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High Expression of Atp7b mRNA in the Peripheral Blood Mononuclear Cells of the Long-Evans Cinnamon Rats: an Animal Model of Wilson's Disease

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Abstract

The Long-Evans Cinnamon (LEC) rat is an animal model of Wilson's disease. The rat has a mutation in the copper (Cu)-transporting P-type ATPase (Atp7b) gene that is homologous to the human Wilson's disease gene, ATP7B. The LEC rat shows all of the biochemical features of the disease. In this study, we focused on the expression levels of mutant Atp7b mRNAs in the peripheral blood mononuclear cells (PBMCs) of the LEC rats. Using quantitative real-time polymerase chain reaction (quantitative RT-PCR), we analyzed the expression levels of Atp7b mRNAs in the PBMCs cells of both the LEC rats and Long-Evans Agouti (LEA) rats, the latter being utilized as a control for the LEC rat. At the ages of 5 and 8 weeks, the inductions of Atp7b mRNA were manifested in the PBMCs of both male and female LEC rats, while their levels in the livers were significantly lower than those of the LEA rats. These results suggest the diversity of cell-physiological and endocrinological Cu metabolisms between the PBMCs and the livers of the LEC rats. Our findings indicate the possibility of a novel Cu metabolism in the cardiovascular network that is concerned with Atp7b of the PBMCs.

Keywords: Wilson's disease (WND); Long-Evans Cinnamon (LEC) Rat; Copper (Cu); Cu-transporting P-type ATPase (ATP7B); Peripheral Blood Mononuclear Cells (PBMCs); Quantitative Real-Time Polymerase Chain Reaction (Quantitative RT-PCR)

Introduction

Wilson's disease (WND, OMIM 277900) is an autosomal recessive disorder of copper (Cu) transport characterized by impaired incorporation of Cu into ceruloplasmin (Cp) and by impaired excretion of Cu via the bile. The disease phenotype includes progressive hepatic degeneration and/or neurological impairment as a result of the toxic effects of accumulated Cu in several tissues, principally the liver and brain. WND is observed with a prevalence of approximately 1:30,000 with a gene frequency of 0.56% and a carrier frequency of 1 in 90 [1-5]. Recently, it was reported that the prevalence for Japan, China, and Sardinia (Italy) is higher than the general population [5,6]. The high prevalence (1:10,000) of WND in Japan strongly supports our previous report [7]: one WND patient at the presymptomatic stage was detected through the analyses of 11,362 child subjects using the automated urinary Cp assay at the mandatory medical health care examination for 3-year-old children. Our attempt was to succeed for the first time in early and presymptomatic diagnosis of WND in conjunction with the mandatory medical health care program at the age of 3 years.

The gene responsible for WND was assigned to chromosome 13q14.3-q21.1 [3,5]. It has been cloned and shown to encode a putative Cu-transporting P-type ATPase [4,8-11], officially designated as ATP7B. The ATP7B is a large molecule with many functional domains required for heavy metal binding, energy transduction from ATP hydrolysis to cation transport, cation channel formation, phosphorylation and ATP binding. Recent studies have shown that the characteristic observation of excessive Cu accumulation in the livers of WND patients can be explained by the functional abnormality of ATP7B. However, there is a wide spectrum of mutations in the ATP7B gene responsible for WND: more than 350 disease-causing mutations have been identified to date [12,13].

The Long-Evans Cinnamon (LEC) rat is an animal model of WND [14,15]. The rat has a mutation in the Atp7b gene that is homologous to the human WND gene [16-18]. It shows all of the biochemical features of the disease, such as elevated hepatic Cu levels, reduced biliary Cu excretion, hemolysis, deficiency of both ceruloplasmin and Cu in the serum and increased hepatic iron (Fe) levels [14,15,19-25]. The LEC rat spontaneously develops acute hepatitis at about 4 months of age and develops hepatoma about 1 year after birth. It has been demonstrated that excess hepatic Cu and/or Fe contributes to the development of liver injury in the LEC rat [14,21,26,27]. In this study, we focused on the expression level of mutant Atp7b mRNA in the peripheral blood mononuclear cells (PBMCs) of the LEC rats at the ages of 5 and 8 weeks. These ages are presymptomatic stages of acute hepatic dysfunction. Using quantitative real-time polymerase chain reaction (quantitative RT-PCR), we analyzed the expression levels of Atp7b mRNAs in the PBMCs and the livers of both the LEC rats and the Long-Evans Agouti (LEA) rats, the latter being utilized as a control for the LEC rat. We compared the data between the LEC and the LEA rats and discussed our findings.

