



# Immunolocalization of $\beta$ -catenin, E-cadherin and N-cadherin in neonate and adult rat kidney

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**ABSTRACT.**  $\beta$ -catenin, E-cadherin and N-cadherin are adhesion molecules that play important roles in organogenesis, tissue homeostasis, renal epithelial integrity and polarity. The present study demonstrated their immunolocalization in adult and neonate rat kidney. Membranous or cytoplasmic expression of  $\beta$ -catenin, E-cadherin and N-cadherin were seen in adult and developing renal tubular epithelial cells. Particularly, in adult kidney, E-cadherin and  $\beta$ -catenin were intensively expressed in distal renal tubules, whereas N-cadherin was expressed in proximal renal tubules. In neonate rat kidney on 1 and 4 days old, developing renal tubular epithelial cells were mainly reacted with E-cadherin and very weakly expressed N-cadherin;  $\beta$ -catenin was expressed in developing renal tubules and mesenchymal blastemal cells. Interestingly,  $\beta$ -catenin-positive renal tubular epithelial cells simultaneously expressed E-cadherin in the kidney of adult and developing rats. Collectively, the adhesion molecules were differentially distributed in the renal tubules of adult rats and  $\beta$ -catenin and E-cadherin are predominant adhesion molecules in developing kidney. The present findings would provide the basic information of evaluating renal tubular toxicity using rats, in addition to renal genesis, in terms of adhesion molecules.

**KEY WORDS:** adhesion molecule, immunohistochemistry, rat, renal tubule

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$\beta$ -catenin is a multi-functional protein involved in cell adhesion, cell signaling and regulation of gene transcription depending on its intracellular localization [6]. At the cell membrane,  $\beta$ -catenin participates in formation, maintenance and function of adherence junctions by linking cadherin to the actin cytoskeleton [6]. The classical cadherins such as E-cadherin and N-cadherin are  $\text{Ca}^{2+}$ -dependent cell adhesion molecules and play crucial roles in the renal epithelial cell integrity and polarity [4, 5]. In addition,  $\beta$ -catenin functions as a component of the wingless or Wnt nuclear signaling pathway and contributes to organogenesis, tissue homeostasis and tumorigenesis or disease development in kidney [3, 8], indicating the biphasic roles of  $\beta$ -catenin. Therefore, the cadherins and cadherin binding protein  $\beta$ -catenin appear to be the key players for maintaining cell-cell adhesion along the nephron, and may be involved in nephrotoxicity. Their essential roles in renal physiology and in the pathogenesis of renal dysplasia and renal injuries have been reported [7, 8], but the cellular distribution in renal tubules of neonates and adult rat kidney are still unclear. To clarify the distribution of  $\beta$ -catenin, N-cadherin and E-cadherin in developing and adult renal tubules, we used rat kidney. The rat is the most widely used laboratory animal, particularly for the renal physiology or renal pathology. The present findings would provide the basic information for evaluating renal toxicity which may be related to renal tubular injury and subsequent regeneration, in addition to fundamental features of adhesion molecules in renal tubules of developing and adult rat kidney

In adult kidney, generally, N-cadherin expression is restricted to the proximal tubule, whereas E-cadherin expression is found in the distal tubule and collecting ducts of pig and human kidney; however,  $\beta$ -catenin immune-expression is not reported in renal tubules [9, 11]. In adult rat and mouse kidneys, N-cadherin is expressed in the proximal renal tubules; E-cadherin is abundant in the distal renal tubules, and its expression is very low in the proximal renal tubules;  $\beta$ -catenin is expressed in both the proximal and distal tubules [7, 13, 14]. In embryonic kidney,  $\beta$ -catenin is expressed in ureteric epithelia and cortical and medullary renal stromal cells of mice, but its expression in developing renal tubules is not clear [1, 3, 10]. In contrast, N-cadherin first appears mainly in the developing proximal tubule, whereas E-cadherin is expressed in the developing distal tubules and collecting duct in human fetal kidney [11]. In newborn mice, E-cadherin is abundantly expressed in the proximal and distal tubules, although the distribution of  $\beta$ -catenin is not reported [4]. The distribution of these adhesion molecules in the developing renal tubules of rats

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has not been reported in detail. In the present study, we investigated the localization of  $\beta$ -catenin, N-cadherin and E-cadherin in neonate and adult rat kidney using immunohistochemistry and dual immunofluorescence, in order to establish the baseline data which is useful for renal pathological study.

