Kyoto Rhino Rats Derived by ENU Mutagenesis Undergo Congenital Hair Loss and Exhibit Focal Glomerulosclerosis

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Abstract: N-ethyl-N-nitrosourea (ENU) mutagenesis is an important tool for studying gene function and establishing human disease models. Here, we report the characterization of a novel hairless mutant rat strain that carries a recessive mutation called Kyoto rhino (krh), which was created by ENU-mutagenesis. We produced a F344-krh strain through inbreeding without backcrossing to F344 rats. The krh/krh rats lost their coat hair by eight weeks of age. They also developed wrinkled skin, cystic hair canals and long curved nails by four months of age. Markedly dilated hair follicles that contained keratin debris were observed during histological analysis of the skin. The krh locus was mapped near the hairless (Hr) gene on chromosome 15. Sequence analysis revealed a nonsense mutation (c. 1238 C>A, p. S413X) in the Hr gene. The truncated HR protein was deduced to lack a zinc-finger domain and repression domains. In aged Hr⁹⁹/Hr⁹⁹ rats, focal glomerulosclerosis (FGS) was observed in which collapsed glomeruli contained protein exudates in Bowman’s capsule. Mesangial matrices that had proliferated in segments and foot processes that were fused in podocytes were also observed. The Hr⁹⁹/Hr⁹⁹ rats also suffered from significant proteinuria. Given its breeding history, the F344-Hr⁹⁹ strain may harbor ENU-induced mutation(s) that underlie FGS in addition to having the Hr mutation. The F344-Hr⁹⁹ rat is a useful model of skin disease and may provide a new model system for the examination of the pathogenesis of FGS.

Key words: disease model, hairless, mutation, nephrosis

Introduction

Hairless mutant rodents are valuable models for studying molecular mechanisms that underlie hair growth control. They are particularly valuable when searching for the genetic basis of hereditary human hair disorders. In mice, 43 mutations are responsible for primary genetic hairlessness [7]. Among them, the most important are allelic mutations of the hairless (Hr) gene. The best characterized allele is the hairless (hr). Hr/hr mice have a striking total alopecia phenotype which appears between three and four weeks of age. The pheno-
type originates in the periorbital region and propagates in a wave-like fashion in the rostral-to-caudal direction [20]. It has been determined, through comparative studies of several distinct mouse Hr mutations, that the Hr gene product plays a key role in controlling hair follicle transformation during the catagen phase [20]. The hairless phenotype of Hr-mutant mice (Hr<sup>+/−</sup>/Hr<sup>−/−</sup>) is similar to that of the human disease atrichia. The disease phenotype comprises papular lesions (APL) and alopecia universalis congenital (ALUNC), complete hair loss after birth. It is a result of human HR mutation [2, 4, 9].

Another important mutant Hr of the mouse is the rhino (rh) mouse. Hr<sup>+/−</sup>/Hr<sup>−/−</sup> mice lose all of their hair by seven weeks of age, possess wrinkled skin and their nails overgrow. Additionally, they develop an autoimmune disease characterized by hypergammaglobulinemia, immunoglobulin deposits in the basement membrane of skin, spleen, liver, and kidney, and the presence of antinuclear antibodies which appear in young mice and increase with age [14].

Several rat hair loss mutations have been described. They are for the Charles River hairless rat [1], the Iffa Credo (IC) rat [6], the Hairless Wistar Yagi rat also known as the HWY/Slc rat [13], the Dundee experimental bald rat also known as the DEBR rat [22], the Bald rat [12], and the Hiroaki hairless rat (HHR) [17]. An intragenic deletion in the desmoglein 4 gene underlies the IC rat skin phenotype [6]. The absence of 80-kb of genomic DNA that contains five basic keratin genes is the cause of the HHR rat hairless phenotype [17].

Rat hair follicles are larger than those of mice. Therefore hairless rat mutants are attractive models for studying hair follicle development, differentiation, and cycling. Rat mutants are also good models for evaluating the effects of new drugs for treating human skin diseases. Therefore, it would be beneficial to establish new hairless rat models for these purposes.

We recently treated rats with N-ethyl-N-nitrosourea (ENU) to obtain different mutants [16]. Several hair loss phenotypes were identified by employing phenotype-driven screening. A hair loss mutant line was established by crossing mutant-type males with wild-type female littermates. Our analysis of the breeding record of this line can be used to prove that the hair loss phenotype is autosomal recessive. Thus, the mutation was named Kyoto rhino (krh).

