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(Pro)renin and (pro)renin receptor expression during kidney development in neonates

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Abstract:	Although a recent study demonstrated that the (pro)renin receptor ((P)RR) was highly expressed in the developing kidney during the mouse embryonic development, the mechanism by which (P)RR supports renal development in humans is not fully understood. In this study, we examined the plasma levels of (pro)renin and soluble (P)RR (s(P)RR) in cord blood and neonates as well as (P)RR expression in human kidney tissues. Samples were collected from 57 preterm and 67 full-term human neonates. (Pro)renin and s(P)RR levels were measured using enzyme-linked immunosorbent assays. Additionally, we performed an immunohistochemical (IHC) analysis of kidney tissues from neonates and minor glomerular abnormalities in order to assess (P)RR expression in the kidney. Plasma (pro)renin and s(P)RR levels in cord blood were significantly higher in preterm neonates than in full-term neonates. Four weeks after birth, these differences were no longer evident for either plasma (pro)renin or s(P)RR levels between the two groups. Importantly, plasma (pro)renin and s(P)RR levels in cord blood were inversely correlated with gestational age. Furthermore, IHC indicated that renal expression levels of (P)RR in neonates was stronger than that in minor glomerular abnormalities. Conclusion: (P)RR may play a pivotal role in prenatal renal development in humans.

Paper Title

(Pro)renin and (pro)renin receptor expression during kidney development in neonates

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Short Title

Soluble (pro)renin receptor in neonates

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List of Key words

Renin-angiotensin; gestational age; cord blood; enzyme-linked immunosorbent assay; immunohistochemistry

What is Known

- Renal renin-angiotensin system (RAS) has several pathophysiologic functions not only in blood pressure regulation but also in pediatric renal disease.
- Renal RAS activation plays a key role of renal development during gestation.

What is New

- Plasma (pro)renin and soluble (pro)renin receptor (s(P)RR) levels in cord blood were significantly higher in preterm neonates than in full-term neonates.
- Plasma (pro)renin and s(P)RR levels in cord blood were inversely correlated with gestational age.
- Immunohistochemical analysis of kidney tissue indicated that renal expression levels of (P)RR in neonates was stronger than in minor glomerular abnormalities.
- (P)RR may play a pivotal role in prenatal renal development in humans.

Abstract

Although a recent study demonstrated that the (pro)renin receptor ((P)RR) was highly expressed in the developing kidney during the mouse embryonic development, the mechanism by which (P)RR supports renal development in humans is not fully understood. In this study, we examined the plasma levels of (pro)renin and soluble (P)RR (s(P)RR) in cord blood and neonates as well as (P)RR expression in human kidney tissues. Samples were collected from 57 preterm and 67 full-term human neonates. (Pro)renin and s(P)RR levels were measured using enzyme-linked immunosorbent assays. Additionally, we performed an immunohistochemical (IHC) analysis of kidney tissues from neonates and minor glomerular abnormalities in order to assess (P)RR expression in the kidney. Plasma (pro)renin and s(P)RR levels in cord blood were significantly higher in preterm neonates than in full-term neonates. Four weeks after birth, these differences were no longer evident for either plasma (pro)renin or s(P)RR levels between the two groups. Importantly, plasma (pro)renin and s(P)RR levels in cord blood were inversely correlated with gestational age. Furthermore, IHC indicated that renal expression levels of (P)RR in neonates was stronger than that in minor glomerular abnormalities.

Conclusion: (P)RR may play a pivotal role in prenatal renal development in humans.

Lists of Abbreviations

IHC	Immunohistochemical
(P)RR	(Pro)renin receptor
RAS	Renin-angiotensin system

Introduction

The role of the renin-angiotensin system (RAS) in the regulation of blood pressure regulation and sodium and fluid homeostasis is well recognized. Recently, the focus of interest in the RAS has shifted toward the role of the local/tissue RAS in specific tissues [14, 30]. Furthermore, recent studies have shown that the RAS plays a role in the development of the mammalian kidney [9, 28]. A large number of studies addressed the importance of an intact RAS cascade during kidney development using both pharmacological inhibition and genetic deletion of various RAS components. The contribution of RAS in kidney development is fully understood, but the temporal and spatial expression of different components of the system suggests a direct action on receptors expressed in the developing structures of the immature kidney [9]. Renin is an aspartyl protease that cleaves angiotensinogen into angiotensin I, the rate-limiting reaction in the cascade generating angiotensin [15]. The existence of a receptor for renin and for its inactive precursor, (pro)renin, was postulated, and a receptor binding renin and (pro)renin, termed the (pro)renin receptor ((P)RR), was cloned in 2002 [15]. (P)RR-bound renin and (pro)renin not only exert enzymatic action, but also induce angiotensin II-independent intracellular signaling [7]. (P)RR has shown its multi-functionality in at least four different aspects [18]. One of these is to enhance angiotensin I production from angiotensinogen by non-proteolytically increasing catalyzing activity of renin or (pro)renin when bound to (P)RR, resulting in enhanced RAS. Another is to induce the MAPK singal transduction pathway when (P)RR is bound to its ligand renin or (pro)renin. Another role is on ATPases in podocytes and elsewhere, and (P)RR is also involved in diabetes [18].

(P)RR mRNA and protein are detected in the whole mouse metanephros on E12.5 [27]. Spatially, (P)RR immunoreactivity is present in the uretic bud epithelia and nascent nephrons on E13.5 [25]. On E16.5 and E18.5, (P)RR immunostaining is mostly detected in the tubules, which morphologically resemble collecting ducts followed by the glomerular mesangium [27].