## MATERIALS AND METHODS

### *Animals and experimental procedures*

Four adult F344/DuCrj male (117–140 g body weight; more than 6–7 weeks old) and eight pregnant F344/DuCrj female rats at 15-day gestation were obtained from Charles River Japan (Hino, Japan). They were housed in an animal room controlled at  $22 \pm 3^\circ\text{C}$  and with a 12 hr light-dark cycle, being allowed free access to a standard commercial diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. After one-week acclimatization, four adult rats were euthanized under deep isoflurane anesthesia and renal samples were collected. Pregnant F344/DuCrj rats gave birth and renal samples from neonatal rats (at least 3 neonate rats in each age group) were collected at aged 1, 4, 8, 12, 15 and 21 days. The animal experiments were conducted under the institutional guidelines approved by the ethical committee of Osaka Prefecture University for the Care and Use of Experimental Animals.

### *Histopathology and immunohistochemistry*

Renal tissues from the left and right kidneys were collected and immediately fixed in 10% neutral buffered formalin (NBF) and periodate-lysine-paraformaldehyde (PLP) solution processed by PLP-AMeX (acetone, methyl benzoate, and xylene). NBF-fixed tissues were dehydrated and embedded in paraffin and sectioned at  $3\text{--}4\ \mu\text{m}$  in thickness. The deparaffinized sections were stained with hematoxylin and eosin (HE) for morphological observations.

Tissue sections fixed in PLP were deparaffinized and used in immunohistochemistry with mouse monoclonal antibodies specific for  $\beta$ -catenin (clone CAT-5H10, isotype IgG<sub>1</sub>- $\kappa$ , 1:1,000, Invitrogen Co., Carlsbad, CA, U.S.A.), E-cadherin (clone 36/E-cadherin, isotype IgG2a, 1:100, BD Transduction Laboratories<sup>TM</sup>, San Jose, CA, U.S.A.), N-cadherin (clone 389, isotype IgG<sub>1</sub>- $\kappa$ , 1:100, Invitrogen Co.), Aquaporin 1 (clone 1/A5F6, isotype IgG<sub>1</sub>, 1:100, GeneTex, Irvine, CA, U.S.A.) and Vimentin (clone V9, isotype IgG<sub>1</sub>, 1:200, Dako Corp., Glostrup, Denmark). After antigen retrieval with Proteinase K (Dako Corp.) or citrate buffer, tissue sections were stained by the Histostainer (Histofine, Nichirei Bioscience Inc., Tokyo, Japan). Briefly, sections were incubated with 5% skimmed milk for 10 min, followed by 1 hr incubation with primary antibody. After treatment with 3% H<sub>2</sub>O<sub>2</sub> for 15 min, horseradish peroxidase-conjugated secondary antibody (Histofine simple stain MAX PO<sup>®</sup>; Nichirei Inc., Tokyo, Japan) was applied for 30 min. Positive reactions were visualized with 3, 3'-diaminobenzidine tetrahydrochloride (DAB substrate kit, Vector Laboratories, Burlingame, CA, U.S.A.) and the sections were lightly counter stained with hematoxylin. For negative controls, tissue sections were treated with mouse non-immunized serum instead of the primary antibody.

### *Double immunofluorescence*

Renal tissues from neonate and adult kidneys were collected and immediately immersed into mounting medium (TISSU MOUNT<sup>®</sup>, Chiba Medical, Soka, Japan) and frozen on deep freezer. Sections were cut at  $10\ \mu\text{m}$  in thickness on a cryostat, thaw mounted onto MAS-coated micro slide glass (Matsunami Glass Ind., Ltd., Osaka, Japan). Double immunofluorescence was carried out using E-cadherin in combination with  $\beta$ -catenin, N-cadherin, aquaporin 1 and vimentin. Briefly, after fixation in cold acetone: methanol (1:1) for 10 min at  $4^\circ\text{C}$ , the sections were incubated with 10% normal goat serum for 30 min. The sections reacted with the primary antibody overnight at  $4^\circ\text{C}$ . After rinsing with PBS, the sections were incubated for 45 min with the secondary antibodies: Alexa568/anti-mouse IgG1, Alexa488/anti-mouse IgG2a (Invitrogen Co.;  $\times 1,000$ ). The sections were visualized with Vectashield mounting medium containing 40, 6-diamidino-2-phenylindole (Vector Laboratories) for nuclear staining, and captured by a laser scanning confocal imaging microscope (Nikon C1Si) and processed with EZ-C1 3.20 Free Viewer (Nikon, Tokyo, Japan).

