**Rat Mutations cvd and hob with Cerebellar Malformations Map to Chromosome 2**

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**Abstract:** In this paper, we executed genome mapping and comparative mapping analyses for cvd and hob, autosomal recessive mutations with cerebellar vermis defect and cerebellar dysplasia in the rat. For the linkage analysis, we produced three sets of backcross progeny, (ACI × cvd)F1 and (F344 × cvd)F1 females crossed to a cvd homozygous male rat, and (HOB × WKY)F1 males crossed to hob homozygous female rats. Analysis of the segregation patterns of simple sequence length polymorphism (SSLP) markers scanning the whole rat genome allowed the mapping of these autosomal recessive mutations to rat Chromosome (Chr) 2. The most likely gene order is D2Mgh12 - D2Rat86 - D2Mit15 - D2Rat185 - cvd - D2Rat66 - D2Mgh13, and D2Mit18 - Fga - D2Mit14 - D2Rat16 - hob - D2Mgh13. Crossing test between a proven cvd heterozygous and a hob heterozygous rats demonstrated their allelism. Furthermore, comparative mapping indicated the cvd locus corresponds to mouse chromosome 3 and a strong candidate gene Unc5h3, a causative gene for the rostral cerebellar malformation mouse, was implicated.

**Key words:** cerebellar vermis defect rat, dysplasia, neurological mutant, hobble behavior rat, rostral cerebellar malformation, Unc5h3

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**Introduction**

In humans, malformations of the central nervous system (CNS) are of major clinical importance, leading to considerable mortality and morbidity, both pre- and post-natally [6]. The etiology of CNS malformations involves both genetic and environmental factors, and despite intensive investigations, the causes of CNS malformations in humans still remains largely undetermined [6, 17]. Cerebellar vermis defect has been reported in human disorders such as rhomboencephalosynapsis [7], Dandy-Walker [3], and Joubert syndromes [8]. The Joubert syndrome is a familial disorder, and most syndromes with cerebellar vermis agenesis are considered...
to be autosomal recessive disorders [3, 6].

Mutants in laboratory animals provide useful tools for investigating the underlying mechanisms of CNS development. Because rats are bred in smaller quantities than mice, much fewer neurological mutations have been reported in this species. However, the rat is more convenient for experimental use because of its larger brain and body. Recently, more neurological and experimental information have been accumulated on the development of the rat brain.

We found two recessive mutations, cvd [9-13] and hob with cerebellar malformations. The cvd mutation was discovered in an inbred colony of LEW rats maintained at Osaka Prefecture University. At the present time, the mutation is maintained in a tentatively named CVD strain which has a hybrid background between Donryu and LEW [10]. From the pathological feature of this mutation, we named it “cerebellar vermis defect” (CVD, symbol cvd). The hobble behavior (hob) mutation was found in a colony of F344 congenic rats with the C locus of the fatty rat, maintained by Sankyo Co., Ltd. The hob mutation has been kept by brother-sister matings between heterozygous (hob/+ ) males and homozygous (hob/hob) females. This mutant strain, named HOB, exhibits cerebellar vermis defect, fused cerebellar hemispheres, and cerebellar dysplasia [9]. Genetic analysis of these mutant genes may provide useful information on cerebellar development, especially in the development of cerebellar vermis. In this paper we performed genetic linkage and comparative mapping of the cvd and hob genes.

**Materials and Methods**

**Rat crosses**

Linkage analyses were performed with three different backcrosses of (ACI/N Jcl × CVD)F₁ and (F344/Crj × CVD)F₁ to a male cvd homozygous rat, and (HOB × WKY)F₁ to female hob homozygous rats. These matings resulted in the production of 96 backcross progeny from a [(ACI × CVD)F₁ × CVD], 57 backcross progeny from a [(F344 × CVD)F₁ × CVD], and 116 backcross progeny from [HOB × (HOB × WKY)F₁] crosses.

For the allelism test, mating between a proven cvd heterozygous (cvd/+) and a hob heterozygous (hob/+ ) was conducted. Clinical behavior and histopathology of CNS were checked in the F₁ rats.

**Pathological observations**

The phenotype for the CVD, HOB, and F₁ rats was determined by the presence or absence of the cerebellar vermis and histopathology. Briefly, the brains were removed and fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin-embedded histologic sections were cut by a microtome at 4 µm and stained with hematoxylin and eosin (HE). We confirmed a dysplastic cerebellum in the affected rats histologically.

