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The *WD* Gene for Wilson's Disease Links to the Hepatitis of LEC Rats

Takao Ono,[1] Shigeyuki Takada[2] and Michihiro C. Yoshida[1,2,3]

[1]*Chromosome Research Unit, Faculty of Science and* [2]*Department of Cytogenetics, Division of Biological Sciences, Graduate School of Environmental Earth Science, Hokkaido University, North 10 West 8, Kita-ku, Sapporo 060*

LEC rats develop an autosomal recessive hepatitis and subsequently liver cancer associated with copper accumulation in the liver similar to that of Wilson's disease. Using 71 backcross [(WKAH × LEC) × LEC] rats, linkage analysis of the hepatitis with the *WD* gene for Wilson's disease revealed identical segregation and no recombination event between these two genes. This result indicates that the *WD* gene is a prime candidate for the *hts* gene responsible for the hepatitis of LEC rats, and suggests that the hepatitis of LEC rats may be caused by a defect in a copper-transporting ATPase expressed in the liver.

Key words: LEC rat — *hts* — Wilson's disease gene

The LEC rat is a mutant strain which develops hereditary hepatitis[1,2] and subsequently liver cancer.[3] The hepatitis of LEC rats is associated with a low level of ceruloplasmin (Cp) in plasma[4] and copper accumulation in the liver,[5,6] indicating that the LEC strain has a disorder of copper transport or abnormal copper metabolism. Although the basic defect in the LEC rats was not known, the pathogenesis of the hepatitis is quite similar to that of Wilson's disease, which is characterized by failure to excrete copper from the liver into bile, resulting in abnormal toxic accumulation of copper in the liver.[7,8] Therefore, the LEC rat is suggested to be a model of Wilson's disease. An autosomal recessive gene is responsible for each disease, namely *WD* for Wilson's disease and *hts* for LEC rats,[9] although the *hts* gene has not been isolated.

Recently, the *WD* gene has been cloned, and its product predicted as a copper-binding P-type ATPase protein.[10–12] Using the human *WD* gene as a probe, we conducted a linkage analysis between *WD* and the hepatitis of LEC rats.

The polymerase chain reaction (PCR) primers, P1 (5′-AGGGTCATGCGGGTGCTCCTGCTG-3′) (nucleotides 3055–3078) and P2 (5′-CCATGCTCTGCAGAGTGTGCACAG-3′) (nucleotides 3557–3580), were synthesized based on the sequence of human *WD* cDNA.[10] PCR amplification was carried out using human liver cDNA (Clonetec) as a template for 30 cycles (each cycle: 94°C, 1 min; 62°C, 1 min; 72°C, 1 min). The amplified product (nucleotides 3055–3580) was labeled with ^{32}P-dCTP by using a multiprime-labeling system kit (Amersham), and used as a probe. This PCR product was confirmed to be the *WD* gene by sequence analysis (data not shown).

Genomic DNAs were extracted from lung tissue of LEC, WKAH, F_1(WKAH×LEC) and backcross [(WKAH×LEC)×LEC] rats. Hepatitis was diagnosed on the basis of Cp ferroxidase activity in plasma of young rats (8–13 weeks old)[4] and subsequently on the basis of copper accumulation in histological sections of older rats (55–68 weeks old) by Timm's modified copper staining method.[13] Each DNA was subjected to restriction fragment length analysis using a number of restriction enzymes to establish a polymorphism within the *WD* gene (data not shown). *Bam*HI was found to be positive in this regard. Therefore, DNA samples digested with *Bam*HI were electrophoresed in a 0.7% agarose gel and transferred to nylon membrane (Hybond-N$^+$: Amersham). The DNA blots were hybridized with radiolebeled *WD* probe for 20 h at 37°C in 5×SSC, 50% formamide, 5×Denhardt and 0.5% SDS.

On Southern blots, LEC exhibited a 6.2 kilobase pair (kbp) fragment and WKAH showed a 9.3 kbp fragment (hereafter each fragment is referred to as a-type for LEC and b-type for WKAH, respectively) (Fig. 1). F_1 rats showed two fragments, a and b (ab-type), while backcross rats showed either an a-type fragment or two fragments, a and b (ab-type). On linkage analysis of 71 backcrosses, all 32 rats with hepatitis gave only an a-type fragment identical to that of the LEC, whereas others (39) without hepatitis showed two fragments of both a- and b-types (Table I). These results clearly exhibited an identical pattern of segregation of the *WD* gene and hepatitis without exception, and no recombination event between the two genes, *WD* and *hts*, was detected. This was statistically confirmed by the Lod score[14] (Z=28.4;

[3] To whom correspondence should be addressed.

Table I. Relationship between *WD* Gene and Hepatitis Characterized by Cp and Copper Accumulation in Backcross [(WKAH×LEC)×LEC] Rats

