Vol. 202, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1994 Pages 512-518

The gene responsible for LEC hepatitis, located on rat chromosome 16, is the homolog to the human Wilson disease gene

Nobuya Sasaki[1, 6], Yoshihide Hayashizaki[1], Masami Muramatsu[1], Yoichi Matsuda[2], Yohsuke Ando[3], Takashi Kuramoto[3], Tadao Serikawa[3], Takachika Azuma[4], Akihiro Naito[4], Takashi Agui[5], Tadashi Yamashita[7], Ichiro Miyoshi[7], Noritoshi Takeichi[6] and Noriyuki Kasai[7]*

[1]Gene Bank, RIKEN Tsukuba Life Science Center, The Institute of Physical and Chemical Research, Tsukuba Science City, Ibaraki 305, Japan

[2]National Institute of Radiological Science, Chiba 263, Japan

[3]Institute of Laboratory Animals, Faculty of Medicine, Kyoto University, Kyoto 606, Japan

[4]Department of Bacteriology and [5]Institute for Experimental Animal Science, Nagoya City University Medical School, Nagoya 467, Japan

[6]Cell Biology, Cancer Institute and [7]Institute for Animal Experimentation, School of Medicine, Hokkaido University, Sapporo 060, Japan

Received April 19,1994

Summary: We identified the rat homolog to the human Wilson disease (WD) gene as the gene responsible for hepatitis (*hts*) in the Long Evans Cinnamon (LEC) rat. A genetic study using fifty-three backcross progenies showed that the rat WD gene detected by Southern blotting using the human WD gene as a probe was tightly linked to the *hts* phenotype of the LEC rat with no recombination. LEC is a transcriptionally deficient mutant because no transcript of the rat WD gene could be found in the LEC rat by Northern blotting. This rat WD gene was mapped to 16q12.23-12.3 by fluorescence in situ hybridization and mouse×rat somatic cell hybrid analysis.

The LEC rat is a mutant strain that develops fulminant hepatitis and severe jaundice at around 4 months of age. About half of the animals die within one week after the onset of jaundice and in all survivors hepatocellular carcinoma spontaneously appears after 12 to 18 months (1, 2). One biochemical feature of the LEC rat that has been reported is an abnormal accumulation of copper in hepatocytes and a marked decrease in serum ceruloplasmin oxidase activity (3). These symptoms are very similar to those of human Wilson disease (WD), which is an autosomal recessive disorder controlled by a single gene located on human chromosome 13 (4). Genetic analysis revealed that hepatitis in the LEC rat is also controlled by a single autosomal recessive gene, called *hts* and another line of genetic analysis also clarified that the rat ceruloplasmin gene was not the gene responsible for the *hts* gene (5, 6). Though the

* To whom correspondence should be addressed. Present address: Institute for Animal Experimentation, School of Medicine, Tohoku University, Sendai 980, Japan.

0006-291X/94 $5.00

deficiency of ceruloplasmin activity is a characteristic biochemical abnormality of human WD, the human ceruloplasmin gene has been mapped to chromosome 3, not to chromosome 13, suggesting that the WD gene is not identical to the ceruloplasmin gene (7). These lines of evidence led us to infer that the rat homolog of the human WD gene might be responsible for the LEC hepatitis. Recently, human WD cDNA was isolated and this product is, as predicted, a copper-binding P-type ATPase protein homologous to the Menkes disease gene (8-10). In this report, we demonstrate that the rat homolog of the human WD gene is the gene responsible for the *hts* phenotype of the LEC rat, which is located on 16q12.23-12.3. Northern blot analysis also indicated that the LEC rat is a mutant with the deficient expression of the rat WD gene.

Materials and Methods

Phenotyping of backcross progenies

Female LEC rat was crossed to male WKAH rat. Then male F1 rats were backcrossed to female LEC rats. Using fifty-three backcross progenies sacrificed at 20~25 weeks of age, the phenotype of hepatitis (*hts*) was determined by measurement of the serum ceruloplasmin oxidase activity (11) and by histopathology, in which livers were fixed in phosphate-buffered formalin, embedded in paraffin and stained with hematoxylin/eosin.

