FULL PAPER *Pathology*

Abnormal Development of Nephrons in *Claudin-16*- Defective Japanese Black Cattle

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ABSTRACT. The kidneys of 37 Japanese Black calves aged 2 to 65 months diagnosed with Claudin 16 (*CL-16*) defect by the DNA-based test were examined pathologically. The animals exhibited clinical symptoms such as growth impairment, renal failure, overgrowth of hooves, and anemia at a young age. There was no correlation between the time of onset and age. Kidney weights relative to body weight were similar to those in normal animals, but both kidney net weights and size were reduced due to atrophy in animals that showed severe renal dysfunction. Histopathological examination of the kidneys showed reduction in the number of glomeruli, compensatory hypertrophy of glomeruli and tubules, and glomerular and tubular atrophy accompanied by interstitial fibrosis and lymphocytic infiltration. Glomeruli were clearly less in number in the kidneys of *CL-16*-defective animals than those of normal animals even in the cases with mild lesions. A small number of immature glomeruli and tubules were also detected, suggesting that there were fewer nephrons developed at birth in *CL-16* -defective animals. It was suggested that a defect of the *CL-16* gene is involved in the "abnormal development of nephrons". Immunohistopathological examination of the kidneys showed that the epithelium of thick ascending limb of Henle was stained with anti-CL-16 antibody in the control animals, but not in the affected animals, suggesting a defect of CL-16 in the epithelium of renal tubules in the affected animals.

KEY WORDS: abnormal development, *Claudin 16*, Japanese Black cattle, nephrons, *Paracellin-1*.

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The *claudin 16* (*CL-16*) defective in Japanese Black cattle is a hereditary disease that causes severe renal failure, growth retardation, and overgrowth of hooves in young animals [5, 23]. It is also known as "renal tubular dysplasia in Japanese Black cattle" [13–17] or "bovine chronic interstitial nephritis with diffuse zonal fibrosis" [5, 8]. This disease is caused by a homozygous deletion of gene and is transmitted by autosomal recessive inheritance [8, 14].

Since it occurs particularly in bulls of breeds with good meat quality, it causes large economic losses to cattle breeders. The *CL-16* gene is considered to correspond to the *Paracellin-1* gene [5], defective of which causes hypomagnesemia, hypercalciuria, calcium deposition in renal parenchyma, and renal failure in humans [20].

Paracellin-1 protein in humans exists in the epithelium of ascending limb of Henle's loop. A lack of it causes a failure of Mg resorption, and results in hypomagnesemia [20]. It is thought that Paracellin-1 protein forms a component of magnesium channel [20, 21]. Although the amino acid has more than 90% homology with Paracelline-1 [5, 13], the *CL-16* defect in cattle exhibits no hypomagnesemia [17] which is a common finding in humans.

In bovines, although studies of the *CL-16* defect in the genetic field have developed considerably [5, 8, 13–18], pathology of the defect remains to be elucidated. The purpose of this study was to clarify the histopathological characteristics of the *CL-16* defect and conditions that lead to renal failure, and to examine the distribution of CL-16 protein immunohistochemically.

MATERIALS AND METHODS

Samples: The kidneys of 37 Japanese Black calves (aged 2–65 months) diagnosed with the *CL-16* defect by DNA-based test [5] were studied (Table 1). Clinical, blood biochemical, and autopsy reports of each animal were evaluated. Among the present cases, 7 animals that underwent autopsy at Iwate University (Nos. 3, 5, 7, 11, 19, 28, 32) were available for body weights and kidney weights and size together with histopathology of all organs (Table 1).

As the control, 16 Japanese Black calves (aged 1–49 months) that had no *CL-16* defect and showed no microscopic lesions in the kidney were examined.

Body weight: Growth of the animals was compared with the control data presented in the archives of Wagyu Registry Association [22].

Histopathological examination: The kidneys of 37 animals and 16 controls were examined. Kidney sections were stained with hematoxylin and eosin (HE), Masson's trichrome, periodic acid-Schiff reaction, periodic acid-methenamine-silver (Yajima's modification), and Watanabe's method for reticulum (silver impregnation).

Glomeruli were counted in 10 low-power fields (object lens: × 4), and the number of glomeruli per field was calculated and compared. Obsolescent glomeruli were included in the counting.