The kidney (P)RR protein levels are high throughout mouse gestation and decline gradually during the postnatal development [27]. A truncated form that is cleaved by furin, referred to as soluble (P)RR (s(P)RR), is secreted into the extracellular space [12]. An accurate measurement of s(P)RR levels *in vivo* is an important issue in elucidating the roles of (P)RR in physiology and pathophysiology [12]. These data prompted us to measure plasma level of (pro)renin and s(P)RR in cord blood of neonates. In addition, we investigated the expression of (P)RR in neonatal kidney tissues. This study was performed to test the hypothesis that (P)RR regulates kidney development in humans.

Material and Methods

Patients and samples

The study's experimental protocol was approved by the Institutional Review Board of Tokushima University. Study participants were recruited in Tokushima University Hospital between April 1, 2013 and March 31, 2014. Informed consent was obtained from the parents. Neonates were excluded if they had known or suspected sepsis, severe respiratory distress syndrome, congenital heart disease, or a renal or chromosomal abnormality. Neonates expected to die within 48 h of recruitment were also excluded. Demographic-perinatal characteristics, including gestational age (GA), birth weight, sex, and Apgar scores at 1 and 5 min were recorded for all neonates. Cord blood samples were obtained from the umbilical vein at delivery. A blood sample was obtained in the few days and at 28 days following birth. The samples were stored at -20°C until biochemical analysis. Tissue samples were obtained at the time of autopsy of newborns that died of pulmonary hypoplasia. We also recruited 6 participants, one to seven years old of age, with minor glomerular abnormalities that showed normal glomerular morphology and negative immunofluorescence, but had mild proteinuria or microscopic hematuria. The use of the tissue samples was approved by the ethical

committees of the Institutional Review Board of Tokushima University.

Measurements

Plasma concentrations of s(P)RR were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (IBL, Takahashi, Gunma, Japan). The soluble form of the (P)RR generated by intracellular cleavage by furin is secreted in the plasma [15]. This ELISA kit allows the determination of s(P)RR concentrations in the blood. Plasma concentrations of (pro)renin were measured using commercially available kits (Innovative Research, Inc,, Novi, MI, USA).

Immunohistochemistry

The tissues obtained at the time of autopsy were fixed in 10 % buffered formalin and embedded in paraffin. The paraffin sections (3-μm thickness) were incubated with an anti-(P)RR antibody (ab40790; Abcam, Cambridge, MA, USA) or without the primary antibody overnight at 4°C, rinsed, and incubated with the biotinylated secondary antibody (Vector Labs, Burlingame, CA, USA). After rinsing, the sections were incubated with the avidin-biotin-peroxidase complex (ABC Elite; Vector Labs), followed by 3,3′-diaminobenzidine (Dojindo, Kumamoto, Japan). Each section was counterstained with Mayer's hematoxylin (Wako, Tokyo, Japan), dehydrated, and cover-slipped. Quantification was performed using the EIS-Elements software (Nikon Corporation, Tokyo, Japan). The immunoreactive area (brown) was assessed by setting a threshold and automatically calculated in arbitrary unit.

Statistical analysis

All data are presented as mean ± standard error of mean (SEM). Significant differences were

determined by using unpaired t test for normally distributed variables, whereas the Mann-Whitney U-test was used for nonparametric test. Pearson's correlation coefficients and Spearman's correlation coefficients were used for parametric data and non-parametric data, respectively. The standard least-squared method was used for multiple regression analysis. A p value < 0.05 was considered statistically significant. All computations, including data management and statistical analyses, were performed using JMP software (SAS Institute, Candler, NC, USA) and GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Subject profiles

The profiles of the study participants are summarized in Table 1. One hundred twenty four neonates, comprising 57 preterm and 67 full-term neonates, were recruited. The averages GAs of preterm and full-term neonates were 32.58 ± 0.52 and 37.99 ± 0.10 weeks, respectively. Preterm and full-term neonates weighed $1{,}750.18 \pm 94.06$ and $2{,}722.72 \pm 58.92$ g, respectively. There were no significant differences between preterm and full-term neonates in term of gender and Apgar score at 5min. The average Apgar score at 1 min was significantly lower in preterm neonates than in full-term neonates.

Plasma (pro)renin and s(P)RR levels in preterm neonates

The plasma (pro)renin and s(P)RR levels in cord blood were significantly increased in preterm neonates compared with those in full-term neonates. While there was no significant change in plasma s(P)RR levels at day 4 after birth between preterm and full-term neonates, the plasma (pro)renin levels were significantly higher in preterm neonates than in full-term neonates. There was no significant change in plasma (pro)renin and (P)RR levels at 28 days

after birth (Table 2).

Single regression analysis

The plasma (pro)renin and s(P)RR levels in cord blood and at day 4 after birth were significantly and inversely correlated with gestational age (Figure 1 and Table 3). However, 28 days after birth, the plasma (pro)renin and s(P)RR levels were not correlated with gestational age (Table 3).

Multiple regression analysis

Multiple regression analysis using the stepwise method was utilized to determine the relationship between the plasma (pro)renin or s(P)RR levels in cord blood and other variables. The plasma (pro)renin or s(P)RR levels in cord blood significantly correlated with gestational age (Table 4).