Linkage analysis

Genomic DNA was prepared from kidneys of backcross progenies and digested with the restriction enzyme *Eco*O1091 (Takara). The strain distribution pattern of each backcross progeny was determined using Southern blot analysis with pB8.3 as a probe. This probe was prepared by PCR amplification using primers TGTAATCCAGGTGACAAGCAG and CACAGCATGGAAGGGAGAG specific for the human WD gene as described (9). This fragment, isolated and cloned in Bluescript II, was designated pB8.3.

Northern blot analysis

Total RNA was isolated from livers of 4-week-old LEC, WKAH and LEA rats by the guanidine-isothiocyanate method. Poly (A) + RNA was isolated using oligotex-dT30 (Roche). Northern blot analysis was performed using human WD cDNA (Y-2) as a probe (8) .

Chromosomal assignment by somatic cell hybrids

Eighteen rat × mouse somatic cell hybrids (YS01-YS18) were used in this study (12). Rat-specific DNA fragments detected on Southern blot analysis by pB8.3 were scored for their presence or absence in the hybrid clones and compared to the segregation patterns of individual rat chromosomes in the clone panel.

Physical mapping by FISH

Genomic DNA prepared from kidney of WKAH rat was digested completely with *Eco*RI, and ligated with *Eco*RI-digested Bluescript II using a vector-to-insert molar ratio of 1:1. Colonies of the genomic library were screened by hybridization with pB8.3 and positive clone was purified and designated prWD4. FISH analysis with prWD4 and the rat chromosome 16-specfic cosmid clone (RCKYO88) was performed according to the method described previously (13, 14).

Results

Preparation of genomic DNA fragments encoding the WD gene

To obtain the genomic DNA fragment of the human WD gene and its rat homolog, we amplified the 250 bp fragment carrying the human WD gene using B8.3 primers (9). This fragment was cloned in Bluescript II vector and designated pB8.3. The Southern blot with

pB8.3 revealed that the 4.4 kbp *Eco*RI genomic DNA fragment encoded the rat counterpart sequence of the WD region carried by pB8.3 and we attempted to clone this fragment at the Bluescript II-*Eco*RI site, which resulted in obtaining prWD4. The insert DNAs of prWD4 and pB8.3 were used for subsequent analyses.

Genetic analysis of the hts locus of the LEC rat

The phenotype of *hts* was assayed by measurement of the serum ceruloplasmin oxidase activity and by histopathology. Fifty-three backcross progenies produced from LEC and WKAH (normal rat strain) progenitors were sacrificed at 20~25 weeks of age. Twenty-three progenies showed low levels of the enzyme activity and suffered from fulminant hepatitis at about 4 months after birth. To examine whether or not the *hts* phenotype was linked to the human WD gene, we first tried to test the restriction fragment length polymorphisms (RFLP) of genomic DNAs from LEC and WKAH rats using Southern blot analysis. Using the 250 bp insert DNA of pB8.3 as a probe, thirty restriction enzymes were tested for RFLP. As shown in Figure 1, Restriction enzyme *Eco*O109I produced a longer fragment in LEC than that in WKAH, both of which can be seen in the F1 pattern. As a consequence of linkage analysis, the strain distribution pattern of the rat WD gene was found to be tightly linked to that of the *hts* phenotype with no recombination in the fifty-three backcross progenies. In combination with the similarity of the