In this study, we identified the krh mutation using a positional candidate approach and characterized the krh/krh rats. krh is a nonsense mutation of the rat Hr gene. krh/krh rats develop renal failure with massive proteinuria and focal glomerulosclerosis (FGS).

**Materials and Methods**

**Animals**

ENU-treated F344/NSlc male rats were mated with F344/NSlc female rats to generate G<sub>1</sub> offspring [16]. The ENU-mutagenized G<sub>1</sub> rats (n=42) were used as founders for the phenotype-driven screening of recessive mutations. Briefly, the G<sub>1</sub> rats were crossed with two F344 rats to generate G<sub>2</sub> offspring. The female G<sub>2</sub> offspring were then backcrossed with their parental G<sub>1</sub> rats to generate G<sub>3</sub> offspring. The recessive mutations induced by ENU in the G<sub>1</sub> rats became homozygous in the G<sub>3</sub> rats. Among the G<sub>3</sub> offspring (n=11) from a G<sub>1</sub> male (#E2307), three rats showed a hair loss phenotype; these rats were probands (P generation). We mated the affected rats with the normal littermates to fix the hair loss phenotype. The phenotype was fixed at the F<sub>2</sub> generation and the mutation was called krh. A mutant line was established by employing brother-sister mating (homozygous male × heterozygous female). The generation of inbreeding had reached F<sub>6</sub> at the end of August, 2010. The animal care and experimental procedures that were used were approved by the Animal Research Committee, Kyoto University and were carried out according to the Regulation on Animal Experimentation at Kyoto University.

**Genetic mapping**

Twenty N<sub>2</sub> rats were produced from a (BN/SsNSlc × F344-krh/krh)F<sub>1</sub> × F344-krh/krh backcross. The genotypes for the krh locus were identified on the basis of coat phenotype at four to five weeks of age. Genomic DNA was prepared from tail biopsies using an automatic DNA purification system (PI-200, Kurabo, Japan) and genotypes for D15Rat10, D15Rat13, and D15Rat85 were determined. Linkage relationship was evaluated using the chi-square test of the Excel statistical package.
Confidence intervals (\(P<0.05\)) were calculated according to the method of a previous report [11].

**RT-PCR and direct sequencing**

Total RNA was isolated from the skin of five-week-old animals using ISOGEN (NIPPON GENE, Tokyo, Japan). RT-PCR and direct sequencing of the PCR product was carried out as described previously [15]. Rat Hr cDNA was amplified using the following eight primer sets: rHr-01&02 CACCTTGGAAAGGCTGCT and ACAGGGTCACCTTGGGATG; rHr-03&04 AGGGACTACGCT-GGAAGGA and CCCAAACGTTACCGAGTG; rHr-05&06 GCAGGCAGCAGAATCTTTG and TCCTGTGAGTGTCCTGGTG; rHr-07&08 ACTCAAGAG-GGCAGAGCAGT and GGTGTTGAAGAGTCGCTGGT; rHr-09&10 CTTCCATCAACAGGGCGCTA and CTG- GGCTCTCTGGAGCTT; rHr-11&12 GGTAGCAGAAGGGACAG and TTTCCAGATGCTGCTGTC; rHr-13&14 GACCTAGCCTGTCGGGAATG and CTCCAAGGTCTCCTGACG; rHr-15&16 GTCTC- CAGTGAGCCAGACCA and GTTCCTCCTGGTACACCAAA. The PCR products overlapped each other and spanned the entire 3,624 bp Hr coding sequence (CDs).

**Morphological analysis**

Dorsal and ventral skin samples were collected from krh/krh and krh/+ littermates at two, nine and seventeen weeks of age. Mouse anti-cytokeratin (AE1/AE3, Dako Japan, Tokyo, Japan) was used for immunohistochemical analysis of the skin samples. Bound antibody was detected using horseradish peroxidase conjugated anti-mouse antibody (Histofine Simplestain MAX-PO; Nichirei, Tokyo, Japan) and 3,3’-diaminobenzidine as a chromogen (Vector Laboratories, Burlingame, CA, USA). To detect lipids, frozen sections were made from specimens that had been fixed with formalin and stained with oil red O. Organ samples of the heart, lungs, liver, pancreas, kidneys, spleen, lymph nodes, salivary glands, lacrimal glands, thyroid gland, adrenal glands, small and large intestines, and knee and foot joints, were collected from three krh/krh rats and three F344 rats at 40 weeks of age. They were fixed using 10% neutral buffered formalin, embedded in paraffin, cut at 4 \(\mu\text{m}\) in thickness, and then stained with hematoxylin and eosin (HE). To study glomerular lesions, periodic acid-Schiff (PAS) or periodic acid-methenamine-silver (PAM) staining was employed. For immunofluorescence studies, kidney samples were frozen in 22-oxacalcitriol compound (Miles Inc., Elkhart, IN, USA).

