# Absence of Linkage between the Retinoblastoma Gene and *hts* Gene in the LEC Rat: A Model of Human Wilson's Disease

Nobuya Sasaki,[1] Yoshihide Hayashizaki[2] and Noriyuki Kasai[3, 4]

[1]Department of Laboratory Animal Science, Faculty of Veterinary Medicine, Hokkaido University, Kita 18 Nishi 9, Kita-ku, Sapporo 060, [2]Gene Bank, RIKEN Tsukuba Life Science Center, The Institute of Physical and Chemical Research, 3-1-1 Takanodai, Tsukuba, Ibaraki 305 and [3]Institute for Animal Experimentation, Hokkaido University School of Medicine, Kita 15 Nishi 7, Kita-ku, Sapporo 060

The LEC rat is an authentic model of human Wilson's disease (WD) with an autosomal recessively inherited hepatitis. We investigated linkage between the hepatitis gene (*hts*) and the rat retinoblastoma gene (RB), that is closely linked to the WD gene in humans, to see whether or not the *hts* gene is located on the syntenic region of WD and is the counterpart of the WD gene. Polymerase chain reaction-single strand conformation polymorphism analysis with backcross progenies from LEC and TM strains showed that the recombination rate between these two loci was 55.6%, demonstrating that the *hts* and RB genes are not linked to each other. These data indicate that the *hts* gene is not the counterpart of the WD gene and that the human syntenic region on which the WD locus and human RB gene are located, is not conserved in the rat genome.

Key words:    LEC rat — Wilson's disease — PCR-SSCP — *hts* gene — Retinoblastoma gene

The LEC rat was established as a mutant strain that develops fulminant hepatitis and severe jaundice at around 4 months of age.[1-3] About half of the animals die within a week after the onset of jaundice and in all survivors hepatocellular carcinoma spontaneously appears after 12 to 18 months.[4] As biochemical features of this rat model, it has been reported that copper accumulates in the liver, and that there is a low level of ceruloplasmin in the serum and a high level of copper in the urine.[5, 6] These symptoms are quite similar to those of Wilson's disease (WD) which is also a recessively inherited disease controlled by a single gene.[7-9] Because the hepatitis in the LEC rat is also controlled by a single autosomal recessive gene, named *hts*,[10] efforts have been made to map this locus and to clarify the syntenic relationship between *hts* in the rat and WD in man. Bowcock *et al.* reported that the WD locus is located in chromosomal region 13q14-q21, which is in the proximal region at 9.4 centimorgans distance from the red cell enzyme esterase D (ESD) and at 4.4 centimorgans distance from the retinoblastoma gene locus (RB).[11] Because deficiency of serum ceruloplasmin is a characteristic biochemical abnormality of WD, it is tempting to speculate that the ceruloplasmin gene could be the gene responsible for WD in humans and for *hts* in the LEC rat. However, the human ceruloplasmin gene has been mapped to chromosome 3, but not chromosome 13, by somatic cell hybrid

analysis and precisely to 3q25 by *in situ* hybridization,[12] indicating that the WD gene is not identical to the ceruloplasmin gene. Recently, a genetic study also demonstrated that the ceruloplasmin gene is not responsible for the LEC phenotype (*hts* gene) in the rat.[13] These results lead us to speculate that the LEC rat could be an animal model of human WD, and the *hts* gene the rat counterpart of the human WD gene. In the comparative map of human, mouse and rat genomes, the syntenic region of human chromosome 13 including the region of WD is partially located on mouse chromosome 14[14] and rat chromosome 15.[15, 16] In this study, we investigated genetic linkage between the *hts* gene and the rat RB gene and examined whether or not the *hts* gene is located in the rat syntenic region of WD to test this speculation.