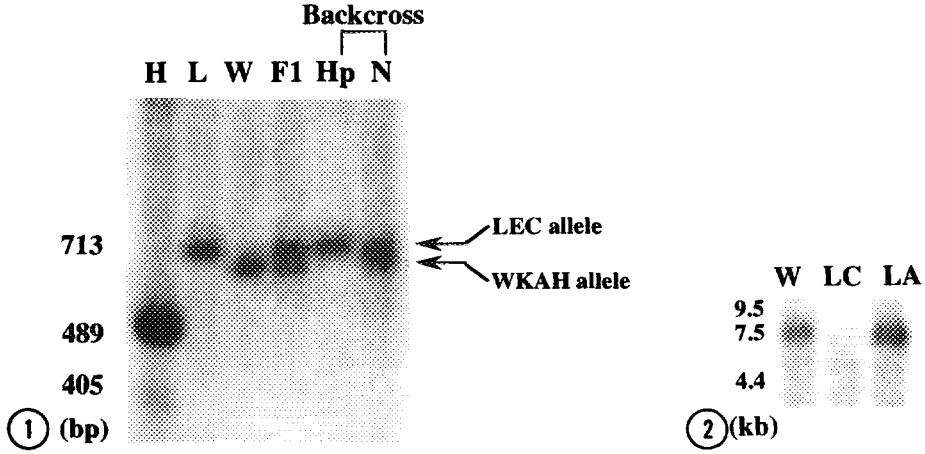

Figure 1. Southern blot analysis of rat WD locus
Southern blot analysis of kidney genomic DNA from human (H), LEC rat (L), WKAH rat (W), F1 (L ×W) and backcross progenies (L ×F1). Hp and N of backcrosses indicate hepatitic and normal phenotypes, respectively. DNA digested with *Eco*O109I and hybridized with radioactive pB8.3. This probe was PCR products from the human WD gene locus described in (9). The size marker is *Msp*I-digested Bluescript II.

Figure 2. Northern blot analysis of the rat WD gene.
Five microgram of poly (A)+RNA prepared from livers of each of the rat strains, WKAH (W), LEC (LC), and LEA (LA) was electrophoresed on 0.7% agarose gel and blotted to a nylon membrane. The membrane was probed with human WD cDNA (Y-2). RNA marker sizes are shown on the left.

rat LEC phenotype to the human WD, this strongly suggested that the rat homolog to the human WD gene is the gene responsible for *hts*.

Expression of the rat WD gene in the LEC rat

To analyze the expression of the rat WD gene in the LEC rat, Northern blotting was done using human WD cDNA (Y-2) to hybridize to liver poly (A)+ RNA of LEC, WKAH and LEA rats. LEA is a control strain which originated from the same closed colony as LEC. As shown in Figure 2, only a 7.5kb transcript, which is the same size as the human WD mRNA, could be detected in WKAH and LEA rats (lane 1,3). On the other hand, no trace of this band could be seen in the LEC pattern (lane 2), indicating that the rat WD gene is not expressed in the LEC mutant. Therefore, we concluded that the rat homolog to the human WD gene (rat WD gene) is responsible for the *hts* phenotype in the LEC rat.

Chromosomal assignment by somatic cell hybrids and FISH

To identify the chromosomal location of the rat WD gene, rat×mouse hybrid cells (YS01-YS18) were used for Southern blotting with pB8.3. With *Eco*RI digestion, rat-specific fragment and mouse fragment were detected at 4.4 kb and 7.6 kb, respectively (Figure 3). The presence or absence of the 4.4 kb rat-specific DNA band was examined in the hybrid clones and we scored its concordance and discordance with each chromosome. Consequently, chromosome 16 gave the highest concordance with the score for the rat 4.4kb fragment (Table 1). However, in one hybrid cell (YS03), chromosome 16 showed discordance with the rat WD gene. We suspected that this discordance might have been due to a chromosome aberration in chromosome 16 carried in YS03. Then, we performed FISH analysis to identify the regional location of the rat WD gene. As an anchor clone, we used the rat chromosome 16-specfic cosmid (RCKYO88) (15). As shown in Figure 4-a, the signals of the rWD4 (red signal) and RCKYO88 (green signal) were localized on chromosome 16q and 16p, respectively. The G-banding analysis showed that the chromosome positive to the both probes was assigned to chromosome 16 (Figure.4-b) (16). For futher precise assignment of the rWD4, the direct