Renal expression levels of (P)RR in neonates

We next examined (P)RR expression in kidney tissues from neonates and from children presenting minor glomerular abnormalities. (P)RR mainly localized in the glomeruli, proximal tubules, collecting ducts, and arteries in neonates. On the other hand, positive, but weak, (P)RR staining was observed in those in minor glomerular abnormalities. Renal (P)RR expression was significantly increased in neonates compared to that in minor glomerular abnormalities (Figure 2A and B). In addition, the levels of (P)RR expression in neonate kidneys were significantly and inversely correlated with gestational age (Figure 2C).

Discussion

We compared plasma (pro)renin and s(P)RR levels between preterm and full-term neonates.

Plasma (pro)renin and s(P)RR levels in cord blood were significantly higher in preterm neonates compared with those in full-term neonates. Notably, plasma (pro)renin and s(P)RR levels in cord blood were inversely correlated with the gestational age. Furthermore, (P)RR expression in the kidney was significantly increased in neonates when compared to children. These data suggest that (pro)renin and (P)RR might be essential for embryogenesis and kidney development.

In human embryos, all components of the RAS are expressed in the kidney as early as 5 weeks of gestation [5, 23]. Angiotensinogen was detected in the proximal tubules, and renin was expressed in capillaries within glomeruli as well as in the wall of arteries in the interstitium and in arterioles up to the aorta in the mesonephros and become confined to the juxtaglomerular apparatus at the vascular pole of the glomerulus in the metanephros [23]. Angiotensin converting enzyme (ACE) was detected in the apical membrane of the mesonephric tubule cells and glomerular endothelial cells, and angiotensin II type 1 receptor was observed in the glomeruli and proximal tubular epithelium [23] [13]. Their production is precisely time-regulated, suggesting that angiotensin II could also exert its effects as a growth-promoting agent during kidney development [29]. Additionally, levels of circulating renin and angiotensin II are higher during fetal life than during postnatal life [5]. During the gestation, the RAS of the fetal lamb responds to the same stimuli such as blood volume depletion, furosemide, hypoxemia, and RAS blockade [3, 19]. Similarly, human fetuses exposed *in utero* to RAS blockers are severely hypotensive at birth and, sometimes, develop irreversible renal lesions in response to renal failure and anuria [11, 20]. On the other hand, inappropriate activation of the RAS during fetal life may have deleterious consequences [10]. Thus, RAS plays an important role in kidney development.

During adult life, (P)RR is abundantly expressed in the kidney, heart, and brain [27]. Recent studies indicate that (P)RR plays an important role in organogenesis and development

[27]. Global (P)RR knockout is lethal in mice, indicating an essential role of the (P)RR during the embryonic development [24]. Cardiomyocyte-specific ablation of the (P)RR in mice results in early mortality due to heart failure [8]. Studies using zebrafish demonstrated that (P)RR mutations result in brain malformations and early embryonic lethality [1]. In human studies, (P)RR mutations are associated with a high blood pressure, left ventricular hypertrophy, and X-linked mental retardation [6] [21]. Furthermore, levels of s(P)RR are increased in pregnant women and in blood cord of neonates [31] [32]. Consistent with this concept, the plasma (pro)renin and s(P)RR levels in cord blood are higher than in preterm neonates when compared to those in full-term neonates, and were inversely correlated with gestational age in the present study. On the other hand, plasma (pro)renin and s(P)RR levels did not differ between preterm and full-term neonates 28 days after birth. Therefore, (P)RR may play an important role in embryonic and fetal development.

Recently, evidence from multiple studies indicated that (P)RR is critical for normal kidney development and function [26]. In the mouse kidney, (P)RR mRNA and protein expression is detected from E12.5 [25]. (P)RR mRNA is expressed in the intact uretic bud isolated from E11.5 wild-type mouse kidneys [27]. (P)RR immunostaining is present in the uretic bud and the cap mesenchyme on E13.5 [27]. Furthermore, it has been shown that (P)RR is localized in glomerular mesangial cells, the subendothelium of renal arteries, podocytes, and distal nephron cells in the human and rat normal and diseased kidney [4] [16]. Indeed, in this study, we demonstrated that renal (P)RR expression in the glomeruli, proximal tubules, collecting ducts and arteries are enhanced in neonates. Additionally, an inverse co-relation between the limited measures of (P)RR expression levels in neonates kidney that we could obtain and gestational age was found, even though the number was small. Previous studies showed that renin was expressed in capillaries within the glomeruli as well as in the wall of arteries in the interstitium and in arterioles up to the aorta and become confined to the

juxtaglomerular apparatus at the vascular pole of the glomerulus in the metanephros in embryos [2] [23]. These findings suggest that (P)RR, at least in part, co-localizes with renin in embryonic kidneys and (P)RR-bound renin may induce kidney development. Targeted genetic inactivation of the (P)RR in the podocytes in mice causes podocyte foot process retraction, nephrotic syndrome, and death from renal failure during early postnatal life [17] [22]. (P)RR maintains nephron progenitors and promotes the differentiation of nascent nephrons by regulating the expression of key genes critical for both populations [26]. These findings, along with the findings reported here, reveal the physiological significance of (P)RR, regulating nephron development during gestation.

The relatively small sample size in this study is a potential limitation. Furthermore, the study was cross-sectional and, therefore, it might be difficult to draw any causal conclusions. However, our observations indicate that the plasma (pro)renin and (P)RR levels in cord blood are increased in preterm neonates compared with those in full-term neonates, accompanied by enhanced expression of renal (P)RR in neonates. These data strongly support the hypothesis that (P)RR plays an important role in nephrogenesis. Furthermore, measuring (pro)renin and (P)RR levels in neonates might become a useful tool to evaluate kidney development. This pilot study provides new insights into the understanding of human kidney development that requires further prospective analyses in large multicenter studies.