Animal	Sex	Phenotype of *WD* gene[a]	Level of Cp[b] (mU/ml)	Copper[c]	Animal	Sex	Phenotype of *WD* gene[a]	Level of Cp[b] (mU/ml)	Copper[c]
Without hepatitis					With hepatitis[d]				
n-1	F	ab	22.79	−	h-1	F	a	6.31	+
n-2	F	ab	12.27	−	h-2	M	a	3.68	+
n-3	M	ab	16.48	−	h-3	F	a	4.55	+
n-4	M	ab	18.23	−	h-4	F	a	2.20	+
n-5	M	ab	16.13	−	h-5	M	a	3.47	+
n-6	F	ab	18.34	−	h-6	M	a	4.63	+
n-7	F	ab	21.28	−	h-7	M	a	5.01	+
n-8	F	ab	20.98	−	h-8	M	a	0.96	+
n-9	F	ab	15.85	−	h-9	F	a	5.89	+
n-10	F	ab	21.42	−	h-10	F	a	2.45	+
n-11	M	ab	21.79	−	h-11	M	a	3.38	+
n-12	M	ab	20.64	−	h-12	M	a	7.87	+
n-13	F	ab	18.82	−	h-13	M	a	2.92	+
n-14	F	ab	20.25	−	h-14	M	a	2.47	+
n-15	M	ab	16.53	−	h-15	M	a	10.80	+
n-16	M	ab	21.82	−	h-16	F	a	3.92	+
n-17	F	ab	19.78	−	h-17	F	a	5.02	+
n-18	F	ab	23.23	−	h-18	M	a	8.16	+
n-19	F	ab	21.35	−	h-19	M	a	4.87	+
n-20	M	ab	18.06	−	h-20	M	a	0.87	+
n-21	M	ab	20.21	−	h-21	F	a	9.83	+
n-22	M	ab	21.87	−	h-22	M	a	7.76	+
n-23	M	ab	18.55	−	h-23	F	a	4.91	+
n-24	M	ab	16.55	−	h-24	M	a	3.21	+
n-25	F	ab	35.85	−	h-25	F	a	4.44	+
n-26	F	ab	27.58	−	h-26	F	a	2.04	+
n-27	F	ab	57.40	−	h-27	F	a	3.60	+
n-28	F	ab	29.65	−	h-28	F	a	4.56	+
n-29	M	ab	24.48	−	h-29	F	a	3.55	+
n-30	M	ab	29.30	−	h-30	M	a	6.06	+
n-31	M	ab	24.30	−	h-31	M	a	4.81	+
n-32	M	ab	20.77	−	h-32	M	a	2.03	+
n-33	M	ab	19.07	−					
n-34	M	ab	19.45	−					
n-35	F	ab	21.12	−					
n-36	F	ab	18.72	−					
n-37	F	ab	19.44	−					
n-38	M	ab	19.41	−					
n-39	M	ab	17.01	−					

a) a, LEC type; ab, hybrid(WKAH×LEC) type (see text).
b) Plasma Cp ferroxidase activity.
c) Hepatic copper accumulation detected by Timm's method for histological preparations. +, accumulation: −, non-accumulation.
d) Significant difference ($\chi^2=71.02$, $P<0.001$) between observed and predicted numbers of rats with hepatitis plus a-type *WD* gene.

$\theta=0.0$) and by a two-autosomal-recessive-gene model[6] ($\chi^2=71.02$, $P<0.001$). Therefore, the *WD* gene is a prime candidate for the *hts* gene, and the disorder of copper metabolism in LEC rats may be caused by mutation of the *WD* gene, as in Wilson's disease.

Although the present study did not allow us to assign *WD* on rat chromosomes, we found no linkage of the *hts* gene with *ESD* (unpublished results) or *RB1* on rat chromosome 15.[15] This contrasts to Wilson's disease, in which the *WD* gene is linked to *ESD* and *RB1*.[16,17] This

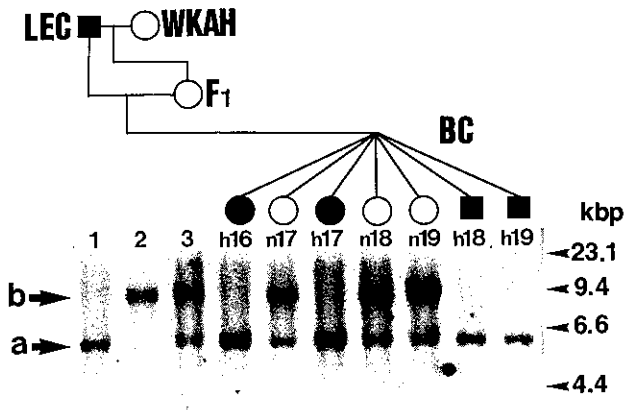

Fig. 1. A pedigree of backcross [(WKAH×LEC)×LEC] rats and Southern blot analysis of the *WD* gene. Closed and open circles indicate female animals with and without hepatitis, respectively. Closed and open squares indicate male animals with and without hepatitis, respectively. Each individuals' genomic DNAs were digested by *Bam*HI. The DNA size marker is shown on the right side (kbp: kilobase-pair). Arrow 'a' indicates a fragment of 6.2 kbp of LEC (lane 1) and 'b' indicates a fragment of 9.3 kbp of WKAH (lane 2). F_1 (lane 3) and backcross rats without hepatitis (n) showed two fragments of a and b type, while backcross rats with hepatitis (h) showed a fragment of a type. Numbers of individuals in backcross rats correspond to those in Table I. BC=backcross rats.

may be due to lack of synteny of these genes on rat chromosome 15, at least on the region including *ESD*, *RB1* and *hts*.

Although five different mutations in the *WD* gene have been found in Wilson's disease,[10,11] a non-functional *WD* gene leads to defective biliary excretion of copper and reduction in the rate of incorporation of copper into Cp, associated with copper accumulation in the liver. Investigation of mutations of the *WD* gene using LEC rats may provide further insights into mechanisms of copper transport and a better understanding of the pathogenesis of the hepatitis of the LEC rats and Wilson's disease. Cloning of the rat *WD* gene using a PCR-based strategy is in progress.

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