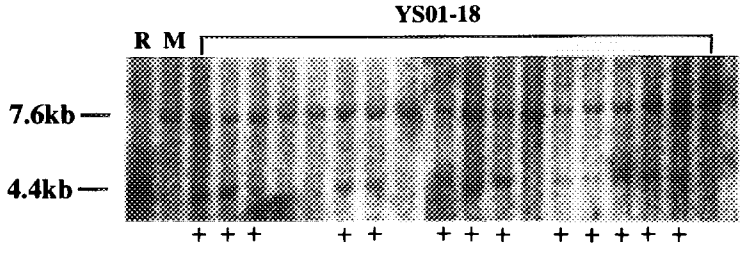

Figure 3. Somatic cell hybrid mapping of the rat WD gene.
Rat × mouse somatic cells (YS01-18) were analyzed by Southern blot with pB8.3. DNA was digested with *Eco*RI. Rat (R) and mouse (M) indicate the 7.6kb fragment and 4.4kb fragment of the WD gene, respectively. The plus (+) sign indicates the presence of a rat signal.

Table 1. Segregation discordance between Rat chromosomes and the human WD gene

	Rat chromosome																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	X	Y
Locus WD	10	11	5	8	8	11	6	7	9	12	3	5	3	6	7	1	4	6	9	6	3	7

The bar (-) indicates the chromosome with the highest concordance of segregation patterns.

R-banding FISH was performed with the biotin-labeled DNA probe, resulting in detecting the yellow signals on chromosome 16q12.23-12.3 (Figure.4-c) (17).

Discussion

Our data demonstrate that the rat homolog to the human WD gene (rat WD) is the gene responsible for the *hts* phenotype in the LEC rat. This conclusion is based on the following data. First, a genetic study with fifty-three backcross progenies indicated that the rat WD gene

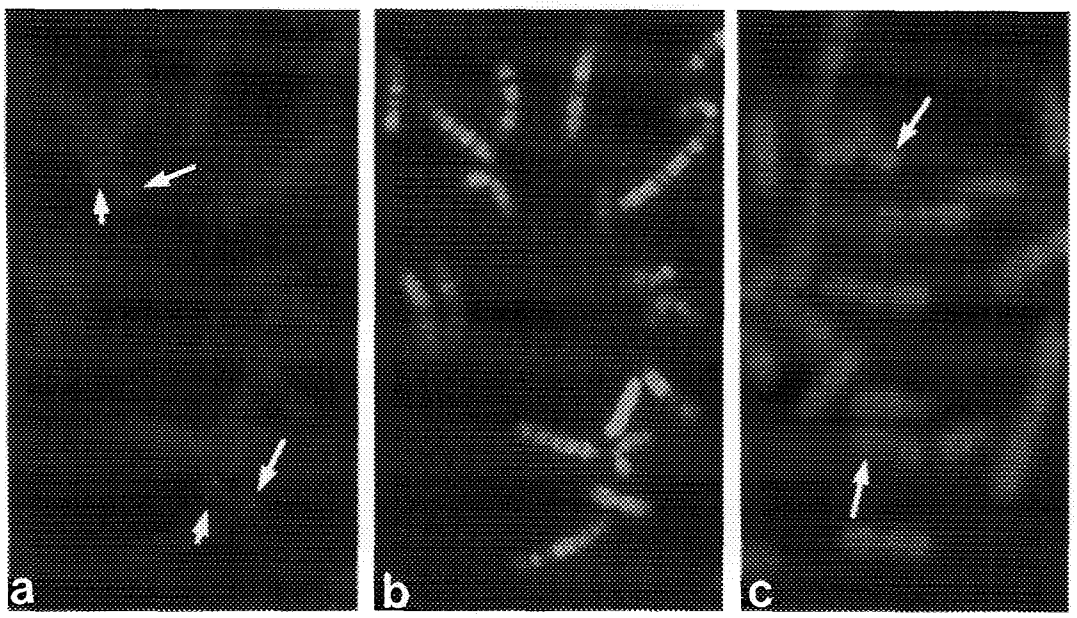

Figure 4. FISH mapping of the rat WD gene on chromosome 16.
(a, b) Chromosomal localization of the rWD4 and RCKYO88 labeled with digoxigenin 11-dUTP and biotin 16-dUTP, respectively. The hybridization signals of the rWD4 and RCKYO88 are indicated by long and short arrows, respectively (a). The G-banded pattern of the same metaphase is shown in (b). (c) Direct R-banding FISH pattern of the rWD4 as biotinylated probe. The signal is located on chromosome 16q12.23-12.3. The metaphase spreads were photographed with FITC/Texas Red filter set (a), Nikon UV-2A (b) and B-2A (c) filters.