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Author's contribution

TT performed literature search, data collection, data analysis, data interpretation and wrote the first draft of the manuscript. MU designed the study, analyzed the data, wrote the manuscript and submitted. TS and RN were member of medical team involved in therapeutic process and reviewed the manuscript. SK contributed to the conception, design of the study and critical review of the manuscript.

Compliance with ethical standard

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and tis later amendments or comparable ethical standards.

Conflict of Interest

The authors declare that they have no conflict of interest.

Informed consent

Informed consent was obtained from the parents including in the study.

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Figure Legends

Figure 1. Single regression analyses for plasma (pro)renin (A) and (pro)renin receptor ((P)RR) levels (B) in cord blood. The plasma (pro)renin and (P)RR levels in cord blood were inversely correlated with gestational age.

Figure 2. Renal tissue (pro)renin receptor ((P)RR) immunoreactivity in neonates and minor glomerular abnormalities. (A) Representative images of (P)RR immunostaining in 33-week gestation neonates (a), 35-week gestation (b), 3 years old (c) and 7-year-old with minor glomerular abnormalities (d), and negative control (e). Original magnification x400. (B) (P)RR levels in renal tissues are expressed in arbitrary units (AU). (C) Single regression analysis of (P)RR expression levels in neonate renal in function of gestational age.

Table 1. Subject profiles

Parameters	Preterm N = 57	Full-term N = 67	P values	$\chi 2$
Gestational age, weeks	32.58 +/- 0.52 **	37.99 +/- 0.10	< 0.0001	
Birth weight, g	1750.18 +/- 94.06 **	2722.72 +/- 58.92	< 0.0001	
Gender, F/M	26/31	34/33	0.5687	0.325
Apgar score, 1 min	6.11 +/- 0.38 **	7.97 +/- 0.17	< 0.0001	
Apgar score, 5 min	8.96 +/- 0.19	9.08 +/- 0.09	0.5733	

F; Females, M; Males, *; $P < 0.05$, **; $P < 0.01$ vs. full-term.

Table 2. (Pro)renin and s(P)RR

	(Pro)renin (ng/mL)			s(P)RR (ng/mL)		
	Preterm N = 57	Full-term N = 67	P values	Preterm N = 57	Full-term N = 67	P values
Cord blood	4.13 +/- 0.38**	2.02 +/- 0.15	< 0.0001	91.36 +/- 5.14 **	75.90 +/- 3.23	0.0097
Day 4	6.00 +/- 0.84**	2.13 +/- 0.25	< 0.0001	74.61 +/- 3.19	71.15 +/- 2.92	0.4274
Day 28	3.61 +/- 0.82	2.04 +/- 1.41	0.5631	83.80+/- 3.49	79.15 +/- 7.09	0.5188

**; P < 0.01 vs. full-term.

Table 3. Gestational age and correlation

	(Pro)renin		s(P)RR receptor	
	R value	P values	R value	P values
Cord blood	-0.5598	< 0.0001 **	-0.2241	0.0123 *
Day 4	-0.4904	< 0.0001 **	-0.2558	0.0155 *
Day 28	0.2859	0.1971	-0.3353	0.1013

*; P < 0.05, **; P < 0.01

Table 4. Multiple regression analysis of (pro)renin and s(P)RR in Cord Blood

(Pro)renin

Parameters	Estimate	SE	t	P values
Intercept	21.57	6.94	3.11	0.0061 **
(Pro)renin Day 28	-0.21	0.17	-1.18	0.2523
s(P)RR Cord Blood	-0.02	0.01	-1.24	0.2293
Gestational Age	-0.44	0.20	-2.22	0.0394 *

s(P)RR

Parameters	Estimate	SE	t	P values
Intercept	510.25	144.29	3.54	0.0020 **
(Pro)renin cord blood	-3.89	2.85	-1.36	0.1874
s(P)RR Day 28	-1.00	0.65	-1.53	0.1404
Gestational Age	-9.35	3.02	-3.10	0.0054 *

*; $P < 0.05$, **; $P < 0.01$

Paper Title

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Short Title

Soluble (pro)renin receptor in neonates

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List of Key words

Renin-angiotensin; gestational age; cord blood; enzyme-linked immunosorbent assay; immunohistochemistry

What is Known

- Renal renin-angiotensin system (RAS) has several pathophysiologic functions not only in blood pressure regulation but also in pediatric renal disease.
- Renal RAS activation plays a key role of renal development during gestation.

What is New

- Plasma (pro)renin and soluble (pro)renin receptor (s(P)RR) levels in cord blood were significantly higher in preterm neonates than in full-term neonates.
- Plasma (pro)renin and s(P)RR levels in cord blood were inversely correlated with gestational age.
- Immunohistochemical analysis of kidney tissue indicated that renal expression levels of (P)RR in neonates was stronger than in minor glomerular abnormalities.
- (P)RR may play a pivotal role in prenatal renal development in humans.