In Nos. 3 and 28, samples were collected from the anterior margin, anterior part, middle part, posterior part, and posterior margin of the kidney, and the number of glomeruli

Table 1. Kidney size, weight and results of blood biochemical examinations in *CL-16* defect calves

Animal No.	Age (month)	Size of kidney (cm)		Weight of kidney (g)		Cre (mg/dl)	BUN (mg/dl)	RBC (10^4/ul)	WBC (10^2/ul)	Ht (%)	Alb (g/dl)	Ca (mg/dl)	Corrected Ca level* (mg/dl)	IP (mg/dl)	Mg (mg/dl)
		Left	Right	Left	Right										
1	2	9.5×5×3	9×5×4	100	95	10.6	153	NE	NE	NE	2.5	5.4	6.9	18.2	1.8
2	3	NE	NE	NE	NE	6.7	140	1150	148	33	3.4	6.3	7.5	20	2.2
3	4	10.5×4×3	12×5.5×2.8	104	106	3.1	58.8	NE	NE	NE	NE	9.2	NE	5.5	2.9
4	4	NE	NE	NE	NE	21.1	146.5	813	108	25.6	4.01	3.5	3.49	17.7	1.5
5	4	11.5×5.5×4.5	12×6.5×3.2	150	148	NE	127.4	881	169	29	NE	NE	NE	NE	NE
6	5	NE	NE	195	150	2.9	45.3	664	64	25	3.3	8.9	9.6	11.3	1.7
7	5	13.5×6×4	11×7×4.5	165	163	2.99	87.5	1313	74	41	4.212	8.4	8.188	14.3	NE
8	6	NE	NE	NE	NE	1.7	35.7	841	57	27	3.24	8.5	9.26	6.1	2.2
9	6	NE	NE	NE	NE	2.8	70	893	120	25	3.2	11.1	11.7	11	2.1
10	6	NE	NE	NE	NE	7	45	584	42	24	4.1	7	7.6	5.9	NE
11	7	9.5×5.5×3	9.7×5.5×3	93	86	5.63	57.5	457	76	16	4.66	7.2	6.54	4.9	NE
12	8	NE	NE	NE	NE	3	64.7	617	84	23	4	7.8	7.8	6.7	4.9
13	8	10×6×5	10×6×5	NE	NE	4.7	52	686	87	23	3.6	8.8	8.5	4.7	NE
14	8	7×14×?	6×12×?	NE	NE	6.8	53	594	7.3	22	4	9.1	9.6	5.9	3.7
15	9	NE	NE	NE	NE	7.4	88.9	605	66	23	3.1	9.9	10.2	8.5	2.4
16	9	NE	NE	NE	NE	9.8	76	755	203	38	4.3	6.8	6.8	10.7	3.5
17	10	NE	NE	NE	NE	3.6	29.1	948	102	42	3.7	11.3	12.1	7.8	2.2
18	10	NE	NE	NE	NE	5.4	72	677	83	29	3.7	8.9	9.2	11.9	6.7
19	11	13×7×4.5	13.5×6×3.2	193	192	2.15	43.3	1158	109	42	4.28	11.7	11.42	6.3	NE
20	13	NE	NE	NE	NE	7.7	135	482	NE	20	NE	8.8	NE	12.6	NE
21	14	NE	NE	NE	NE	3.4	73.4	646	NE	27	2.8	9.6	10	9.3	2.9
22	15	NE	NE	NE	NE	4.3	82.5	850	71	40	3.6	8.5	9.4	5.9	2.1
23	15	NE	NE	NE	NE	11.6	116.7	538	32	25	4.2	NE	NE	19	NE
24	16	NE	NE	NE	NE	2.7	56.9	700	64	32	3.2	9.3	10.4	9.1	1.9
25	16	NE	NE	NE	NE	16.1	187.8	621	72	27	3.5	4.3	4.1	23.1	NE
26	21	NE	NE	NE	NE	3.9	68.6	896	202	42	2.74	NE	NE	NE	NE
27	21	NE	NE	NE	NE	4.4	43.1	497	54	23	3.1	NE	NE	NE	NE
28	21	13×6×6	14×8×4.5	268	224	7.65	123.3	533	71	21	4.39	7	6.61	19.5	NE
29	22	NE	NE	NE	NE	16.4	150	424	NE	19	NE	5.7	NE	15.5	2.2
30	23	NE	NE	NE	NE	8.1	73.7	747	35	33	3.1	8.4	9.3	9	4.7
31	25	NE	NE	NE	NE	3.6	28.8	NE	NE	40	NE	8.1	NE	13.1	2.4
32	25	16×10×7	18×9×5	413	430	9.98	104.4	406	47	20.1	3.96	3.4	3.44	8.7	NE
33	25	NE	NE	NE	NE	10.6	80.3	460	56	21	3.4	7.2	7.7	16.9	NE
34	26	NE	NE	NE	NE	4.2	148	775	60	32	2.9	6.1	6	11.6	1.6
35	26	NE	NE	NE	NE	11.1	83.9	1140	117	56.4	4.1	4.6	4.5	10.5	1.7
36	28	NE	NE	NE	NE	5.4	130	530	6.2	21	3.5	7.8	8.6	9.1	2
37	65	NE	NE	NE	NE	3.1	98.4	455	NE	26	NE	6.9	NE	8.7	2.5

* Corrected serum Ca level (mg/dl)=Measured serum Ca level (mg/dl) + [4-serum albumin level (g/dl)]. NE: Not examined.

was compared among the various parts.