**Electron microscopy**

Perfusion fixation through the left ventricle was conducted with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Kidneys that had been excised were stored in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PB. They were fixed with 2% osmic acid for 2 h and embedded in epoxy resin. Ultra-thin sections were double-stained with uranyl acetate and lead citrate and examined using a Hitachi H-7500 electron microscope (Hitachi, Tokyo, Japan).

**Urine protein measurement**

To collect urine, six male krh/krh rats and six F344/NSlc (+/+) rats, 40 weeks of age, were caged individually in metabolic chambers after they had been orally loaded with physiological saline at 2.5 ml/100 g body weight. Six-hour urine samples were collected and their volumes, and protein concentrations were determined. Statistical differences were determined using the Mann-Whitney U test.

**Results**

**krh/krh rat hair loss phenotype and skin morphology**

For the krh/krh rats, hair loss first occurred around the nose around 2 weeks after birth and extended gradually from the anterior to the posterior of the body (Fig. 1A and 1B). At around four months of age they had wrinkled skin, cystic hair canals and long curved nails. Through histopathological analysis, markedly dilated cystic follicles were observed. These cystic follicles contained a lot of keratin debris (Fig. 1C), and they stained positive for cytokeratin (Fig. 1D). The cysts were lined by a thin layer of squamous epithelium and an easily identifiable granular cell layer. The sebaceous glands that surrounded the dilated cysts were hyperplastic. Staining with Oil red O revealed that a large amount of lipids was present in the lumen of each cyst and on the
surface of the epidermis (Fig. 1E). These findings are indicators that the krh/krh skin and hair phenotypes are similar to those of rh at the Hr locus of the laboratory mouse [7].

krh is an Hr nonsense mutation

Hr on Chr 15 was believed to be the best candidate for krh and therefore the genotype of the backcross progeny was determined using genetic markers for Chr 15. We obtained 12 krh/krh and 8 krh/+ rats from the (BN/SsNSlc × F344-krh/krh)F1 × F344-krh/krh backcross. A significant linkage relationship was observed between krh and D15Rat10 (42.7 Mb) with no recombination (χ²=21.6, P<0.01), which is indicative that krh is located <13.9 cM away from D15Rat10 with 95% probability [11]. krh was expected to span from 28.8 Mb to 56.6 Mb of Chr 15, within which the Hr locus (50.9 Mb) was mapped (RGSC v3.4).

Sequencing analyses of Hr cDNA obtained from krh skin samples revealed that adenine (A) had been substituted for cytosine (C) at nucleotide position 1,238 from the start of the CDS (c. 1,238 C>A). This substitution resulted in a stop codon at amino acid 413 of the HR protein (p. Ser413Ter) (Fig. 2A). The truncated HR protein lacked a zinc-finger domain, a part of repression domain (rD) 1, and all of rD2 and rD3 (Fig. 2B). We characterized krh as an Hr nonsense mutation and called it Hrkrh.

Focal glomerulosclerosis and proteinuria in the aged Hrkrh/Hrkrh rat

Histopathological examinations of organs that were taken from Hrkrh/Hrkrh rats at 40 weeks of age were performed. No lesions that are associated with autoimmune diseases were observed, however, prominent glomerular lesions were noted in the kidneys of the Hrkrh/Hrkrh rats. These lesions were focal lesions that had collapsed glomeruli and protein exudates in Bowman capsule and the renal tubules (Fig. 3A and 3C), and segmental prolifera-
There was no inflammatory cell infiltration into the glomeruli and interstitium. In the renal medulla, protein casts were notable in the collecting tubules (E) (arrows), but those in a wild-type F344 rat (+/+) were limited only to Henle’s loop (F) (arrows). A: HE; B: PAM; C–F: PAS staining.