Abstract

Although a recent study demonstrated that the (pro)renin receptor ((P)RR) was highly expressed in the developing kidney during the mouse embryonic development, the mechanism by which (P)RR supports renal development in humans is not fully understood. In this study, we examined the plasma levels of (pro)renin and soluble (P)RR (s(P)RR) in cord blood and neonates as well as (P)RR expression in human kidney tissues. Samples were collected from 57 preterm and 67 full-term human neonates. (Pro)renin and s(P)RR levels were measured using enzyme-linked immunosorbent assays. Additionally, we performed an immunohistochemical (IHC) analysis of kidney tissues from neonates and minor glomerular abnormalities in order to assess (P)RR expression in the kidney. Plasma (pro)renin and s(P)RR levels in cord blood were significantly higher in preterm neonates than in full-term neonates. Four weeks after birth, these differences were no longer evident for either plasma (pro)renin or s(P)RR levels between the two groups. Importantly, plasma (pro)renin and s(P)RR levels in cord blood were inversely correlated with gestational age. Furthermore, IHC indicated that renal expression levels of (P)RR in neonates was stronger than that in minor glomerular abnormalities.

Conclusion: (P)RR may play a pivotal role in prenatal renal development in humans.

Lists of Abbreviations

IHC	Immunohistochemical
(P)RR	(Pro)renin receptor
RAS	Renin-angiotensin system

Introduction

The role of the renin-angiotensin system (RAS) in the regulation of blood pressure regulation and sodium and fluid homeostasis is well recognized. Recently, the focus of interest in the RAS has shifted toward the role of the local/tissue RAS in specific tissues [14, 30]. Furthermore, recent studies have shown that the RAS plays a role in the development of the mammalian kidney [9, 28]. A large number of studies addressed the importance of an intact RAS cascade during kidney development using both pharmacological inhibition and genetic deletion of various RAS components. The contribution of RAS in kidney development is fully understood, but the temporal and spatial expression of different components of the system suggests a direct action on receptors expressed in the developing structures of the immature kidney [9]. Renin is an aspartyl protease that cleaves angiotensinogen into angiotensin I, the rate-limiting reaction in the cascade generating angiotensin [15]. The existence of a receptor for renin and for its inactive precursor, (pro)renin, was postulated, and a receptor binding renin and (pro)renin, termed the (pro)renin receptor ((P)RR), was cloned in 2002 [15]. (P)RR-bound renin and (pro)renin not only exert enzymatic action, but also induce angiotensin II-independent intracellular signaling [7]. (P)RR has shown its multi-functionality in at least four different aspects [18]. One of these is to enhance angiotensin I production from angiotensinogen by non-proteolytically increasing catalyzing activity of renin or (pro)renin when bound to (P)RR, resulting in enhanced RAS. Another is to induce the MAPK singal transduction pathway when (P)RR is bound to its ligand renin or (pro)renin. Another role is on ATPases in podocytes and elsewhere, and (P)RR is also involved in diabetes [18].

(P)RR mRNA and protein are detected in the whole mouse metanephros on E12.5 [27]. Spatially, (P)RR immunoreactivity is present in the uretic bud epithelia and nascent nephrons on E13.5 [25]. On E16.5 and E18.5, (P)RR immunostaining is mostly detected in the tubules, which morphologically resemble collecting ducts followed by the glomerular mesangium [27].

The kidney (P)RR protein levels are high throughout mouse gestation and decline gradually during the postnatal development [27]. A truncated form that is cleaved by furin, referred to as soluble (P)RR (s(P)RR), is secreted into the extracellular space [12]. An accurate measurement of s(P)RR levels *in vivo* is an important issue in elucidating the roles of (P)RR in physiology and pathophysiology [12]. These data prompted us to measure plasma level of (pro)renin and s(P)RR in cord blood of neonates. In addition, we investigated the expression of (P)RR in neonatal kidney tissues. This study was performed to test the hypothesis that (P)RR regulates kidney development in humans.

Material and Methods

Patients and samples

The study's experimental protocol was approved by the Institutional Review Board of Tokushima University. Study participants were recruited in Tokushima University Hospital between April 1, 2013 and March 31, 2014. Informed consent was obtained from the parents. Neonates were excluded if they had known or suspected sepsis, severe respiratory distress syndrome, congenital heart disease, or a renal or chromosomal abnormality. Neonates expected to die within 48 h of recruitment were also excluded. Demographic-perinatal characteristics, including gestational age (GA), birth weight, sex, and Apgar scores at 1 and 5 min were recorded for all neonates. Cord blood samples were obtained from the umbilical vein at delivery. A blood sample was obtained in the few days and at 28 days following birth. The samples were stored at -20°C until biochemical analysis. Tissue samples were obtained at the time of autopsy of newborns that died of pulmonary hypoplasia. We also recruited 6 participants, one to seven years old of age, with minor glomerular abnormalities that showed normal glomerular morphology and negative immunofluorescence, but had mild proteinuria or microscopic hematuria. The use of the tissue samples was approved by the ethical

committees of the Institutional Review Board of Tokushima University.

Measurements

Plasma concentrations of s(P)RR were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (IBL, Takahashi, Gunma, Japan). The soluble form of the (P)RR generated by intracellular cleavage by furin is secreted in the plasma [15]. This ELISA kit allows the determination of s(P)RR concentrations in the blood. Plasma concentrations of (pro)renin were measured using commercially available kits (Innovative Research, Inc,, Novi, MI, USA).