Immunohistochemical examination: The kidney samples of Nos. 4, 5, 11, 18, 19, 21, 35, 36, 52, 53 were examined. Samples of the liver, spleen, kidney, heart, lung and intestine collected from Nos. 18, 22 and 53 were examined. These samples were embedded in OCT compound, and frozen sections made with a cryostat and fixed in acetone. The sections were examined with the avidin-biotin-complex (ABC) method using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, U.S.A.). We used the anti-C-terminal of CL-16 peptide rabbit serum (at a dilution of 1:10) [7] as a primary antibody. Briefly, the polypeptide CRSHAIPRTQTAKMYAVDTRV corresponding to the COOH-terminal cytoplasmic domain of bovine claudin-16 was synthesized and coupled via cystein to keyhole limpet hemocyanin. The conjugated peptide was used as the antigen to generate polyclonal antibody in a rabbit. The specificity of the antibody was confirmed using total lysates of *Escherichia coli* expressing glutathione S-tansferase fusion proteins with cytoplasmic domains of claudin-1 to -16. Finally, the sections were developed with a 0.02% 3,3'-diaminobenzidin tetrahydrochloride solution.

RESULTS

Clinical and blood biochemical findings: Retarded growth (Fig. 1), diarrhea, and elongated hooves were observed in most affected animals. The animals showed no abnormality at birth and grew normally for a period, but with aging they exhibited growth retardation and refractory diarrhea, although activity and appetite were relatively good. Almost all affected animals showed overgrowth of hooves. On blood biochemical examination (Table 1), increased levels of blood urea nitrogen (BUN) and creati-

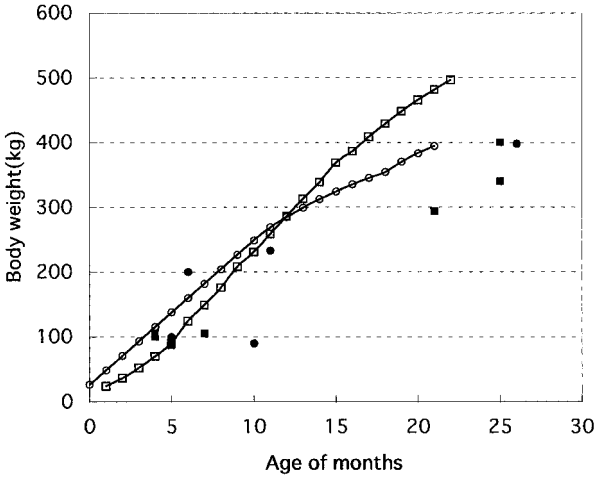

Fig. 1. Age and body weight of calves examined. ⊖: Normal growth curve (♀): Lower limit. ⊟: Normal growth curve (♂, castrated commercial cattle): Lower limit. ●: *CL-16*-defective calves ♀, animal Nos. 6–8, 18, 19, 35. ■: *CL-16*-defective calves ♂, animal Nos.3–5,11,28,31,32.

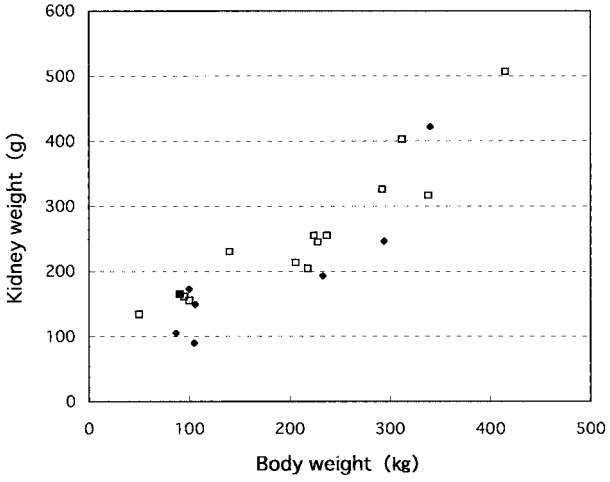

Fig. 2. Body weight and kidney weight of calves examined. ◆: *CL-16*-defective calves, animal Nos. 3, 5–7, 11, 19, 28, 32. □: Control calves, animal Nos. 38–42, 44–48, 50–53.