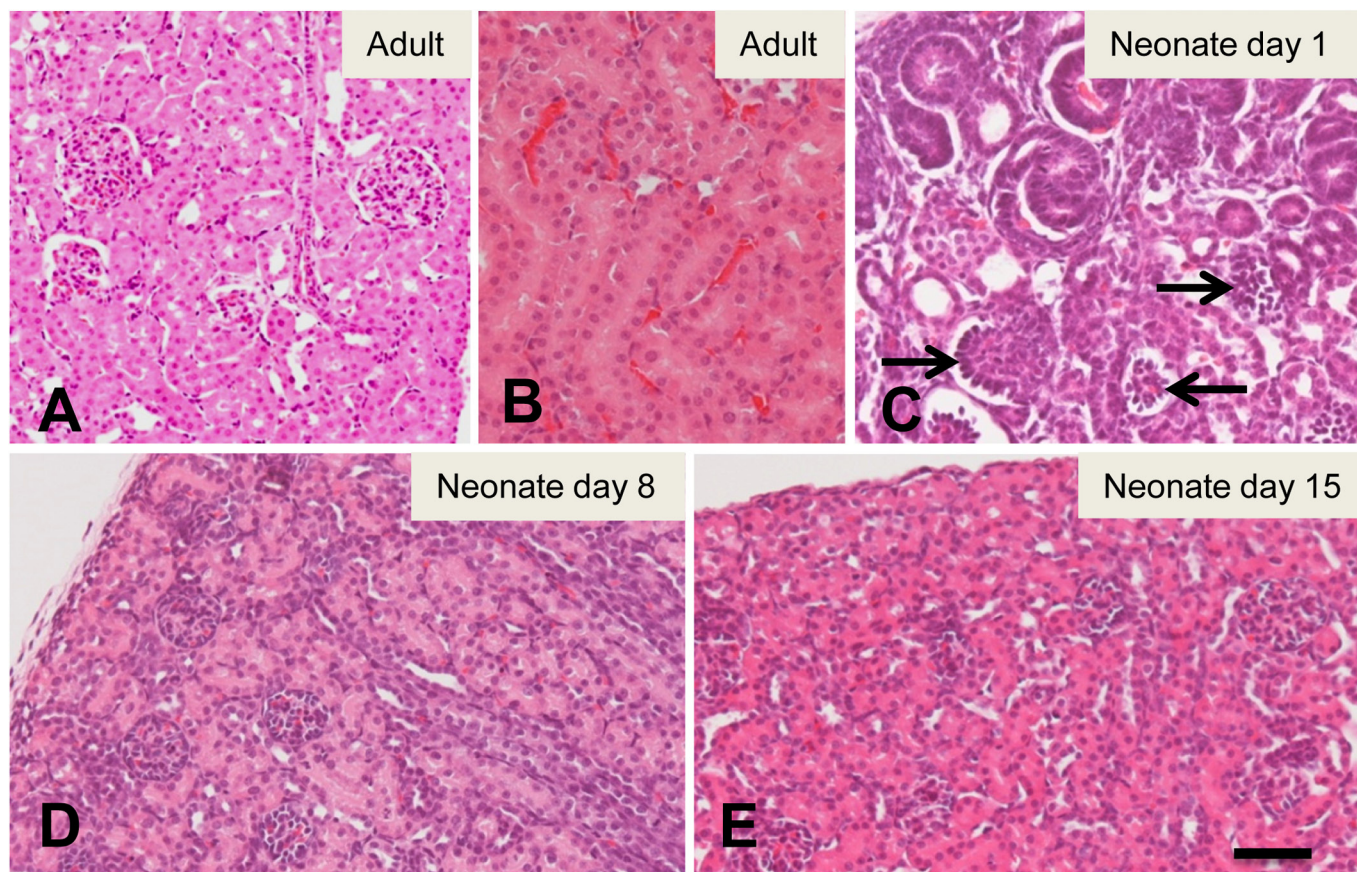
## RESULTS

### *Histology of adult and neonate rat kidney*

In adult kidney, the renal cortex comprises glomeruli, renal tubule segments and collecting tubules (Fig. 1A). The cortical stromal cells were seen in the tubulointerstitium. In cortico-medullary junction, the proximal renal tubules were predominant, and the distal renal tubules were occasionally present (Fig. 1B). Neonate kidney on days 1 and 4 demonstrated loosely-arranged blastemal cell-derived mesenchymal cells among developing renal tubules and glomeruli in the cortical areas (Fig. 1C). At neonate days 8 and 15, the mesenchymal cells were decreased and matured renal tubules and glomeruli become predominant (Fig. 1D and 1E). At neonate days 21, renal tissues were completely formed with similar histology to those of adult rat kidney. These histological observations indicated that nephrogenesis finished around 21 days of age in rats.

### *Immunohistochemical localization of $\beta$ -catenin, E-cadherin and N-cadherin in adult and neonate rat kidney*

In adult rat kidney, strong membranous and weak cytoplasmic localization of  $\beta$ -catenin was seen in distal renal tubular epithelial cells (Fig. 2A and 2E, insets). Localization of E-cadherin and N-cadherin were also seen in renal tubular epithelial cells (Fig. 2B, 2C and 2F). However, E-cadherin was intensively expressed in the distal renal tubular epithelial cells as membranous or cytoplasmic localization (Fig. 2B, inset), whereas N-cadherin expression was seen in the proximal renal tubular epithelial cells