Immunohistochemistry

The tissues obtained at the time of autopsy were fixed in 10 % buffered formalin and embedded in paraffin. The paraffin sections (3-μm thickness) were incubated with an anti-(P)RR antibody (ab40790; Abcam, Cambridge, MA, USA) or without the primary antibody overnight at 4°C, rinsed, and incubated with the biotinylated secondary antibody (Vector Labs, Burlingame, CA, USA). After rinsing, the sections were incubated with the avidin-biotin-peroxidase complex (ABC Elite; Vector Labs), followed by 3,3′-diaminobenzidine (Dojindo, Kumamoto, Japan). Each section was counterstained with Mayer's hematoxylin (Wako, Tokyo, Japan), dehydrated, and cover-slipped. Quantification was performed using the EIS-Elements software (Nikon Corporation, Tokyo, Japan). The immunoreactive area (brown) was assessed by setting a threshold and automatically calculated in arbitrary unit.

Statistical analysis

All data are presented as mean ± standard error of mean (SEM). Significant differences were

determined by using unpaired t test for normally distributed variables, whereas the Mann-Whitney U-test was used for nonparametric test. Pearson's correlation coefficients and Spearman's correlation coefficients were used for parametric data and non-parametric data, respectively. The standard least-squared method was used for multiple regression analysis. A p value < 0.05 was considered statistically significant. All computations, including data management and statistical analyses, were performed using JMP software (SAS Institute, Candler, NC, USA) and GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Subject profiles

The profiles of the study participants are summarized in Table 1. One hundred twenty four neonates, comprising 57 preterm and 67 full-term neonates, were recruited. The averages GAs of preterm and full-term neonates were 32.58 ± 0.52 and 37.99 ± 0.10 weeks, respectively. Preterm and full-term neonates weighed $1{,}750.18 \pm 94.06$ and $2{,}722.72 \pm 58.92$ g, respectively. There were no significant differences between preterm and full-term neonates in term of gender and Apgar score at 5min. The average Apgar score at 1 min was significantly lower in preterm neonates than in full-term neonates.

Plasma (pro)renin and s(P)RR levels in preterm neonates

The plasma (pro)renin and s(P)RR levels in cord blood were significantly increased in preterm neonates compared with those in full-term neonates. While there was no significant change in plasma s(P)RR levels at day 4 after birth between preterm and full-term neonates, the plasma (pro)renin levels were significantly higher in preterm neonates than in full-term neonates. There was no significant change in plasma (pro)renin and (P)RR levels at 28 days

after birth (Table 2).

Single regression analysis

The plasma (pro)renin and s(P)RR levels in cord blood and at day 4 after birth were significantly and inversely correlated with gestational age (Figure 1 and Table 3). However, 28 days after birth, the plasma (pro)renin and s(P)RR levels were not correlated with gestational age (Table 3).

Multiple regression analysis

Multiple regression analysis using the stepwise method was utilized to determine the relationship between the plasma (pro)renin or s(P)RR levels in cord blood and other variables. The plasma (pro)renin or s(P)RR levels in cord blood significantly correlated with gestational age (Table 4).

Renal expression levels of (P)RR in neonates

We next examined (P)RR expression in kidney tissues from neonates and from children presenting minor glomerular abnormalities. (P)RR mainly localized in the glomeruli, proximal tubules, collecting ducts, and arteries in neonates. On the other hand, positive, but weak, (P)RR staining was observed in those in minor glomerular abnormalities. Renal (P)RR expression was significantly increased in neonates compared to that in minor glomerular abnormalities (Figure 2A and B). In addition, the levels of (P)RR expression in neonate kidneys were significantly and inversely correlated with gestational age (Figure 2C).

Discussion

We compared plasma (pro)renin and s(P)RR levels between preterm and full-term neonates.

Plasma (pro)renin and s(P)RR levels in cord blood were significantly higher in preterm neonates compared with those in full-term neonates. Notably, plasma (pro)renin and s(P)RR levels in cord blood were inversely correlated with the gestational age. Furthermore, (P)RR expression in the kidney was significantly increased in neonates when compared to children. These data suggest that (pro)renin and (P)RR might be essential for embryogenesis and kidney development.

In human embryos, all components of the RAS are expressed in the kidney as early as 5 weeks of gestation [5, 23]. Angiotensinogen was detected in the proximal tubules, and renin was expressed in capillaries within glomeruli as well as in the wall of arteries in the interstitium and in arterioles up to the aorta in the mesonephros and become confined to the juxtaglomerular apparatus at the vascular pole of the glomerulus in the metanephros [23]. Angiotensin converting enzyme (ACE) was detected in the apical membrane of the mesonephric tubule cells and glomerular endothelial cells, and angiotensin II type 1 receptor was observed in the glomeruli and proximal tubular epithelium [23] [13]. Their production is precisely time-regulated, suggesting that angiotensin II could also exert its effects as a growth-promoting agent during kidney development [29]. Additionally, levels of circulating renin and angiotensin II are higher during fetal life than during postnatal life [5]. During the gestation, the RAS of the fetal lamb responds to the same stimuli such as blood volume depletion, furosemide, hypoxemia, and RAS blockade [3, 19]. Similarly, human fetuses exposed *in utero* to RAS blockers are severely hypotensive at birth and, sometimes, develop irreversible renal lesions in response to renal failure and anuria [11, 20]. On the other hand, inappropriate activation of the RAS during fetal life may have deleterious consequences [10]. Thus, RAS plays an important role in kidney development.