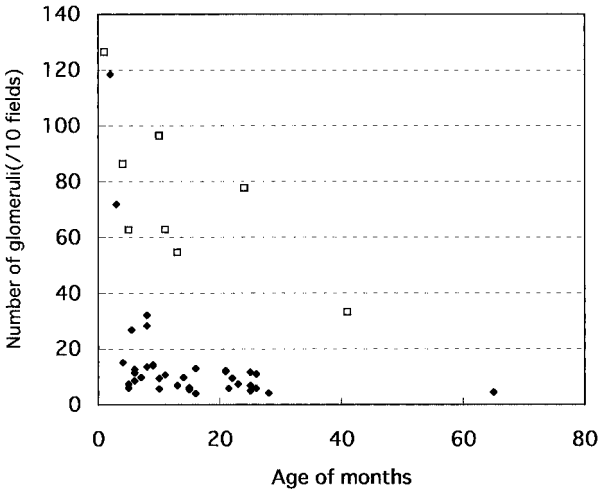

Fig. 3. Age and number of glomeruli of calves examined. ◆: *CL-16*-defective calves, animal Nos. 1–37. □: Control calves, animal Nos. 38, 40, 42, 44, 45, 48, 50, 52.

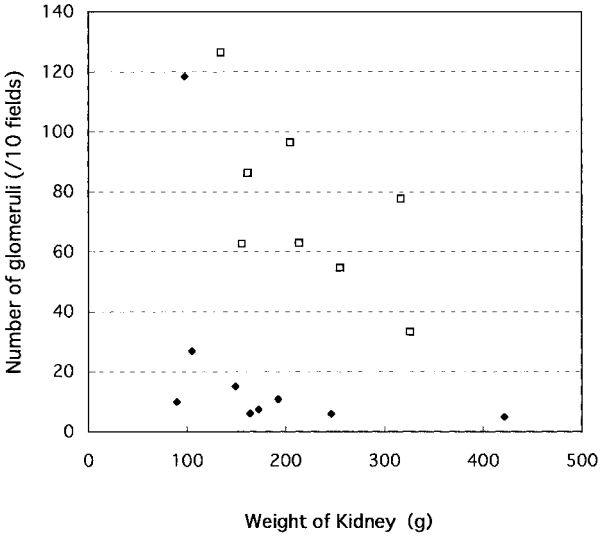

Fig. 4. Kidney weight and number of glomeruli of calves examined. ◆: *CL-16*-defective calves, animal Nos. 1, 3, 5, 7, 11, 19, 28, 32. □: Control calves, animal Nos. 38, 40, 42, 44, 45, 48, 50, 52.

nine (Cre) were observed in all animals, suggesting renal failure. Anemia (13/37), decreased levels of calcium (16/29), and increased levels of inorganic phosphate (26/34) were also noted. The magnesium (Mg) level increased in 8 and decreased in 4 of the 25 animals. No correlation was observed between the appearance of clinical symptoms and age.

Gross changes: Grossly, coarse granular changes on the surface and discoloration of the cortex were noted in the bilateral kidneys. Thinning of the parenchyma and small size of kidney were observed in severe renal failure cases. No marked difference was observed in either size or weight between the right and left kidneys. And no marked differences were observed between 8 affected animals examined (aged 4–25 months) and 14 controls (aged 1–49 months) concerning ratio of body weight and mean weight of right or left kidney (Fig. 2).

Hypoplasia of the pituitary gland (5/7, about 1 g) and thyroid gland (5/8, 6–12 g) was noted. There were no significant changes in other organs.

Histopathological findings: The number of glomeruli in the affected animals was markedly less than that in the con-