Fig. 3. Focal glomerular sclerotic lesions in 40-week-old $Hr^{krh}/Hr^{krh}$ rat. Note that a collapsed glomerulus with protein exudates in Bowman’s capsule and protein casts in renal tubules (A, C), and segmental proliferation of mesangial matrices (B, D) were seen. In the renal medulla, protein casts were notable in the collecting tubules (E) (arrows), but those in a wild-type F344 rat (+/+) were limited only to Henle’s loop (F) (arrows). A: HE; B: PAM; C–F: PAS staining.

From the electron microscopic observations, the segmental glomerular sclerotic lesions were characterized as having proliferating mesangial matrices (Fig. 5A). The proliferation was associated with the dendritic processes of mesangial cells and on rare occasions with dense deposits in the mesangial regions. Foot process fusion was often observed in these glomeruli (Fig. 5B).

**Discussion**

The $krh$ mutation was identified as an $Hr$ nonsense mutation and therefore called $Hr^{krh}$. Protein HR is a nuclear receptor co-repressor for multiple nuclear receptors, such as the thyroid hormone receptor and the vitamin D receptor [23]. In the hair follicle (HF), the absence of functioning HR proteins results in the synthesis of premature and dysregulated catagen. This results in the destruction of the normal HF architecture and abrogates the HF’s ability to cycle [20]. The $Hr^{krh}/Hr^{krh}$ rat has cystic hair follicles and suffers from a premature hair cycle (Fig. 1). The truncated HR protein that is encoded by the $Hr^{krh}$ nonsense mutation is caused by a lack of functional domains which play important roles in regulating target genes [23]. Additionally, the mutation may cause nonsense mediated mRNA decay. Therefore, it is likely that $Hr^{krh}$ may be a loss-of-function mutation. In humans, $HR$ mutations are associated with congenital alopecia, such as ALUNC and APL [2, 4, 9]. Because rats are suitably sized for handling and manipulating [5, 21], the $Hr^{krh}/Hr^{krh}$ rat may be a useful animal model for developing therapies for these human diseases.

The aged $Hr^{krh}$ homozygous rat has FGS which is as-
FGS has a heterogeneous etiology and that it may manifest through multiple genetic factors [10]. The F344-\(Hr^{krh}\) rats were derived by employing ENU mutagenesis. The founder animals (G1 generation) were expected to carry no more than four ENU-induced mutations in their CDS, if the CDS occupies 1% of the genome [16]. The F344-\(Hr^{krh}\) rats were mated by inbreeding without backcrossing to F344 rats to eliminate ENU-induced mutations other than the \(Hr^{krh}\) mutation. Thus, it is likely that the F344-\(Hr^{krh}\) rat may harbor mutation(s) that may play a role in the pathogenesis of FGS. FGS in \(Hr^{krh}/Hr^{krh}\) rats might be caused by unidentified mutation(s) that were induced by ENU or the combined effects of such mutation(s) with the \(Hr^{krh}\) mutation.

The \(Hr^{Rh}/Hr^{Rh}\) mouse has the nonsense mutation (R597X) [8] and develops hypergammaglobulinemia. The excess immunoglobulins that are produced due to this disease are deposited in the basement membranes of the skin, spleen, liver, and kidney, and antinuclear antibodies are produced. These symptoms appear in young mice and increase in severity with age [14]. Although the F344-\(Hr^{krh}/Hr^{krh}\) rat has a nonsense mutation (S413X), the mutation is not associated with an autoimmune disease or IgM, IgG, and C3 deposition in the kidneys (data not shown). Generally, pathological phenotypes that are associated with this disease are often influenced by a predisposed genetic background [18, 19]. Therefore, genes that are predisposed to causing autoimmune diseases in \(rh/rh\) mice may be absent in F344-\(Hr^{krh}\) rats. By replacing the genetic background of F344-\(Hr^{krh}\) with those of other rat strains, we might find autoimmune disease in \(Hr^{krh}/Hr^{krh}\) rats.

In summary, a novel rat mutant strain, F344-\(Hr^{k}\), was established that carries an \(Hr\) nonsense mutation (S413X). In addition to the hair loss phenotype, \(Hr^{k}\) homozygous rats suffer from proteinuria and FGS. Therefore, F344-\(Hr^{k}\) may have potential as a model of skin disease as well as nephritic FGS.

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