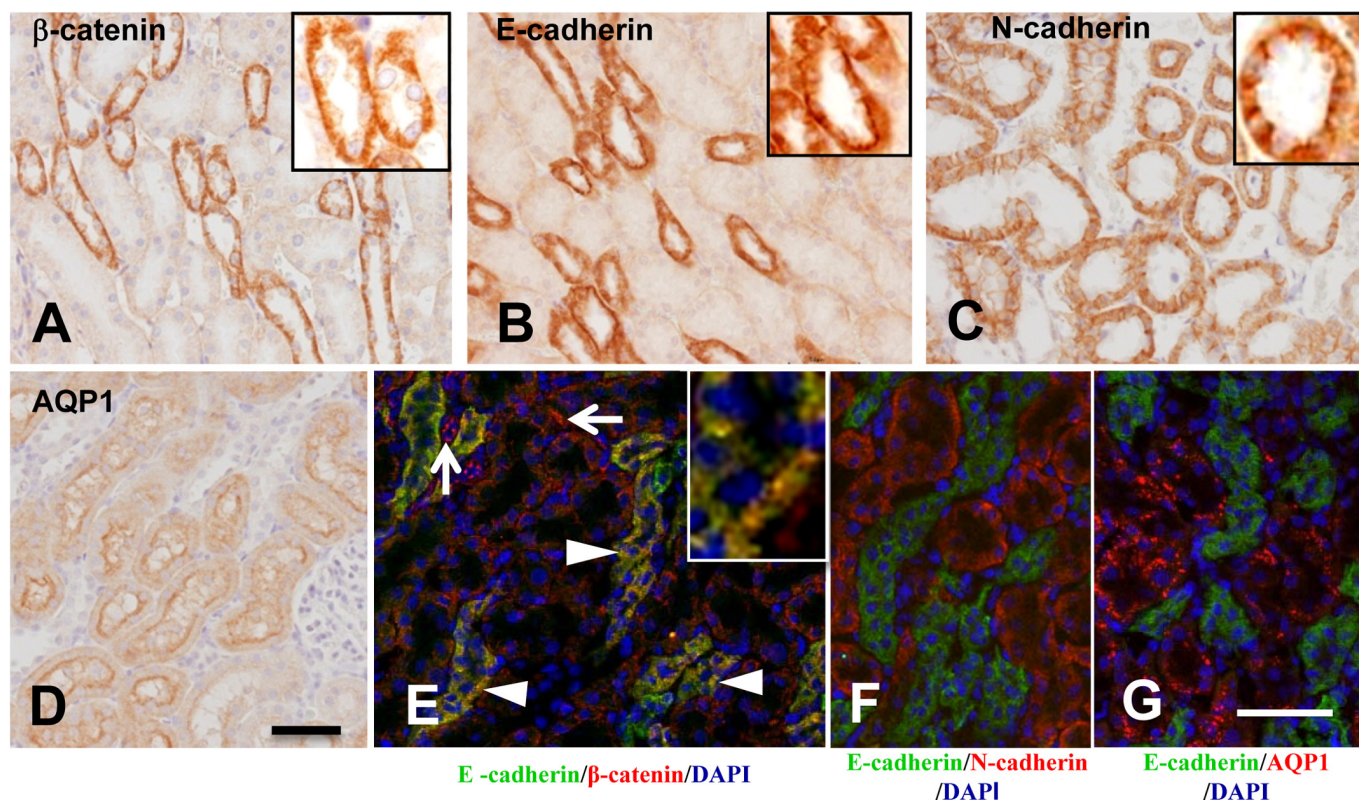


**Fig. 1.** Histology of adult (A and B) and neonates (C–E) rat kidney. A: In the adult cortical area, mature glomeruli and renal tubules are seen. B: In the cortico-medullary junction, the proximal renal tubules are predominant, and the distal renal tubules are occasionally present. C: In the cortical area on neonate day 1, loosely-arranged blastemal cell-derived mesenchymal cells are seen among developing renal tubules and glomeruli. The developing glomeruli, forming vesicles are indicated by arrows. D: Neonate day 8, the mesenchymal cells are decreased and developing renal tubules and glomeruli become predominant. E: Neonate day 15, developing renal tubules and glomeruli are surrounded by blastemal cell-derived mesenchymal cells. HE stain. Bar=50  $\mu$ m.

as lateral plasma membrane localization (Fig. 2C, inset). The proximal renal tubule marker protein, aquaporin 1 (AQP1), was expressed in epithelial cells of the proximal renal tubules (Fig. 2D). The localization of  $\beta$ -catenin, E-cadherin and N-cadherin were not detected in glomeruli or blood vessels of kidney. In the renal medulla, the tubular structures showed intense immunoreactivity for  $\beta$ -catenin and E-cadherin and were devoid of N-cadherin. In double immunofluorescence, almost all E-cadherin-expressing cells simultaneously expressed  $\beta$ -catenin in the distal renal tubules (Fig. 2E), whereas N-cadherin- or AQP1-expressing epithelial cells did not correspond to E-cadherin-expressing cells in the distal renal tubules (Fig. 2F and 2G). These findings indicated that  $\beta$ -catenin and E-cadherin are expressed exclusively in the distal renal tubules; the proximal renal tubules express N-cadherin and AQP1, but did not  $\beta$ -catenin.