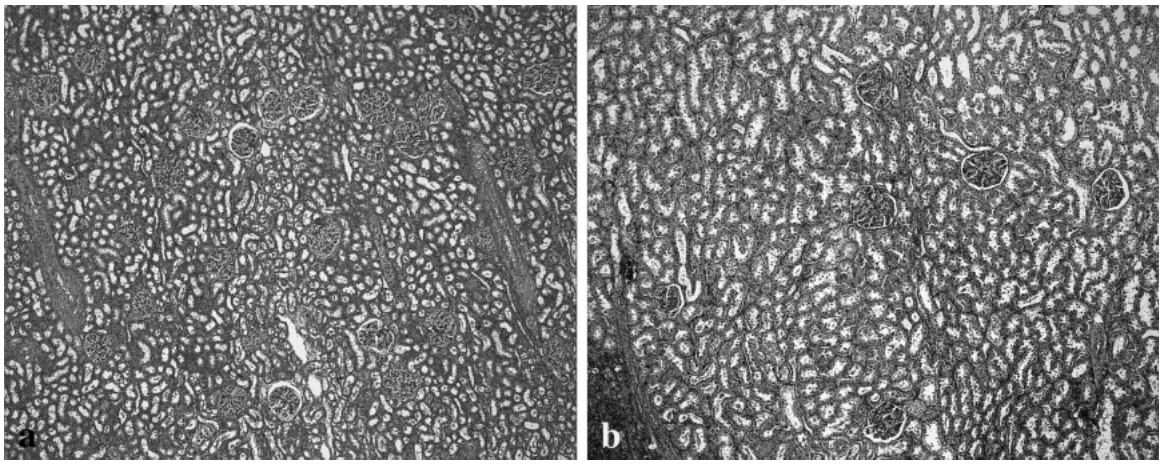

Fig. 5a. Normal renal cortex. Animal No. 42. No abnormality is observed in the number of glomeruli or tubular distribution. HE, × 40. b. Renal cortex of a *CL-16*-defective animal. HE, × 40. Animal No. 19. Glomeruli are markedly reduced in number compared with the normal kidney.

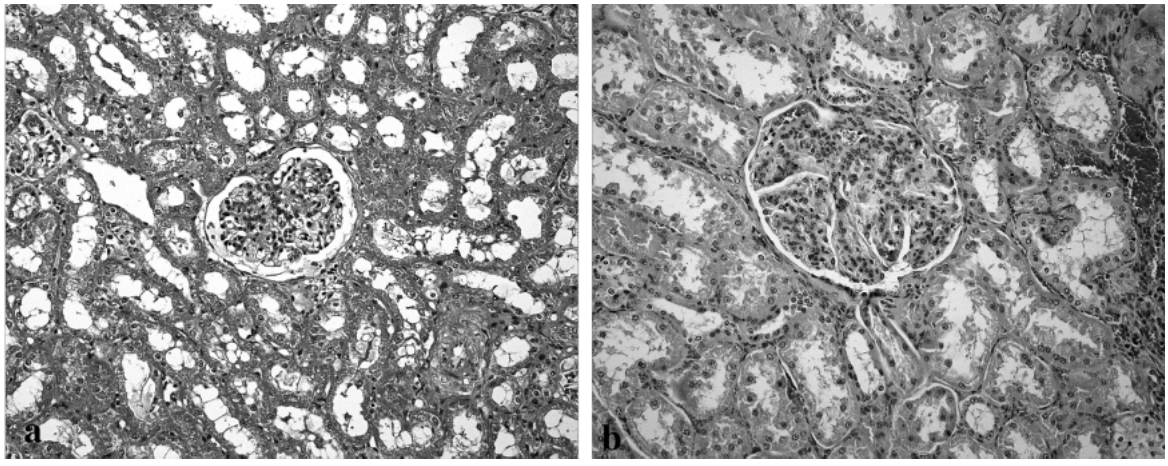

Fig. 6a. A glomerulus of a normal calf. HE, × 100. b. A glomerulus of a *CL-16*-defective calf, × 100. The glomerulus and tubules are large in size showing compensatory hypertrophy. HE, × 100.

trols of the same age or same body weight except in 2 animals (Figs. 3 and 4; Figs. 5a and b). No differences were observed in the number of glomeruli between the parts of the kidney examined. Some glomeruli were atrophic, others were hypertrophic (Figs. 6a and b), and a small number of immature glomeruli were also noted (Fig. 7). Atrophic glomeruli were accompanied with periglomerular fibrosis and thickening of the basement membrane. Around atrophic glomeruli, regressive changes of tubules such as narrowing of the tubular lumen and thickening of the basement membrane of the proximal convoluted tubules, were notable. And marked interstitial fibrosis and lymphocytic infiltration were observed. Such atrophic glomeruli appeared to be more frequent in the superficial layer of the cortex beneath the capsule. In hypertrophic glomeruli, on the other hand, an enlargement of tufts due to increases in mesangial cells and mesangial matrix and dilation of Bowman's capsules were noted, and tubules around them were markedly hypertrophic. No correlation was observed between the Cre level and the total number of glomeruli. An increase in the level of Cre was observed in the animals with many atrophic glomeruli and few hypertrophic glomeruli. These kidneys showed marked interstitial fibrosis and lymphocytic infiltration, and regressive changes of renal tubules.

Three major patterns of fibrosis were observed: cord-like (fibrous tissue proliferated as bundles in the medullary rays), focal (fibrous tissue proliferated as aggregates in the interstitium), and diffuse (fibrous tissue proliferated nearly equally in the interstitium between the tubules). Fibrosis looked to start as cord-like in the cortex, extended to the medullary rays, and progressed in the outer zone of medulla. However, diffuse fibrosis of the entire inner zone of medulla was observed also in the animals with low Cre levels. As fibrosis progressed, the cortical labyrinth was segmented

into lobules by fiber bundles. And interstitial fibrosis and tubular atrophy progressed in each lobule, suggesting that degeneration and loss of tubules were promoted by individual nephrons.

