEC rat hepatocarcinogenesis.

If LEC rats have a carcinogenesis susceptibility gene(s), they should develop tumors early in life. In LEC rats of over one year old, tumors of the liver and kidney develop spontaneously, and parenchymal cells in both the liver and kidney accumulate copper.[9, 10)] The incidence of hepatocellular tumors in LEC rats was 15% in males of 51–60 weeks old, all being adenomas, and the incidence of hepatocellular carcinomas was 50% even in males of 111–120 weeks old. In addition, copper chelating agents (D-penicillamine and trientine dihydrochloride) inhibited the development of liver tumors as well as hepatitis of LEC rats.[20–22)] Iron-deficient diet also inhibits the development of liver tumors.[23)] These data suggest that tumor development in LEC rats is a later event caused by chronic copper and/or iron toxicity.

We established a new congenic strain, WKAH.C-*Atp7b* rats, and first evaluated the features of the rats. In the present study, we demonstrated that, 1) hepatocellular tumors developed spontaneously in WKAH.C-*Atp7b* rats, but the incidence was slightly lower than that of LEC rats, 2) LEC rats were more susceptible to DEN than WKAH.C-*Atp7b* rats in the medium-term bioassay, but progression of the preneoplastic foci to hepatocellular tumors in LEC rats was worse than that in WKAH.C-*Atp7b* and WKAH rats as shown in the long-term carcinogenicity study, 3) incidences of spontaneous liver tumors in LEC, WKAH.C-*Atp7b* and WKAH rats correlated with hepatic copper and iron concentrations, and 4) spontane-

ous renal cell tumors, which are common in aged LEC rats,[10] were not found in WKAH.C-*Atp7b* rats, though LEC and WKAH.C-*Atp7b* rats, both of which have high renal copper accumulation, were more susceptible to DEN-induced renal carcinogenesis than WKAH rats with low renal copper concentration. These data suggest that the susceptibility of LEC rats to liver and kidney carcinogenesis could be explained by excess copper and/or iron accumulation in these organs.

In a recent carcinogenicity study, we found that LEC rats were more resistant to BBN-induced bladder carcinogenesis than F344 or Long-Evans Agouti rats.[24] Therefore, LEC rats are not necessarily a carcinogen-susceptible strain. In the present study, LEC rats were resistant to DEN-induced lung carcinogenesis, and WKAH.C-*Atp7b* and WKAH rats were susceptible. Our recent experiment also showed that LEC rats were more resistant to DEN and *N*-nitrosobis-(2-hydroxypropyl)amine-induced lung carcinogenesis than F344 rats (unpublished data). These data indicate that lung carcinogenesis is controlled by gene(s) other than *Atp7b*.

In the present study, the survival rate of DEN-treated WKAH.C-*Atp7b* rats was markedly higher than that of 0.9% NaCl-treated ones. This may have been due to hepatocyte renewal after a necrogenic dose of DEN, as was observed on repeated s.c. injections of D-galactosamine hydrochloride, a hepatotoxic agent, into LEC rats, and on partial hepatectomy in our recent study.[4] In the case of partial hepatectomy, the hepatic copper concentration of male LEC rats was 45% of that of sham-operated rats on day 14 and none of the rats died of hepatic injury.

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