**DNA sampling**

DNA was extracted from the spleen of homozygous cvd/cvd, hob/hob and heterozygous cvd/+ , hob/+ animals as well as from the parental strains ACI, F344 and WKY by standard procedures [2, 19]. DNA samples were diluted in water to a concentration of 1 mg/ml and stored at –20°C.

**Molecular markers**

Forty-one simple sequence length polymorphism (SSLP) markers, evenly distributed over the whole rat genome and revealing polymorphisms were selected for linkage analysis. These SSLP markers were used for the systematic analysis of haplotype segregations in the cvd/cvd or hob/hob progeny of the backcross.

All SSLP markers were amplified from 100 ng of template DNA in a final reaction volume of 25 µl containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 125 µM of each deoxynucleotide, 0.1 % Tween 20, 0.1 µM of each primer, and 0.6 unit of Taq DNA polymerase. PCR reactions were carried out in a programmable thermal cycler (M.J. Research™) with 35 cycles (denaturation at 94°C for 30 s, annealing at 55°C for 60 s, extension at 72°C for 45 s) with a final extension at 72°C for 3 min. Amplification products were scored on 4 % NuSieve agarose gel with ethidium bromide staining.

**Results**

**Phenotypes of CVD and HOB rats**

The cvd homozygotes could be identified from 14 days old, because they showed difficulties in moving especially in the hindlimbs. With advancing age, they
exhibited abnormalities in hindlimbs involving wide-based stance, stretching and ataxia resulting in recumbency. At the necropsy, all homozygote rats lacked the cerebellar vermis, and cerebellar hemispheres were fused in the midline. Histologically, the brains of the affected rats had dysplastic lamination and cell positioning throughout the cerebellum. In the cerebello-pontine junctional areas, the Purkinje and granular cells were haphazardly distributed, showing dysplastic arrangement of the cerebellar cortex. Detailed histopathology of the CVD rat has been described previously [9]. The HOB rat showed clinical behavior and pathological changes indistinguishable from the CVD rat.

**Analysis of the breeding records**

Analysis of the breeding records confirmed that the cvd mutation is transmitted as a recessive autosomal condition as reported in a previous paper [10]. All F1 progeny of (ACI × CVD), (F344 × CVD) and (HOB × WKY) were phenotypically normal. From the backcross matings between the F1 animals and their cvd homozygous fathers or hob homozygous mothers, we obtained 43 affected and 53 normal rats in the ACI cross ($\chi^2=1.042, 0.30<P<0.40$), and 24 affected and 33 normal rats in the F344 cross ($\chi^2=1.421, 0.20<P<0.30$), and 54 affected and 62 normal rats in the WKY cross ($\chi^2=1.042, 0.30<P<0.40$). This number seems less than the expected 50% but can probably be explained by the death of some affected rats before weaning. Both sexes were involved in the affected backcross progeny in the ACI cross (23 females and 20 males), in the F344 cross (11 females and 13 males), and in the WKY cross (31 females and 23 males).

**Genetic localization of the cvd and hob mutations**

Checking the segregation ratio of homozygous versus heterozygous haplotypes in the progeny from the backcrosses between the ACI, F344 and CVD, and between the WKY and HOB, we found a clear-cut distortion for several markers of rat Chr 2 indicative of genetic linkage. Typing of the haplotypes for an additional subset of rat Chr 2 specific SSLP markers indicated that the cvd locus localized between the D2Rat185 and the D2Rat66 locus, and the hob locus between the D2Rat16 and the D2Mgh13 (Figs. 1, 2). Analysis of the haplotype distribution pattern indicated that the cvd locus is located 2.3 cM from the D2Rat185 and the hob locus is located 3.4 cM from the D2Mgh13 at the 5% risk level.

To confirm the relationship between the hob and cvd locus, we crossed a male hob/+ rat, proven to be its genotype from a previous breeding record, with a female cvd/+ rat. Out of 6 offspring, 3 rats showed ataxia indistinguishable from that found in HOB or CVD rats. Three F1 rats lacked cerebellar vermis (Fig. 3) and histopathology revealed cerebellar dysplasia (Fig. 4).