A comparative map has been presented of the relative locations of the RB and ESD genes located on chromosomes 13, 14, and 15 in the human, mouse[14] and rat,[15, 16] respectively. From this map, the *hts* locus is presumed to be located close to the RB gene on chromosome 15, if the *hts* gene is the rat homologue to the human WD gene. To detect the linkage of the RB gene to the *hts* locus, polymerase chain reaction (PCR) was employed to amplify the rat RB gene using RB2957A and RB2957B primers. However, because the PCR product of rat RB from TM and LEC genomic DNA produced a single band (123 bp) and did not show polymorphism, we tried single strand conformation polymorphism (SSCP) electrophoresis of PCR products to distinguish the TM and LEC alleles, resulting in the separation of these alleles, as

---

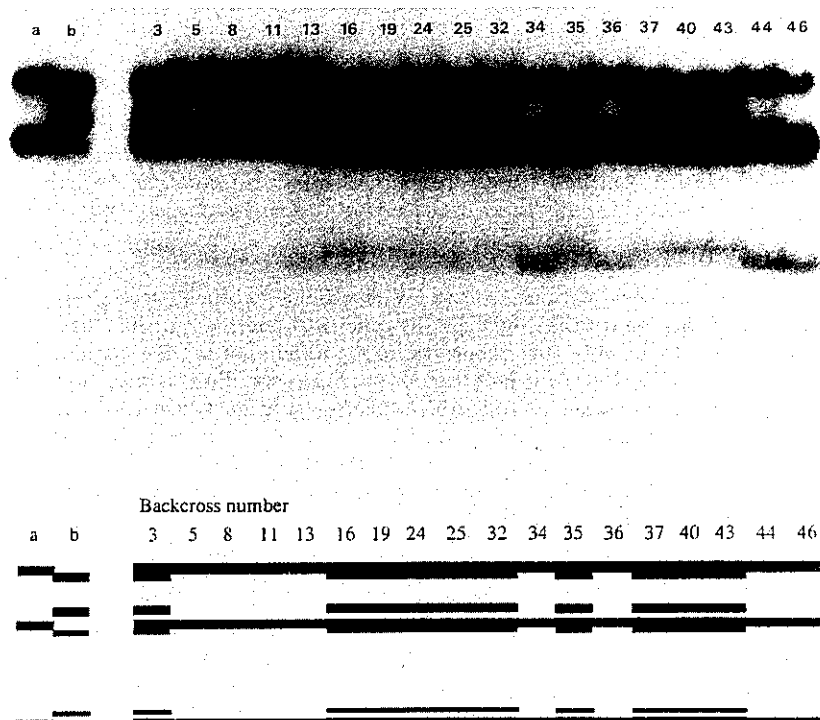[4] To whom correspondence should be addressed.

Fig. 1. Comparison of the strain distribution pattern revealed by PCR-SSCP analysis in exon 27 at the retinoblastoma gene between TM and LEC. lane a, LEC; lane b, TM; lenes 3–46, backcross progeny (*hts* phenotype). DNAs were extracted from the livers of backcross progenies. To amplify the 3′ untranslated region of RB exon 27,[20] a set of primers, RB2957A (5′ATAAAATGTGCAGGTACAAGCT3′) and RB2957B (5′AATGGCATGATC TGCACAAGATT3′), was synthesized on a DNA synthesizer (MilliGene/Biosearch). Amplification of the RB exon 27 sequence yields a 123 bp product. PCR-SSCP was carried out as described by Orita *et al.*[21] with minor modifications. The target sequences in the RB gene were amplified by PCR in 25 $\mu$l of solution containing 2.5 $\mu$l of PCR buffer (10 m$M$ Tris-HCl, pH 8.3, 50 m$M$ KCl, 1.5 m$M$ MgCl$_2$, 0.01% gelatin), 10 $\mu M$ dATP, dGTP and dTTP respectively, 1 $\mu M$ dCTP, 1 $\mu$l of [$\alpha$-$^{32}$P]dCTP (6000 Ci/mmol, 10 mCi/ml, Amersham), 1 $\mu M$ of each primer, 0.1 $\mu$l of genomic sample DNA, and 0.125 unit of Ampli*Tag* DNA polymerase (Perkin-Elmer Cetus). The reaction was started by denaturing at 95°C for 5 min, and 35 reaction cycles were run in a Thermal Cycler (MJ Research). A single cycle consisted of three reactions, i.e., denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. Then 3 $\mu$l of reaction mixture was mixed with 27 $\mu$l of formamide dye (95% formamide, 10 m$M$ EDTA, 0.05% bromphenol blue and 0.05% xylene cyanol). Samples were heated to 80°C for 5 min, and immediately applied to 10% polyacrylamide gel without glycerol. Electrophoresis was performed at a constant power of 30 W for 6 h with the apparatus enclosed by a water jacket circulating water at 10°C. The gel was dried on filter paper and exposed to X-ray film at −80°C for 3–12 h using an intensifying screen (QuantaIII; DuPont).