In animals over 10 months, the cystic dilation of tubules and flattening of the epithelium were noted in all areas (Fig. 8), and detachment and regeneration of the epithelium of proximal tubules were noted frequently and particularly in the deep cortex and the outer zone of medulla.

Immature tubules were composed of cells resembling the epithelial cells of proximal tubules with a rich cytoplasm and a clear, large nucleus, and their lumens were markedly narrowed or absent, appearing like an endocrine gland-like structure. Many of the narrowed tubular lumens contained proteinaceous casts. Most of these tubules forming cords or foci were present in the cortex, and also in the outer zone of medulla in severely affected cases. There were also many tubules that showed deposition of a large amount of lipofuscin and thickening of the basement membrane (Fig. 9a). Another type of tubules was composed of cells that had scant cytoplasm and a small, intensely stained nucleus and resembled to the epithelial cells of Henle's loop (Fig. 9b). These tubules were observed in large numbers in the outer zone of medulla. They were observed in small number also in the inner zone of medulla but not in the cortex.

Kidneys of control animals: No immature glomerulus or

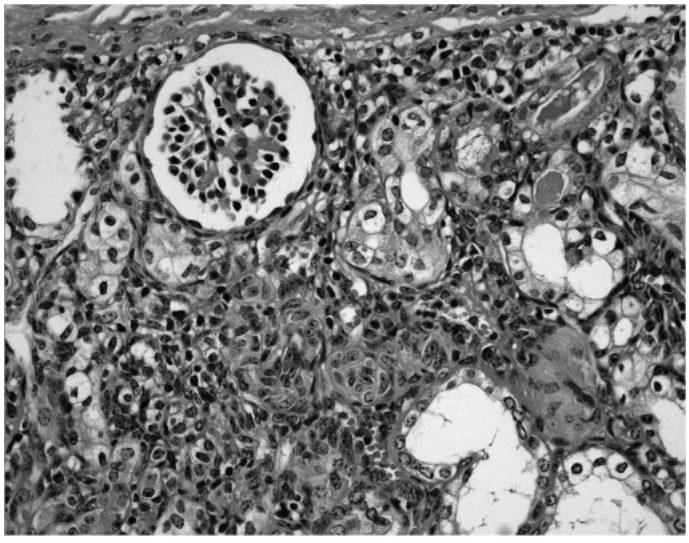

Fig. 7. An immature glomerulus. Animal No. 3. Immature nephrons are observed under the capsule. Glomeruli are small, the vasculature is poorly developed. Narrowing of the tubular lumen and marked interstitial fibrosis and lymphocytic infiltration are seen. HE, × 400.

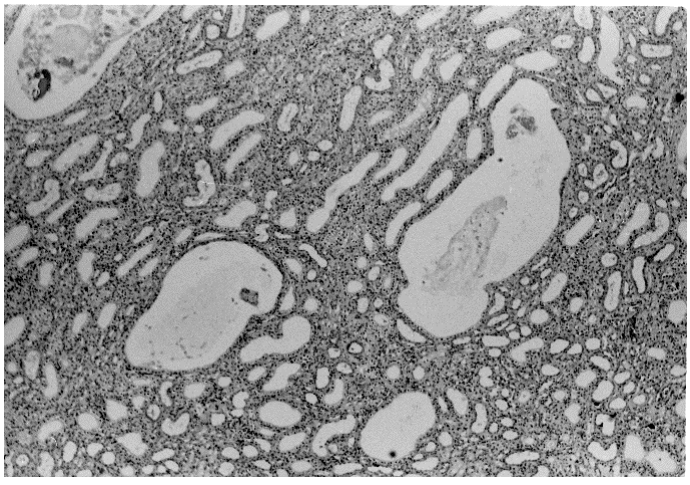

Fig. 8. Cystic ectasia of renal tubules. Animal No. 17. HE, × 50.