In neonate kidney on 1 and 4 days, the developing renal tubules expressed  $\beta$ -catenin in varying degrees mainly as lateral plasma membrane localization (Fig. 3A and 3E, insets). Similar to  $\beta$ -catenin, E-cadherin expression was seen in developing renal tubules on days 1 and 4 (Fig. 3B and 3E–G, insets).  $\beta$ -catenin and E-cadherin were not detected in developing glomeruli (Fig. 3A and 3B). Mesenchymal blastemal cells surrounding the developing renal tubules expressed  $\beta$ -catenin (Fig. 3A and 3E, arrows). Additionally, N-cadherin was expressed in renal epithelia of some parts of developing tubules as lateral plasma membrane localization (Fig. 3C and 3F, insets); however, the reaction for N-cadherin was weaker than that of E-cadherin or  $\beta$ -catenin. Double immunofluorescence showed that many double positive cells for  $\beta$ -catenin and E-cadherin were seen in developing renal tubules (Fig. 3E and F, inset), indicating that immature, developing renal tubules in nephrogenesis can express simultaneously  $\beta$ -catenin and E-cadherin. In neonates on day 21, the distribution of  $\beta$ -catenin, E-cadherin and N-cadherin corresponded to that in adult rat kidneys. These findings showed that the immature, developing renal tubules expressed mainly  $\beta$ -catenin and E-cadherin and weakly N-cadherin, and that as renal tubules developed, the expression of  $\beta$ -catenin, E-cadherin and N-cadherin became similar to that seen in adult rat kidney.

In addition, the developing renal tubules in neonates on days 1, 4, 8 and 12 did not express vimentin, whereas interstitial spindle-shaped cells (blastemal cell-derived mesenchymal cells) reacted to vimentin (Fig. 3D). These findings were confirmed in dual immunofluorescence with vimentin and E-cadherin (Fig. 3G).