Materials and Methods

Animals

Five- and eight-week-old male and female LEC rats were utilized. Age-matched male and female Long-Evans Agouti (LEA) rats were

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used as controls. There were 4 rats in each group. The animals were from the domestic line of the Division of Animal Research Resources, Institute of Health Biosciences, the University of Tokushima Graduate School (Tokushima, Japan). Both five-week-old and eight-week-old LEC rats were studied to represent the stage before the onset of acute hepatitis. They show Cu and Fe accumulation in the liver but not in the kidneys [14,15,19,21-24,28-32]. In this early stage several biochemical parameters related to hepatic dysfunction, such as the plasma aspartate transaminase and glutamic-pyruvic transaminase levels, are still within the normal ranges (data not shown [14,15,21,33-35]. All of the rats were fed a laboratory diet (CMF; Oriental Yeast Co. Ltd. Tokyo, Japan) and distilled water ad libitum, and were housed in a facility that was maintained at 22°C with a 12-h light-dark cycle. All animal procedures were approved by the Animal Care and Use Committees of Hokkaido Institute of Public Health. All procedures using rats were carried out according to the regulations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Extraction of total RNAs from the PBMCs

The 1.5 mL blood samples were collected by using retro-orbital bleeding technique under ether-anesthesia. The collected samples were put into 15 mL conical tubes (BD Falcon™, Franklin Lakes, NJ, USA) containing 100 µL heparin solution (10,000 Units, FUSO Pharmaceutical Industries, Ltd. Osaka, Japan) and were vortexed well for approximately 15 sec. The total RNAs in the blood samples were extracted according to the protocol of QIAamp RNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). Concentration of each RNA sample was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The RNA samples with 260/280 ratio (an indication of protein contamination) between 1.9 and 2.1 and 260/230 ratio (an indication of reagent contamination) greater than 2.0 were used for the analysis. The total RNA between 50 ng/µL and 500 ng/µL of could be obtained from 1.5 mL blood.

Extraction of total RNAs from the liver tissue

The animals were perfused transcardially with a solution of 40 mM Tris-20 mM HCl containing 152 mM NaCl (500 ml/kg) under ether-anesthesia. Their livers were quickly removed, shredded to the scale of about 1.0 × 1.0 × 1.0 mm, and put into RNAlater® solution (Ambion®, Applied Biosystems). They were stored at 4°C until their total RNAs were extracted. The tissue pieces of the livers were homogenized by a micro-tube homogenizer (Model: 23M, Nippon Genetics, and Tokyo, Japan). The total RNAs from the rat livers were extracted using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The total RNA between 1000 ng/µL and 2000 ng/µL could be obtained from about 20 mg tissues treated with RNAlater® solution. cDNA synthesis. cDNA syntheses were performed using a TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, Branchburg, NJ, USA) in a total 100 µL reaction mixture containing 1.0 µg of the total RNA samples. The RT-PCR mixtures were incubated at 25°C for 10 min and at 48°C for 30 min, and the reaction were terminated by heating at 95°C for 5 min. The cDNAs were kept at 4°C while quantitative RT-PCR analyses were performed for one week.

Quantitative RT-PCR

The primer and fluorescent-labeled probe sets for Atp7b and metallothionein-1a (Mt-1a) were designed by Biosearch Technologies Japan (Tokyo, Japan). The 5′-fluorogenic reporter of the probe was FAM, and the 3′-fluorogenic quencher was Black Hole Quencher (BHQ-1).