During adult life, (P)RR is abundantly expressed in the kidney, heart, and brain [27]. Recent studies indicate that (P)RR plays an important role in organogenesis and development

191 [27]. Global (P)RR knockout is lethal in mice, indicating an essential role of the (P)RR during
192 the embryonic development [24]. Cardiomyocyte-specific ablation of the (P)RR in mice
193 results in early mortality due to heart failure [8]. Studies using zebrafish demonstrated that
194 (P)RR mutations result in brain malformations and early embryonic lethality [1]. In human
195 studies, (P)RR mutations are associated with a high blood pressure, left ventricular
196 hypertrophy, and X-linked mental retardation [6] [21]. Furthermore, levels of s(P)RR are
197 increased in pregnant women and in blood cord of neonates [31] [32]. Consistent with this
198 concept, the plasma (pro)renin and s(P)RR levels in cord blood are higher than in preterm
199 neonates when compared to those in full-term neonates, and were inversely correlated with
200 gestational age in the present study. On the other hand, plasma (pro)renin and s(P)RR levels
201 did not differ between preterm and full-term neonates 28 days after birth. Therefore, (P)RR
202 may play an important role in embryonic and fetal development.
203 Recently, evidence from multiple studies indicated that (P)RR is critical for normal
204 kidney development and function [26]. In the mouse kidney, (P)RR mRNA and protein
205 expression is detected from E12.5 [25]. (P)RR mRNA is expressed in the intact uretic bud
206 isolated from E11.5 wild-type mouse kidneys [27]. (P)RR immunostaining is present in the
207 uretic bud and the cap mesenchyme on E13.5 [27]. Furthermore, it has been shown that
208 (P)RR is localized in glomerular mesangial cells, the subendothelium of renal arteries,
209 podocytes, and distal nephron cells in the human and rat normal and diseased kidney [4] [16].
210 Indeed, in this study, we demonstrated that renal (P)RR expression in the glomeruli, proximal
211 tubules, collecting ducts and arteries are enhanced in neonates. Additionally, an inverse
212 co-relation between the limited measures of (P)RR expression levels in neonates kidney that
213 we could obtain and gestational age was found, even though the number was small. Previous
214 studies showed that renin was expressed in capillaries within the glomeruli as well as in the
215 wall of arteries in the interstitium and in arterioles up to the aorta and become confined to the

juxtaglomerular apparatus at the vascular pole of the glomerulus in the metanephros in embryos [2] [23]. These findings suggest that (P)RR, at least in part, co-localizes with renin in embryonic kidneys and (P)RR-bound renin may induce kidney development. Targeted genetic inactivation of the (P)RR in the podocytes in mice causes podocyte foot process retraction, nephrotic syndrome, and death from renal failure during early postnatal life [17] [22]. (P)RR maintains nephron progenitors and promotes the differentiation of nascent nephrons by regulating the expression of key genes critical for both populations [26]. These findings, along with the findings reported here, reveal the physiological significance of (P)RR, regulating nephron development during gestation.

The relatively small sample size in this study is a potential limitation. Furthermore, the study was cross-sectional and, therefore, it might be difficult to draw any causal conclusions. However, our observations indicate that the plasma (pro)renin and (P)RR levels in cord blood are increased in preterm neonates compared with those in full-term neonates, accompanied by enhanced expression of renal (P)RR in neonates. These data strongly support the hypothesis that (P)RR plays an important role in nephrogenesis. Furthermore, measuring (pro)renin and (P)RR levels in neonates might become a useful tool to evaluate kidney development. This pilot study provides new insights into the understanding of human kidney development that requires further prospective analyses in large multicenter studies.

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Author's contribution

TT performed literature search, data collection, data analysis, data interpretation and wrote the first draft of the manuscript. MU designed the study, analyzed the data, wrote the manuscript and submitted. TS and RN were member of medical team involved in therapeutic process and reviewed the manuscript. SK contributed to the conception, design of the study and critical review of the manuscript.

Compliance with ethical standard

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and tis later amendments or comparable ethical standards.

Conflict of Interest

The authors declare that they have no conflict of interest.

Informed consent

Informed consent was obtained from the parents including in the study.

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Figure Legends

Figure 1. Single regression analyses for plasma (pro)renin (A) and (pro)renin receptor ((P)RR) levels (B) in cord blood. The plasma (pro)renin and (P)RR levels in cord blood were inversely correlated with gestational age.

Figure 2. Renal tissue (pro)renin receptor ((P)RR) immunoreactivity in neonates and minor glomerular abnormalities. (A) Representative images of (P)RR immunostaining in 33-week gestation neonates (a), 35-week gestation (b), 3 years old (c) and 7-year-old with minor glomerular abnormalities (d), and negative control (e). Original magnification x400. (B) (P)RR levels in renal tissues are expressed in arbitrary units (AU). (C) Single regression analysis of (P)RR expression levels in neonate renal in function of gestational age.

Table 1. Subject profiles

Parameters	Preterm N = 57	Full-term N = 67	P values	$\chi 2$
Gestational age, weeks	32.58 +/- 0.52 **	37.99 +/- 0.10	< 0.0001	
Birth weight, g	1750.18 +/- 94.06 **	2722.72 +/- 58.92	< 0.0001	
Gender, F/M	26/31	34/33	0.5687	0.325
Apgar score, 1 min	6.11 +/- 0.38 **	7.97 +/- 0.17	< 0.0001	
Apgar score, 5 min	8.96 +/- 0.19	9.08 +/- 0.09	0.5733	

F; Females, M; Males, *; $P < 0.05$, **; $P < 0.01$ vs. full-term.