is tightly linked to the *hts* phenotype. Second, Northern blot analysis showed that the transcription of the rat WD gene is defective in the LEC rat. Third, the phenotype, the biochemical data and the histopathology of the LEC rat are very similar to those of human WD. In human WD, various mutations in the WD gene have been reported and one of them showed complete deficiency of transcription of this gene (8). Therefore, the LEC rat is considered to be a good model for this type of the human WD.

Copper taken up by hepatocytes is usually incorporated into ceruloplasmin. The required amount of copper carried by ceruloplasmin is then secreted from the hepatocytes into plasma, whereas excess copper is discharged into bile (4). It has previously been reported that ceruloplasmin in the LEC rat lacks copper binding activity, although ceruloplasmin protein levels and the expression of mRNA are normal (18). In the LEC rat, ceruloplasmin without copper is secreted, whereas copper accumulates in hepatocytes. Therefore, ceruloplasmin was considered to be the gene responsible for *hts*, however, this was found to be untrue because genetic study revealed no linkage between *hts* and the ceruloplasmin gene in the LEC rat (5). Taken together with our results, the function of the WD product is hypothesized to be as follows. Since the human WD gene product has six copper-binding sites, it may function as a carrier that transports copper to ceruloplasmin or helps copper to bind to ceruloplasmin. Futhermore, as the WD gene product has putative transmembrane regions, mature ceruloplasmin may excrete excess copper from the liver into the bile only when a WD product-copper-ceruloplasmin complex is formed. The failure of the WD gene product to function as a carrier may result in the copper accumulation in the liver of the LEC rat and in human WD patients.

One clear difference in phenotype in the two species is that all LEC rats that survived more than 18 months developed hepatocellular carcinoma. Generally, cancer is caused by multistep processes. It has been reported that the LEC rat is highly sensitive to X ray-irradiation and carcinogenesis (19, 20). This evidence is in agreement with the fact that the amount of DNA damage in the LEC rat was higher than in the control rat. As reasons for the excess accumulation of DNA damage in the LEC rat, two possibilities may be suggested. First, in the LEC rat, DNA damage is produced more frequently than in the control rat because oxygen radicals, which more produced by copper accumulation, induce DNA damage in the LEC rat (21). Second, the repair process of DNA double-strand breaks is disturbed compared to that in the WKAH rat. This impairment of DNA repair is controlled by a single autosomal recessive gene, designated *xhs* (19). In addition to the DNA repair impairment of the LEC rat and the tendency for mutation, hyperregeneration of hepatocytes resulting from fulminant hepatitis should promote the frequency of DNA mutation. Therefore, we suppose that the difference in carcinogenic phenotype between the LEC rat and human WD patients is due to the susceptibility to DNA damage and impairment of DNA repair. Thus, cells of the LEC rat may naturally be mutable due to the defect of a DNA repair gene and DNA mutation caused by excess copper, which tends to cause DNA mutation through the generation of oxygen free radicals, may eventually accumulate. Then, following regeneration and proliferation of hepatocytes, the accumulated DNA mutation may promote carcinogenesis. Thus, the LEC rat should provide a useful tool for understanding not only the pathogenesis of WD, but also the mechanism of oncogenesis in hepatocytes.

Acknowledgments

We thank Dr. Jonathan D. Gitlin for human WD cDNA (Y-2). This work was supported by a Research Grant for Aging and the Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare, a Grant-in-Aid for Human Genome Program and a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture, Japan, a Research Grant for Human Genome Project from the Science Technology Agency, a Special Grant for Promotion of Research from the Institute of Physical and Chemical Research (RIKEN) to Y. H.