To compare the cvd region in the mouse, we conducted comparative mapping of the region by use of a mouse SSLP marker in the rat. Several SSLP markers, which were designed for mouse Chr 3 were examined for applicability to the rat. A D3Mit16 was applicable and showed polymorphism in the F344 and CVD cross. This marker was closely linked with rat D2Mgh12, D2Rat86, D2Mit15, D2Rat185, D2Rat66, and D2Mgh13 markers that were close to the cvd locus. The result of comparative mapping is shown in Fig. 2. This result indicates that the cvd region is homologous to mouse Chr 3.

**Discussion**

In this paper, we introduce a new mutant rat with abnormal hobble behavior, named as HOB. The pathology of the HOB rat is very similar to the previously reported CVD rat. A crossing test between these strains clearly demonstrated the allelism between hob and cvd.

To identify the causative gene for the rat cvd and hob mutations, we genetically mapped these mutations to the rat chromosomal region by linkage analysis, rat-mouse comparative mapping. The cvd and hob mutations were mapped on rat Chr 2, a region of which is homologous to mouse Chr 3. Furthermore, by searching the web site of Mouse Genome Informatics (http://www.informatics.jax.org/), we found a rostral cerebellar malformation (rcm) mouse whose causative gene, Unc5h3 (unc5 homolog (C. elegans) 3) is located in the vicinity of D3Mit16.

Mice homozygous for either the spontaneous Unc5h3 mutation or the transgenic insertion allele have abnormalities in the rostral cerebellum and caudal midbrain, consisting of reduction in cerebellar size, abnormal foliation pattern and ectopic cerebellar Purkinje and granule cells [1]. Severe anatomic malformation of
Fig. 1. a) Distribution of Chr 2 haplotypes in 43 rats homozygous for the cvd mutation (cvd/cvd) derived from a (ACI × CVD)F1 × CVD backcross. Black boxes represent homozygosity for the paternal allele. White boxes represent heterozygosity for the ACI allele. b) Distribution of Chr 2 haplotypes in 24 rats homozygous for the cvd mutation (cvd/cvd) resulting from the (F344 × CVD)F1 × CVD backcross. Black boxes represent homozygosity for the paternal allele. White boxes represent heterozygosity for the F344 allele. c) Distribution of Chr 2 haplotypes in 54 rats homozygous for the hob mutation (hob/hob) resulting from the HOB × (HOB × WKY)F1 backcross. Black boxes represent homozygosity for the paternal allele. White boxes represent heterozygosity for the WKY allele.

Fig. 2. Diagrammatic representation of rat Chr 2 indicating the most likely localization of the hob mutation (a) and cvd mutation (b). c) Comparative mapping of the cvd region in the rat and mouse. Distances from the centromere for mouse loci are based on the Mouse Genome Database, and are shown on the left in cM.
Unc5h3rcm/Unc5h3rcm mice is restricted to the rostral cerebellum, in particular the parafloccular lobe [14]. This pathological phenotype of the Unc5h3rcm/Unc5h3rcm mice is similar to that of the CVD and HOB rats. There is crucial difference in pathology between the CVD/HOB rats and Unc5h3rcm/Unc5h3rcm mice in that cerebellar vermis is present in the Unc5h3rcm/Unc5h3rcm mice, whereas vermis is absent from the CVD and HOB rats [4]. There is no gene considered as a cause of cerebellar lesion similar to that shown by CVD and HOB rats adjacent to the mapped region on rat Chr 2 and its homologous region in mouse Chr 3. Unc5h3 is a receptor for netrin 1 and the netrin signal may play important roles in axonal guidance and in development of the cerebellar anlage [5, 15, 16, 18]. Homozygous mice for the Unc5h3 mutation have cerebellar granule and Purkinje cells within the inferior colliculus and mid brain. This lesion is attributed to an apparent disruption of cerebellar boundaries [1, 16]. Our previous studies on neurogenesis of the CVD rat indicated that bromodeoxyuridine-labelled neurons migrated into the pons [12, 13]. This abnormal migration correlates well with the finding in the Unc5h3rcm/Unc5h3rcm mouse. UNC5h3 is considered as a strong candidate gene of the cerebellar vermis defect in the rat.

Cerebellar vermis defect and cerebellar dysplasia in humans are due to a number of complicated syndromes, most of which are genetically controlled. Thus the CVD and HOB rats may provide a valuable opportunity to study the mechanism of the cerebellar vermis defect and cerebellar dysplasia. Further molecular biological study of the cvd and hob critical region is currently underway.

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References