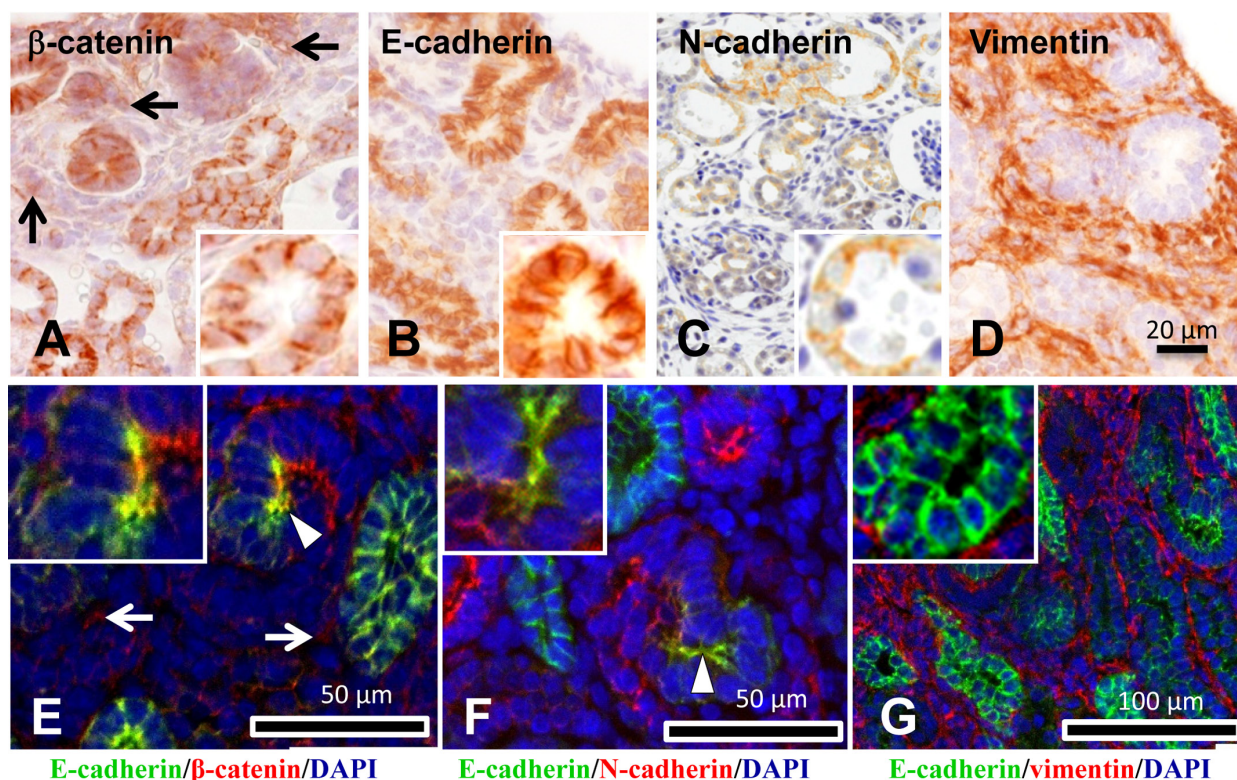


**Fig. 2.** A–D: Immunohistochemistry in cortico-medullary junction of adult kidney. Membranous or cytoplasmic localization of  $\beta$ -catenin (A) and E-cadherin (B) are observed in distal renal tubular epithelial cells (inset), but not in the proximal renal tubules. N-cadherin (C) is localized as a lateral membrane in the proximal renal tubules (inset). AQP1 (Aquaporin 1) (D) is expressed in epithelial cells of the proximal renal tubules. E–G: Double immunofluorescence in cortico-medullary junction of adult kidney. E:  $\beta$ -catenin (red) and E-cadherin (green) both are co-expressed in the distal renal tubules (arrowheads). Inset shows yellow color double positive reaction in renal epithelial cells of higher magnification. Cortical stromal cells show positive reaction of  $\beta$ -catenin (arrows). N-cadherin (F, red)- or AQP1 (G, red)-expressing epithelial cells are not corresponding to E-cadherin (green)-expressing cells in the distal renal tubules. Bar=50  $\mu$ m.