References

1) Masuda, R., Yoshida, M.C., Sasaki, M ., Dempo, K. and Mori, M. (1988) Jpn. J. Cancer. Res. 79, 828-835.
2) Kasai, N., Osanai, T., Miyoshi, I., Kamimura, E., Yoshida, M.C. and Dempo, K. (1990) Lab. Anim. Sci. 40, 502-505.
3) Li, Y., Togashi, Y., Sato, S., Emoto, T., Kang, J.H., Takeichi, N., Kobayashi, H., Kojima, Y., Une, Y. and Uchino, J. (1991) J. Clin. Invest. 87, 1858-1861.
4) Dankes, D.M. Metabolic Basis of Inherited Disease. (1989) 1411-1413. McGraw-Hill, New York.
5) Masuda, R., Yoshida, M.C., Sasaki, M., Dempo, K. and Mori, M. (1988) Lab. Anim. 22, 166-169 .
6) Yamada, Y., Muramatsu, Y., Agui, T. and Matsumoto, K. (1992) Biochem. Int. 27, 243-249.
7) Yang, F., Naylor, S.L. Lum, J.B., Cutshaw, S., McCombs, J,L., Naberhaus, K.H., McGill, J.R., Adrian, G.S., Moore, C.M., Barnett, D.R. and Bowman, B.H. (1986) Proc. Natl. Acad. Sci. USA 83, 3257-3261.
8) Yamaguchi, Y., Heiny, M.E. and Gitlin, J.D. (1993) Biochem. Biophys. Res. Commun. 197, 271-277.
9) Bull, P.C., Thomas, G.R., Rommens, J.M., Forbes, J.R. and Cox, D.W. (1993) Nature Genet. 5, 327-337.
10) Petrukhin, K., Fisher, S.G., Pirastu, M., Tanz, R.E., Chernov, I., Devoto, M., Brzustowicz, L.M., Cayanis, E., Vitale, E., Rosso, J.J., Matseoane, D., Boukhgalter, B., Wasco, W., Figus, A.L., Loudianos, J., Gao, A., Sternlieb, I., Evgrafov, O., Parano, E., Pavone, L., Warburton, D., Ott, J., Penchaszadeh, G.K., Scheinberg, I.H. and Gilliam, T.C. (1993) Nature Genet. 5, 327-337.
11) Schosinsky, K.H., Lehmann, H.P. and Beeler, M.F. (1974) Clin. Chem. 20, 1556-1563.
12) Yasue, M., Serikawa,T. and Yamada, J. (1991) Cyotgenet. Cell Genet. 57, 142-148.
13) Matsuda, Y., Harada, Y-N., Natsuume-Sakai, S., Lee, K., Shiomi, T. and Chapman, V.M. (1992) Cytogenet. Cell Genet. 61, 282-285.
14) Takahashi, E., Hori, T., O'Connell, P., Leppert, M. and White, R. (1990) Hum. Genet. 86, 14-16.
15) Kuramoto, T., Mori, M., Saburi, S., Yamada, J. and Serikawa,T. (1993) Acta. Histochem. Cytochem. 26, 325-332.
16) Satoh, H., Yoshida, M.C. and Sasaki, M. (1989) Cytogenet. Cell Genet. 50, 151-154.
17) Rønne, M., Shibasaki, Y., Poulsen, B.S. and Andersen, O. (1987) Cytogenet. Cell Genet. 26, 113-117.
18) Yamada, T., Agui, T, Suzuki, Y, Sato, M. and Matsumoto, K. (1993) J. Biol. Chem. 268, 8965-8971.
19) Hayashi, M., Okui, T., Endoh, D., Sato, F., Kasai, N. and Namioka, S. (1994) Mutat. Res.,in press.
20) Sakamoto, H., Sawada, N., Kamimura, Y., Enomoto,K. and Mori, M. (1993) Jpn. J. Cancer. Res.,84, 966-971.
21) Yamamoto, F., Kasai, H., Togashi, Y., Takeichi, N., Hori, T. and Nishimura, S. (1993) Jpn. J. Cancer. Res. 84, 508-511.