shown in Fig. 1. We confirmed that multiple bands were derived from a single band by SSCP analysis of the PCR-product, which was eluted from a slice of gel containing a single band. Then PCR-SSCP assay was applied to 48 backcross progenies between LEC and TM for linkage analysis. Independently, the phenotype of *hts* was assayed by measurement of the serum ceruloplasmin oxidase activity and histopathology using these backcross progenies killed at 20–25 weeks of age. Eighteen progenies showed low levels of enzyme activity and suffered from fulminant hepatitis at about 4 months of age. The

resulting strain distribution pattern of the RB gene revealed by PCR-SSCP and the LEC phenotype are shown in Table I. The recombination rate between *hts* and rat RB was 55.6%, indicating that *hts* and RB genes are not linked to each other ($\chi^2=0.36$, $P>0.05$). Although it could be expected that the rat RB gene is linked to the *hts* locus if the human WD gene is the counterpart of the *hts* locus in the LEC rat under conditions in which the synteny is conserved in rats and humans, the result was contrary to our expectation. Possible explanations are as follows. First, the *hts* gene as a counterpart of

Table I.  Distribution of *hts* Phenotype and RB

| | Number of backcross progenies |
|---|---|
| | 1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 |
| RB | L  L  T  L  L  L  L  L  L  T  T  L  L  L  L  L  L  T  T  T  T  T  L  T  L  L  T  T  T  T  T  L  L  T  T  T  T  T  L  T  L  T  L  T  T  L  T  T  L  T  L  L  L  T |
| *hts* | T  T  L  T  L  T  T  L  T  T  L  T  L  T  T  L  T  T  L  T  L  T  T  T  T  L  L  T  T  T  T  T  T  T  L  T  L  L  L  L  T  T  L  T  T  L  L  T  L  T  T |

T and L indicate inheritance of the TM allele and the LEC allele, respectively. The F1 progenies were obtained from mating LEC and TM rats, and further backcross progenies of F1 and LEC homozygote were made to analyze segregation or cosegregation between the RB gene and *hts* locus. The *hts* phenotype (hepatitis) was assayed by the measurement of the serum level of ceruloplasmin[19] and histopathology using backcross progenies killed at 20 to 25 weeks of age. Statistical comparisons were made by using the $\chi^2$ test.

human WD is located on a different chromosome or the region on chromosome 15 is far from the RB gene. In this case, the syntenic region would not be conserved in humans and rats, different from the case in humans and mice. Second, the *hts* gene is not the counterpart of the WD gene.

The number of DNA markers known today is very small in rats compared with humans and mice, especially those on chromosome 15.[17] Therefore, it is necessary to find more markers and analyze the polymorphism. In the case of the RB locus, it was difficult to find polymorphism among rat strains, because polymorphisms are usually rare in exons but frequent in introns. However, SSCP analysis has made it possible to detect even one nucleotide substitution. Alternatively, restriction landmark genomic scanning (RLGS) is expected to be able to identify a large number of landmarks.[18]

Since genetic study of WD is likely to remain extremely difficult, knowledge from genetic studies of the LEC rat should provide a useful tool to study the WD gene.

## REFERENCES

1) Kasai, N., Osanai, T., Miyoshi, I., Kamimura, E., Yoshida, M. C. and Dempo, K.  Clinico-pathological studies of LEC rats with hereditary hepatitis and hepatoma in the acute phase of hepatitis. *Lab. Anim. Sci.*, **40**, 502–505 (1990).

2) Sasaki, M., Yoshida, M. C., Kagami, K., Takeichi, N., Kobayashi, H., Dempo, K. and Mori, M.  Spontaneous hepatitis in an inbred strain of Long-Evans rats. *Rat News Lett.*, **14**, 4–6 (1985).

3) Yoshida, M. C., Masuda, T., Sasaki, M., Takeichi, N., Kobayashi, H., Dempo, K. and Mori, M.  New mutation causing hereditary hepatitis in the laboratory rat. *J. Hered.*, **78**, 361–365 (1987).

4) Masuda, R., Yoshida, M. C., Sasaki, M., Dempo, K. and Mori, M.  High susceptibility to hepatocellular carcinoma development in LEC rats with hereditary hepatitis. *Jpn. J. Cancer Res.*, **79**, 828–835 (1988).