The primer sets for Atp7b mRNA were as follows: forward primer: 5′-TGCCTGAACAGGAGAGAAAGGT-3′, reverse primer: 5′-TCGTGTGGGCAAAGCAAGT-3′, fluorescent-labeled probe: 5′-AGCCAAAGAGGCCAGTCGGAAAATCTTATC-3′, respectively. These primer and probe sets were designated with the exon-exon junction between exon-1 and exon-2 of NM_012511 in order to focus on the specific region of Atp7b mRNA using this uniquely designed set. The gene structures of ATP7B in humans and of Atp7b in LEC rats are shown in Figure 1. In humans, the gene structure is composed of 21 exons. However, 6 exons after exon-15 were completely deleted in the LEC rat [36], Professor T. Agui, unpublished data; personal communications). We focused on the exon-exon junction between exon-1 and -2. This is because any mutation is very rarely detected in the region of human exon-1. Therefore, we hypothesized that it was possible to detect mRNA synthesis around the region of ATP7B even with mutation, especially in LEC rats.

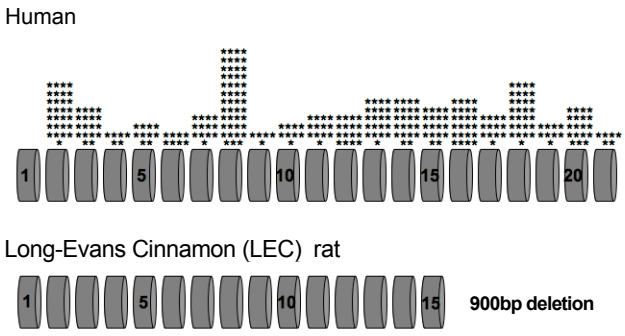

Figure 1: Gene structures on the ATP7B of humans and on the Atp7b of Long-Evans Cinnamon (LEC) rats. In humans, the gene structure is composed of 21 exons. However, 6 exons after exon-15 were completely deleted in the LEC rats. The asterisk (*) marks indicate the mutation points cited from the Wilson disease mutation database in the University of Alberta curated by Bugbee *et. al.* [12].

The primer sets for rat Mt-1a mRNA were as follows: forward primers: 5′-GAACTGCAAATGCACCTCCTG -3′, reverse primers: 5′-ACACAGCCCTGGGCACAT-3′, fluorescent-labeled probe: 5′-AAGAAGAGCTGCTGCTCCTGCTGCC-3′, respectively. These primers and probe sets were located at the exon-exon junction between 2 and 3 of NM_138826.

All the primers and probes were made by Biosearch Technologies, Inc. (Novato, CA, USA). All of them were purchased through Biosearch Technologies Japan. The rat ß-actin was utilized as an endogenous standard mRNA (EC-R-3-1000-C, accession No. NM_031144.2) as follows: forward primer: 5′-TGCCCCGAGGCTCTCTT-3′, reverse primer: 5′-GATGGAATTGAATGTAGTTTCATGGA-3′, fluorescent-labeled probe: 5′- CAgCCTTCCTTCCTGGGTATGGAATCC-3′, respectively. Our quantitative RT-PCR was performed with 2 µL of cDNA sample, 25 µL of 2 X TaqMan Universal PCMaster Mix (Applied Biosystems, Foster City, CA, USA), 900 nM of each primer and 250 nM of probe in a total 50 µL reaction mixture. The reactions were carried out on an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with the following thermal cycling conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 repeats of 15 sec at 95°C and 1 min at 60°C. The data of RT-PCR were analyzed by the sequence detector software version 1.6.3 (Applied Biosystems, Foster City, CA, USA). Relative mRNA levels were determined using the comparative CT (threshold cycle) method. The expression of Atp7b and Mt-1a mRNAs was normalized to ß-actin as an endogenous control gene. CTS was calculated by subtracting the CT value of the endogenous control (CTR) from the CT value of the sample (...CT; CT = CTS - CTR). The relative expression (2-....CT) to a calibrator was

	Male		Female	
	LEA	LEC	LEA	LEC
Atp7b	1.00 ± 0.28	0.31 ± 0.06*	1.00 ± 0.25	0.50 ± 0.14*,+
Mt-1a	1.00 ± 0.36	3.13 ± 0.48*	1.00 ± 0.19	9.18 ± 2.47*,++

*$p < 0.001$ versus sex-matched LEA rats.
+$p < 0.05$ and ++$p<0.005$ versus male LEC rats

Table 1: Relative quantities of mRNA variations in the livers between the LEA and LEC rats at the age of 8 weeks (n=4).

determined by subtracting the...CT (Calibrator) from the...CT value [.... CT = ...CT – ...CT (Calibrator)]. The samples and endogenous controls were run in duplicate.