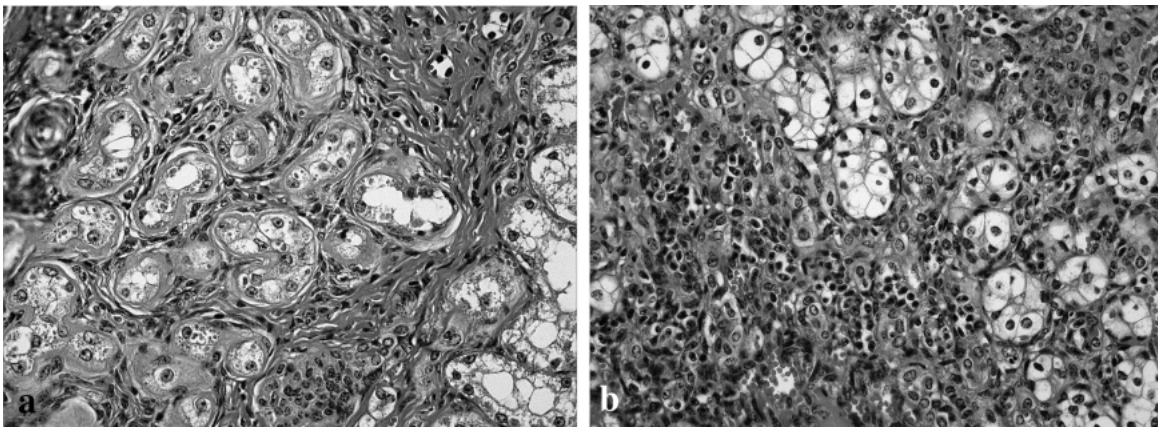

Fig. 9a. Immature tubules observed in the renal cortex. No. 3. Immature epithelium with a rich cytoplasm and large nucleus show lipofuscin deposition. HE, × 400. b. Another type of immature tubules observed in the outer zone of medulla. Animal No. 3. Epithelium have scant cytoplasm and a small, intensely stained nucleus. HE, × 400.

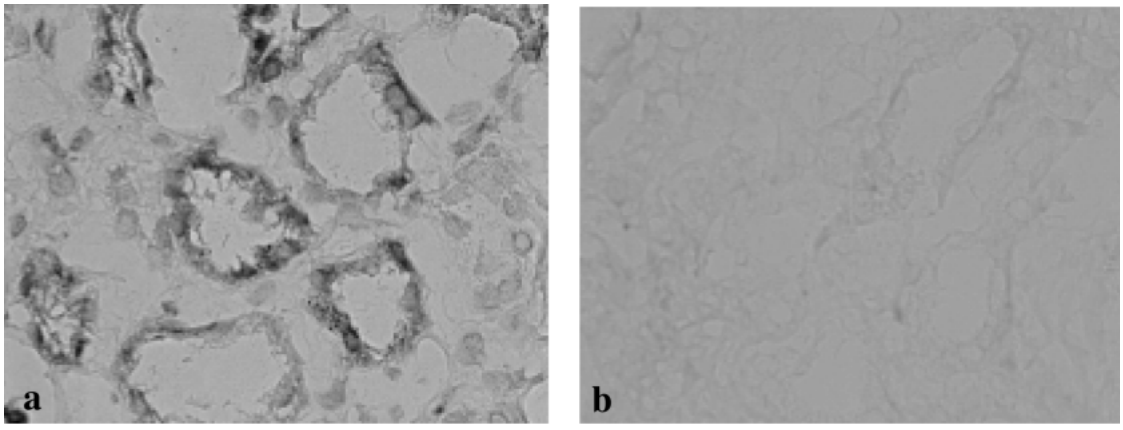

Fig. 10a. Epithelium of thick ascending limb of Henle showed positive staining with anti-CL-16 protein. Kidney of normal control animal No. 52, ABC stains, × 50. b. Epithelium of thick ascending limb of Henle no staining with anti-CL-16 protein. Kidneys of *CL-16*-defective animal No. 21, ABC stains, × 50.

Table 2. The profile of control animals

Animal No.	Age of (month)	Weight of body (kg)	Size of kidney (cm)		Weight of kidney (g)		BUN (mg/d*l*)	Cre (mg/d*l*)	Analysis of DNA	Diagnosis at necropsy
			Left	Right	Left	Right				
38	1	50	12×7×2	11×7×4	121	147	NE	NE	NE	Formation of false joint from coxofemoral luxation
39	4	91	10.5×6.5×4.5	11.5×6.5×4	168	162	14	NE	NE	Duplication of spinal cord
40	4	95	11×7×3	12×7×3	163	160	NE	NE	NE	Hypoplasia of pituitary gland, thyroid gland and atrophy of thymus
41	4	140	14×10×4	16×6×4	218	242	NE	NE	NE	Multiple lipoma
42	5	100	11×7×4	12×6×3	170	141	NE	NE	NE	Normal
43	8	NE	16×8×5	17×9×4	310	318	73	NE	NE	Severe atony of reticulum, omasum, and abomasum
44	10	218	12×7×4	14×8×2.5	184	225	NE	NE	NE	Normal
45	11	206	13×7×4.5	14×7×3	211	216	NE	NE	NE	Cattarhal enteritis of jejunum
46	12	224	14×9×4	15×9.5×3.5	250	260	NE	NE	NE	Normal
47	13	228	14×9×4	15×9.5×3.5	250	260	NE	NE	NE	Normal
48	13	237	14×6.8×5.2	15×8.5×4	242	268	NE	NE	NE	Normal (used for experiment)
49	22	NE	17×10.5×5	18×9×4	470	422	NE	NE	NE	Obstruction in duodenum
50	24	338	16.5×8×4.5	16×7.5×4.5	328	305	NE	NE	NE	Hemophiliac
51	31	415	12×6.5×3.5	17×7.5×4	166.6	193	NE	NE	NE	Heart failure and malnutrition
52	41	292	15×8×4	17×7.5×5	316	336	NE	NE	NE	Normal
53	49	312	17×8.3×5.5	17×7.5×6	402	404	NE	NE	NE	Enzootic bovine leukemia