## DISCUSSION

The present study illustrates the distribution of  $\beta$ -catenin, E-cadherin and N-cadherin expression in renal tubules of adult and neonate rat kidney. These adhesion molecules were detected in plasma membrane or cytoplasm of developing and mature renal tubular epithelial cells which is consistent with their known role in establishing cell-to-cell contact as well as cell signal transduction in response to extracellular stimuli [6, 9]. In adult rat kidney,  $\beta$ -catenin was expressed in epithelial cells of the distal tubules, in which co-expression of E-cadherin was confirmed in dual immunofluorescence, indicating that  $\beta$ -catenin binds to E-cadherin in the epithelial cells as an adhesion molecule [16]. A similar expression pattern of  $\beta$ -catenin has also been reported in the adult mouse kidneys [7]. However, together with present study, N-cadherin and E-cadherin are expressed in proximal or distal tubules respectively in rats, pigs and humans [9, 11, 13], whereas E-cadherin and N-cadherin are detected in both proximal and distal tubules of rabbit kidney [15], indicative of species specificity in adult kidney.

The developing kidney, the metanephric development begins with the mutual induction between mesenchymal cells of the metanephric blastema and epithelial cells of the ureteric bud. In nephrogenesis, tubular formation begins from the S-shaped body. The lower portion of the S-shaped structure differentiates into the renal corpuscle, and the remaining portion forms the proximal and distal tubules [2].  $\beta$ -catenin is expressed in the uteric epithelial cells and plays crucial roles in branching morphogenesis and in the differentiation of ureteric epithelial cells [3, 10]. Similar with present findings,  $\beta$ -catenin is expressed in developing renal tubules in mouse neonates on days 1–8, indicating that  $\beta$ -catenin might have important roles in tubulogenesis [12].  $\beta$ -catenin is also expressed in cortical stromal cells (mesenchymal blastemal cells) associated with developing renal tubules of mice and rats [1], suggesting participation in nephron differentiation. In contrast, N-cadherin was first detected in the lower limb of S-shaped bodies and the developing proximal tubule, whereas E-cadherin is expressed in the collecting duct, the upper limb of S-shaped bodies and the developing distal tubules in human fetal kidney [11]. In the present study, it was found that the developing epithelial cells in renal tubules in neonates, which do not still differentiate into the proximal and distal renal tubules, reacted to  $\beta$ -catenin and E-cadherin in varying degrees. In some parts of these developing tubules, there were N-cadherin-positive epithelial cells (indicative of differentiation toward the proximal renal tubules). On the basis of these findings, it was considered that  $\beta$ -catenin expression



**Fig. 3.** A–D: Immunohistochemistry in renal cortex of neonate kidney on day 1. Expression of  $\beta$ -catenin (A) and E-cadherin (B) are observed in the developing renal tubular epithelial cells (inset). Few blastemal cells react to  $\beta$ -catenin among developing renal tubules (arrows). N-cadherin (C) is weakly expressed in some parts of developing tubules. Inset shows positive reaction in renal epithelial cells of higher magnification. D: The developing renal tubules do not express vimentin, but blastemal cell-derived mesenchymal cells strongly express vimentin. E–G: Double immunofluorescence in renal cortex of neonate kidney on day 1.  $\beta$ -catenin (E, red)- or N-cadherin (F, red)-expressing epithelial cells are corresponding to some E-cadherin (green)-expressing cells in the developing renal tubules (arrowhead, inset). Yellow color indicates double positive reaction. Some blastemal cells show positive reaction with  $\beta$ -catenin among developing renal tubules (arrows). G: Vimentin (red)-expressing blastemal cells are not corresponding to E-cadherin (green)-expressing cells. Inset shows E-cadherin localization in renal epithelial cells of higher magnification.

in rat kidneys was observed in developing renal tubules in nephrogenesis and the distal renal tubules in adult rats. Additionally, N-cadherin and E-cadherin are specifically expressed in the proximal and distal renal tubules, respectively, indicating that the N-cadherin and E-cadherin immunohistochemistry would be useful for the detection of different renal tubules such as the proximal and distal renal tubules in rats [13].

In conclusion,  $\beta$ -catenin and E-cadherin are prominent adhesion molecules in developing kidney of neonate rats. The differential expression of N-cadherin, E-cadherin and  $\beta$ -catenin in mature rat renal tubules indicates the differences in the susceptibility of nephron segments to renal pathology. Therefore, the present finding would provide a significant insight into the normal kidney development and renal pathology.

**CONFLICT OF INTEREST.** The authors declare no conflict of interest.

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