Statistics

In this study, all statistical analyses were performed with the use of statistical software (JMP 7 for Macintosh; SAS Institute Japan Inc., Tokyo, Japan). One-way analysis-of-variance (ANOVA) was employed to identify statistically significant differences between two groups. Differences with P values of less than 0.05 were considered to be statistically significant.

Results and Discussion

The data of quantitative real-time polymerase chain reaction (quantitative RT-PCR) in the livers are indicated in Table 1. Using quantitative RT-PCR, the expression levels of Atp7b and metallothionein 1a (Mt-1a) mRNAs were analyzed in the livers of both the LEC and LEA rats. Concerning the levels of Atp7b mRNA in the LEC rat livers, the two following reports were published. The first, reported by Wu et al. revealed that RT-PCR analyses were detectable as weak bands [17]. The second, reported by Yamaguchi et al. demonstrated that Northern blot analyses indicated that they were absent in the LEC rat livers [18]. Our results support the former because our quantitative RT-PCR analyses detected the expression of Atp7b mRNA. However, the levels in the LEC rat livers at the age of 8 weeks reduced to 0.31 and to 0.50 against the controls in males and females, respectively. These results suggest that a part of mutant Atp7b mRNA exists in the LEC rat livers while it was significantly down-regulated compared to the controls. Metallothioneins (MTs) are low molecular weight proteins. They can capture heavy metals, such as Cu, zinc, cadmium and mercury. In the LEC rat liver, the abnormal accumulated Cu ions bind to MTs [21]. Our results concerning hepatic Mt-1a, one of the MT isoforms, were reasonable because the mRNA levels of Mt-1a were elevated to 3.13 and to 9.18 against the controls in males and females, respectively. We detected a novel gender difference concerning the induction levels of Mt-1a in the LEC rat livers. Several gender differences concerning the LEC rat were found. One of the most important gender differences was crucial at the acute hepatitis stage in the LEC rat [35]. The onset of acute hepatitis in the female LEC rats occurs at a younger age than in the male rats. The mortality of the female rat is higher than that of the male rat. Regarding the gender differences, the severity observed at the acute hepatitis stage might be related to that of the induction levels of Mt-1a in the LEC rat livers. In human, the gender differences were observed that the prevalence of severe hepatic dysfunction is higher in females than in men [37,38]. The expression of MTs might be related to the tolerance of hepatocytes against Cu toxicity [37]. We also revealed gender differences of porphyrin and heme metabolisms in the LEC rats [39].

The data of quantitative RT-PCR in the peripheral blood mononuclear cells (PBMCs) are indicated in Figures 2 and 3. The expression levels of Atp7b (Figure 2) and Mt-1a (Figure 3) mRNAs in the PBMCs of both the LEC and LEA rats were analyzed. Concerning the levels of Atp7b mRNA in the PBMCs of the LEC rats, our results indicated that the levels of the LEC rats at the age of 5 weeks significantly up-regulated to 7.94 and to 13.0 against the controls in males and females, respectively. Their levels at the age of 8 weeks were significantly increased to 13.7 and to 9.82 against the controls in males and females, respectively. At both ages, novel gender differences were detected concerning the induction of the Atp7b mRNA levels in the PBMCs of the LEC rats. Our findings were the first to report the following: 1) the revelation of the high expression of the Atp7b mRNA levels in the PBMCs of the LEC rats; and 2) the confirmation of the existence of novel gender differences concerning their high expression levels. These results suggest that a part of mutant Atp7b mRNA up-regulates in the PBMCs of the LEC rats, although it is significantly down-regulated in their livers.