tubule was noted in any of the control animals (aged 1–49 months).

Immunohistopathological findings: No cells showed positive to anti-CL-16 sera in the liver, spleen, heart, lung or intestine. The epithelial cells of the thick ascending limb of Henle from control cattles were positive to anti-CL-16 serum, but those of affected cattle were negative (Figs. 10a, b, Table 2). Glomeruli and the interstitium of all cattles were negative for staining.

DISCUSSION

Generally, renal hypoplasia is defined as a developmentally small but normally differentiated kidney [1]. In this study, kidney weights relative to body weight in affected animals were nearly same as those in normal animals. Therefore, the present cases were not "renal hypoplasia".

In 8 affected animals (Nos. 3, 5–7, 11, 19, 28, 32), the number of glomeruli was clearly less than that in normal kidneys of the same weight. Moreover, when the relationship between the number of glomeruli and age was examined in all animals, the number of glomeruli was markedly reduced in 35 animals. The degree of kidney growth differed between animals of the same age depending on the degree of development of the whole body. Body weights and kidney weights were not measured in many of the affected animals examined in this study, preventing the accurate comparison of these two parameters. However, it is most likely that the number of glomeruli was readily decreased also in the animals despite of no data available for body weights and kidney weights.

We speculated the following three features as the characteristics of renal lesions in *CL-16*-defective Japanese Black cattle: 1) Animals are born with various degree of maturation of glomeruli. 2) Kidney size is nearly normal in the mid cases, but is reduced in the progressed cases due to parenchymal atrophy accompanied by fibrosis, and 3) the immature nephrons are prone to be degenerated and destroyed while the mature glomeruli become compensatory hypertrophy. Hereditary renal disorders with an early onset have been reported in various species [3, 9, 11, 19]. However, as mentioned above, no disease that exhibits the same histological features as those observed in *CL-16* -defective animals have not reviewed, to our knowledge, in the literatures.

The results of immunohistopathological examination showed that the renal tubular epithelium of *CL-16*-defective cattle lacked CL-16 protein. This is consistent with the previous immunoblot study [5]. The CL family is comprised of more than 18 types of protein and CL-16 is shown in tight junctions (TJs) of the intercellular space between the epithelium of renal tubules [20, 21]. Kiuchi-Saishin *et al.* [7] reported that claudin-16 expresses in the thick ascending limb of Henle in mice. TJs are one of several cell junctions and CL family proteins play a role as a barrier and fence [2, 4, 6, 10, 20]. We speculated that the epithelial cell junction became loosened, resulting in leakage of the tubular fluid through the epithelium. This was thought to cause atrophy of renal tubules and inflammation of the interstitium.

Sasaki *et al.* [18] considered that the renal tubular dysplasia was the primary change, and reduced number of nephrons was secondary change. But we considered that the latter was also primary lesion. Because reduction in number of glomeruli and immature tubules was observed in all defective animals. Therefore this disease might be diagnosed as abnormal development of nephron but not solely of glomerli or tubules. Because degree of interstitial fibrosis and lymphocytic infiltration was varied in each case, these were considered as secondary chages. In future studies, close histological examination of the kidneys of *CL-16*-defective cattle at birth and at a stage in which no clinical renal dysfunction has as yet been observed may be needed to elucidate the pathogenesis of this disease.

A high percentage of *CL-16*-defective animals show growth retardation. Growth impairment is a non-specific symptom observed secondarily to renal failure and many other diseases, and its cause is difficult to identify. In the animals examined in this study, however, hypoplasia of pituitary gland and thyroid were observed frequently. These findings are often observed in bulls and cows [12] that show growth retardation due to causes other than a defect of *CL-16*. It could not be ruled out that, hypoplasia of pituitary gland and thyroid might play a critical role to cause growth impairment in the *CL-16* -defective animals, although more evidences should be accumulated in future to interpret the relationship between the two.

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