

Development of *Mocos*-deficient rat as a model of xanthinuria type II

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Materials and Methods

Animal experiment

All animal experiments were approved by the Animal Research Committee of Tokyo University of Agriculture (TUA) (Approval number: 2022130) and The University of Tokyo (Approval number: 200014) and were conducted according to their regulations on animal experimentation.

Generation of *Mocos* KI rats

Mocos-KI rats were generated by CRISPR/Cas9 system as described previously {Yoshimi, 2016 #1}. Guide RNAs (gRNAs) were designed by Optimized CRISPR Design (crispr.mit.edu) and synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa, USA). Long ssODNs (lsODNs) were prepared using a LsODN Preparation Kit (Biodynamics Laboratory Inc., Tokyo, Japan). Cas9 protein was purchased from Integrated DNA Technologies. Pronuclear-stage embryos of F344/Jcl rats were produced by natural mating. The oviducts of female rats with vaginal plugs were removed after euthanasia by CO₂ and cervical dislocation, and embryos were flushed out from the ampullae with culture medium. Cas9 protein, lssODNs and gRNAs were introduced into the embryos using a super electroporator NEPA 21 (NEPA GENE Co., Ltd., Ichikawa, Chiba, Japan). Embryos that developed to the two-cell stage were transferred into the oviducts of pseudopregnant female rats that were anesthetized using isoflurane. Offspring were genotyped with the following primer set: 5'-GTGGCCAATGTGAAGGAGTT-3' and 5'-AATGCCTCCTACCAACAACG-3'. Founder rats were mated with F344/Jcl rats and F1 heterozygous rats were intercrossed to obtain F2 progeny.

Genotyping of the R419X mutation

CycleavePCR method was used to genotype the R419X mutation. This method detects fluorescent signals from the allele-specific DNA-RNA chimera probe that is digested by thermostable RNaseH following hybridization with amplicon {Watanabe, 2023 #20}. Sequences of primer to obtain amplicon are 5'-CACAACCAGACGTTTCTGAC-3' and 5'-ACTACAGGGTCTTGCACTGA-3', respectively. Probe to detect the wild-type allele is 5'-cacctg(A)gg-3' in which 3' is labelled with FAM. Probe to detect the mutant allele is 5'-cagttc(A)cag-3' in which 3' is labelled with ROX. RNA in the probe is enclosed with parentheses. Real-time PCR is performed with CycleavePCR Reaction Mix (Takara Bio, Inc., Kusatsu, Shiga, Japan).

Body weight measurement and survival rate

Body weight of homozygous (male; n=8, female; n=5), heterozygous (male; n=6, female; n=6) and wild-type (male; n=6, female; n=5) rats was measured every week from 4 to 16 weeks of age. Rats were given

standard chews (CE-2, Japan CLEA, Tokyo, Japan) and tap water *ad libitum*. Survival rates were calculated by recoding of number of rats died during experimental period. Kaplan-Meier Curve and logrank test was performed by R commander plug-in EZR (Easy R) {Kanda, 2013 #21}

Hematology and Urine analysis

Blood was collected in EDTA-2K tubes (Capiject II, Terumo Corporation, Tokyo, Japan) and analyzed by automated veterinary hematology analyzer XN-1000V (Sysmex Corporation, Kobe, Hyogo, Japan). To collect urine and feces, rats at 7 weeks of age were placed individually in metabolic cage (Natsume Seisakusho Co. Ltd., Tokyo, Japan) for three nights and given the chews and water *ad libitum*. Every morning we collected urine and feces and measured the volume of them. Food and water intake were also measured. Urine biochemistry was performed at Nagahama Institute of Bioscience, OrientalBio Co., Ltd. (Nagahama, Shiga, Japan). Xanthine/hypoxanthine levels were determined using the Xanthine/Hypoxanthine Assay Kit (ab155900; Abcam, Cambridge, UK) following the manufacturer's instructions.

Serum biochemistry

Blood samples were collected from the abdominal vein of male rats (9-12 weeks of age) under isoflurane anesthesia and were coagulated in Venoject II tubes (Terumo Corporation, Tokyo, Japan). Serum was collected after centrifuge at $10000 \times g$ for 5 minutes. Serum biochemistry was performed at Nagahama Institute of Bioscience, OrientalBio Co., Ltd. (Nagahama, Shiga, Japan).

Histological analysis

Rats (3, 5, 7, 8, 9, and 10 weeks of age) were euthanized under isoflurane anesthesia. Tissue samples from the kidney were fixed in 10% neutral buffered formalin, and embedded in paraffin. Sections of 4 μm thick were cut and stained with hematoxylin and eosin (HE).

Statistical Analysis

All values were presented in the form of mean $\pm$ SD. Statistical evaluation of differences between the experimental groups was done using the Mann–Whitney U test or log rank test for survival. All tests were performed using the EZR package {Kanda, 2013 #21}. A *P* value <0.05 was considered significant.