In the PBMCs of the LEC rats, the mRNA levels of Mt-1a at the age of 5 weeks were 1.47 and 1.13 against the controls in males and females, respectively (Figure 3). There were no significant differences at this age. Their levels at the age of 8 weeks were significantly increased to 3.32 and to 1.76 against the controls in males and females, respectively. In addition, the gender difference was confirmed concerning the induction levels of Mt-1a in the PBMCs of LEC rats at the age of 8 weeks. Their gender differences suggest the variations in concerning the self-defense mechanisms, for example, against free or loosely binding Cu ions in the blood and/or oxidative stresses in the PBMCs of the LEC rats.

Conclusion

In this study, we focused on the expression level of Atp7b mRNA in the PBMCs of the LEC rats. These analyses were the first trials of the measurement of Atp7b mRNA levels in the blood. At the ages of

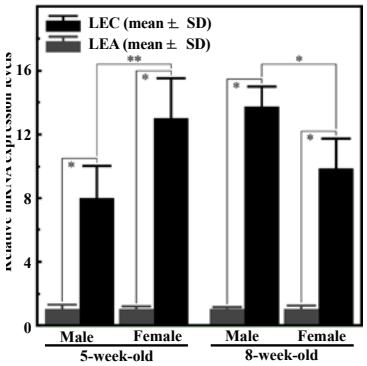

Figure 2: Quantitative RT-PCR analyses of Atp7b mRNA in the peripheral blood mononuclear cells of the LEA and LEC rats (n = 4). The mRNA levels of Atp7b were determined by using real-time RT-PCR and normalized to the expressions of their ß-actin mRNAs. *p < 0.001; **p < 0.05.

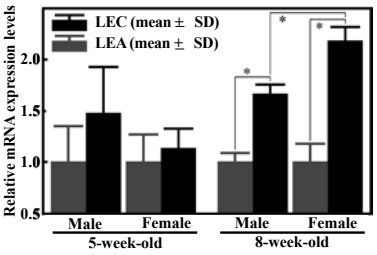

Figure 3: Quantitative RT-PCR analyses of Mt-1a mRNA in the peripheral blood mononuclear cells of the LEA and LEC rats (n = 4). The mRNA levels of Mt-1a were determined by using real-time RT-PCR and normalized to the expressions of their ß-actin mRNAs. *p < 0.001.

5 and 8 weeks, the high expressions of Atp7b mRNA were manifested in the PBMCs of both male and female LEC rats. We consider that the Atp7b mRNA in the PBMCs of the LEC rat is composed of two parts, the normal and deleted parts. However, the synthesis of the normal part in the mutant Atp7b mRNA was accelerated in the PBMCs of the LEC rat. These results are novel and unique because their levels in the livers are significantly lower than those of the LEA rats. These phenomena were discovered for the first time in the LEC rats. At present, we are yet to consider the details of these mechanisms. However, these results suggest the diversity of cell-physiological and endocrinological Cu metabolisms between the PBMCs and the livers of the LEC rats. Our findings indicate the possibility of a novel Cu metabolism in the cardiovascular network that is concerned with Atp7b of the PBMCs. Further studies will need to be done to establish the hypothesis that there is a novel Cu metabolism in the cardiovascular network though Atp7b in the PBMCs.

Upon incubation of PBMCs with the medium containing Cu ions, PBMCs incorporated and accumulated them as Cu-MTs. Our results, showing the increment of MT-1a mRNAs in the PBMCs of the LEC rats rather than those of the controls, agreed with that phenomenon. The Cu-MTs formation was more pronounced in monocytes than in granulocytes [40]. Recently, unique phenomena were discovered concerning the Cu metabolism in the PBMCs [41]. Those were: the mRNA up-regulation of ATP7A (disease-causing gene of Menkes disease) was revealed in the THP-1 monocytes, suggesting a role in the oxidation of low density lipoproteins. The inductively coupled plasma mass spectrometry (ICP-MS) analyses of Cu in murine PBMCs showed markedly increased intracellular Cu levels in the cells isolated from ATP7A-deficient mice versus control mice [42]. Those results are strongly supported our findings. Therefore, those findings are suggesting a possibility that both ATP7B and ATP7A function as a compensatory mechanism concerning Cu excretion from the PBMCs.

Finally, we believe it is necessary to confirm the expression levels of ATP7B mRNAs in the PBMCs of the patients with WND using the primer and fluorescent-labeled probe set designed at the exon-exon junction between exon-1 and 2.

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