5) Li, Y., Togashi, Y., Sato, S., Emoto, T., Kang, J. H., Takeichi, N., Kobayashi, H., Kojima, Y., Une, Y. and Uchino, J.  Spontaneous hepatic copper accumulation in Long-Evans cinnamon rats with hereditary hepatitis. *J. Clin. Invest.*, **87**, 1858–1861 (1991).

6) Li, Y., Togashi, Y., Sato, Y., Emoto, T., Kang, J. H., Takeichi, N., Kobayashi, H., Kojima, Y., Une, Y. and Uchino, J.  Abnormal copper accumulation in non-cancerous and cancerous liver tissues of LEC rats developing hereditary hepatitis and spontaneous hepatoma. *Jpn. J. Cancer Res.*, **82**, 490–492 (1991).

7) Bearn, A. G.  Genetic and biochemical aspects of Wilson's disease. *Am. J. Med.*, **15**, 442–449 (1953).

8) Cumings, J. N.  The copper and iron content of brain and liver in the normal and in hepato-lenticular degeneration. *Brain*, **71**, 410–415 (1948).

9) Wilson, S. A. K.  Progressive lenticular degeneration: a familial nervous disease associated with cirrhosis of the liver. *Brain*, **34**, 295–508 (1912).

10) Masuda, R., Yoshida, M. C., Sasaki, M., Dempo, K. and Mori, M.  Hereditary hepatitis of LEC rats is controlled by a single autosomal recessive gene. *Lab. Anim.*, **22**, 166–169 (1988).

11) Bowcock, A. M., Farrer, L. A., Hebert, J. M., Agger, M., Sternlieb, I., Scheinberg, I. H., Buys, C. H. C. M., Scheffer, H., Frydman, M., Chajek-Saul, T., Bonne-Tamir, B. and Cavalli-Sforza, L. L. Eight closely linked loci place the Wilson disease locus within 13q14-q21. *Am. J. Hum. Genet.*, **43**, 664–674 (1988).

12) 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. Characterization, mapping and expression of the human ceruloplasmin gene. *Proc. Natl. Acad. Sci. USA*, **83**, 3257–3261 (1986).

13) Yamada, Y., Muramatsu, Y., Agui, T. and Matsumoto, K. A new restriction fragment length polymorphism of the ceruloplasmin gene in rat. *Biochem. Int.*, **27**, 243–249 (1992).

14) Nadeau, J. H., Davisson, M. T., Doolittle, D. P., Grant, P., Hillyard, A. L., Kosowsky, M. R. and Poderick, T. H. Comparative map for mice and humans. *Mamma. Genome*, **3**, 480–536 (1992).

15) Levan, G., Szpirer, J., Szpirer, C., Klinga, K., Hanson, C. and Islam, M. Q. The gene map of Norway rat (*Rattus norvegicus*) and comparative mapping with mouse and man. *Genomics*, **10**, 699–718 (1991).

16) Yasue, M., Serikawa, T. and Yamada, J. Chromosomal assignments of 23 biochemical loci of the rat by using rat × mouse somatic cell hybrids. *Cytogenet. Cell Genet.*, **57**, 142–148 (1991).

17) Serikawa, T., Kuramoto, T., Hilbert, P., Mori, M., Yamada, J., Dubay, C. J., Lindpainter, K., Ganten, D., Guenet, J-L., Lathrop, G. M. and Beckmann. J. S. Rat gene mapping using PCR-analyzed microsatellites. *Genetics*, **131**, 701–721 (1992).

18) Hatada, I., Hayashizaki, Y., Hirotsune, S. and Komatsubara, H. A genomic scanning method for higher organisms using restriction sites as landmarks. *Proc. Natl. Acad. Sci. USA*, **88**, 9523–9527 (1991).

19) Schoşinsky, K. H., Lehmann, H. P. and Beeler, M. F. Measurement of ceruloplasmin from its oxidase activity in serum by use of o-dianisidine dihydrochloride. *Clin. Chem.*, **20**, 1556–1563 (1974).

20) Roy, N. K., Ballesteros, A. and Garte, S. J. Cloning and sequence of the rat retinoblastoma (RB) gene cDNA. *Nucleic Acids Res.*, **21**, 170 (1993).

21) Orita, M., Suzuki, Y., Sekiya, T. and Hayashi, K. Rapid sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics*, **5**, 874–879 (1989).