Table 2. (Pro)renin and s(P)RR

	(Pro)renin (ng/mL)			s(P)RR (ng/mL)		
	Preterm N = 57	Full-term N = 67	P values	Preterm N = 57	Full-term N = 67	P values
Cord blood	4.13 +/- 0.38**	2.02 +/- 0.15	< 0.0001	91.36 +/- 5.14 **	75.90 +/- 3.23	0.0097
Day 4	6.00 +/- 0.84**	2.13 +/- 0.25	< 0.0001	74.61 +/- 3.19	71.15 +/- 2.92	0.4274
Day 28	3.61 +/- 0.82	2.04 +/- 1.41	0.5631	83.80+/- 3.49	79.15 +/- 7.09	0.5188

**; P < 0.01 vs. full-term.

Table 3. Gestational age and correlation

	(Pro)renin		s(P)RR receptor	
	R value	P values	R value	P values
Cord blood	-0.5598	< 0.0001 **	-0.2241	0.0123 *
Day 4	-0.4904	< 0.0001 **	-0.2558	0.0155 *
Day 28	0.2859	0.1971	-0.3353	0.1013

*; P < 0.05, **; P < 0.01

Table 4. Multiple regression analysis of (pro)renin and s(P)RR in Cord Blood

(Pro)renin

Parameters	Estimate	SE	t	P values
Intercept	21.57	6.94	3.11	0.0061 **
(Pro)renin Day 28	-0.21	0.17	-1.18	0.2523
s(P)RR Cord Blood	-0.02	0.01	-1.24	0.2293
Gestational Age	-0.44	0.20	-2.22	0.0394 *

s(P)RR

Parameters	Estimate	SE	t	P values
Intercept	510.25	144.29	3.54	0.0020 **
(Pro)renin cord blood	-3.89	2.85	-1.36	0.1874
s(P)RR Day 28	-1.00	0.65	-1.53	0.1404
Gestational Age	-9.35	3.02	-3.10	0.0054 *

*; $P < 0.05$, **; $P < 0.01$

Figure

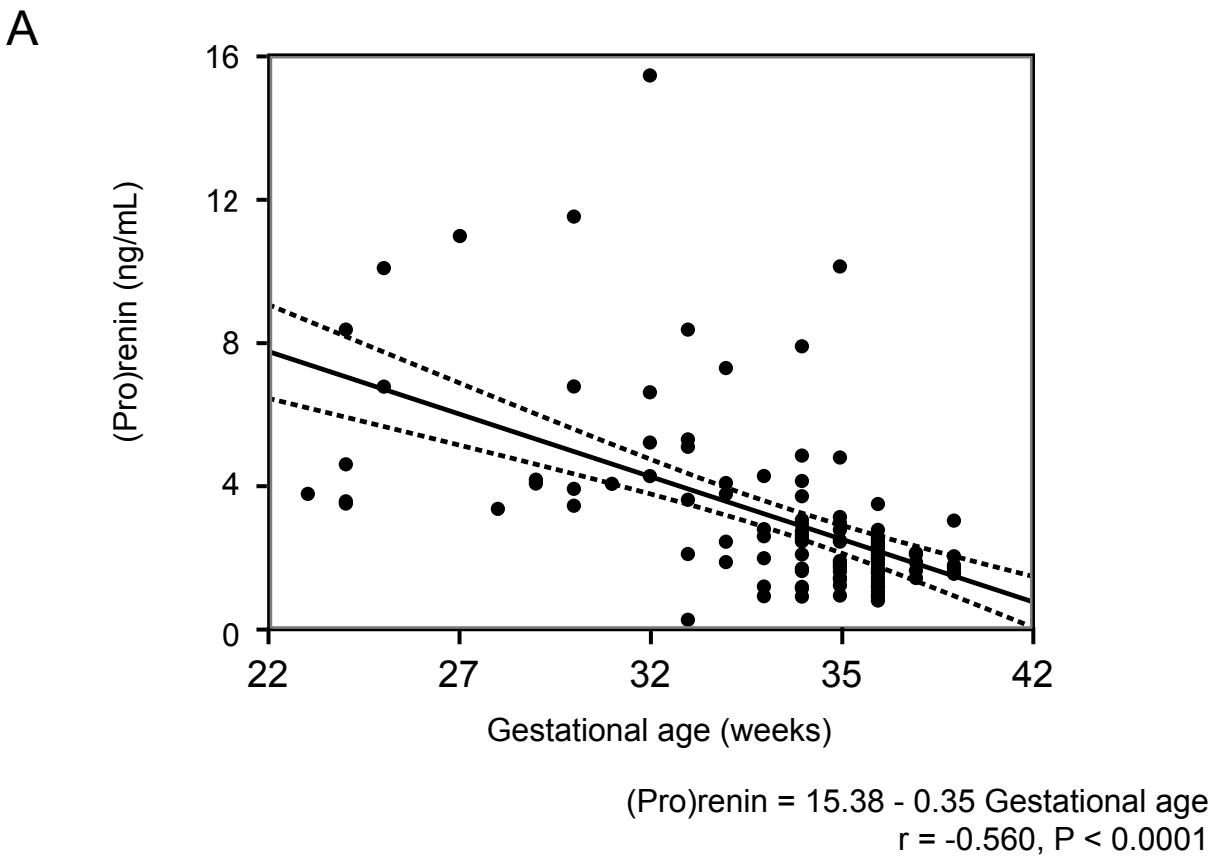

Figure 1

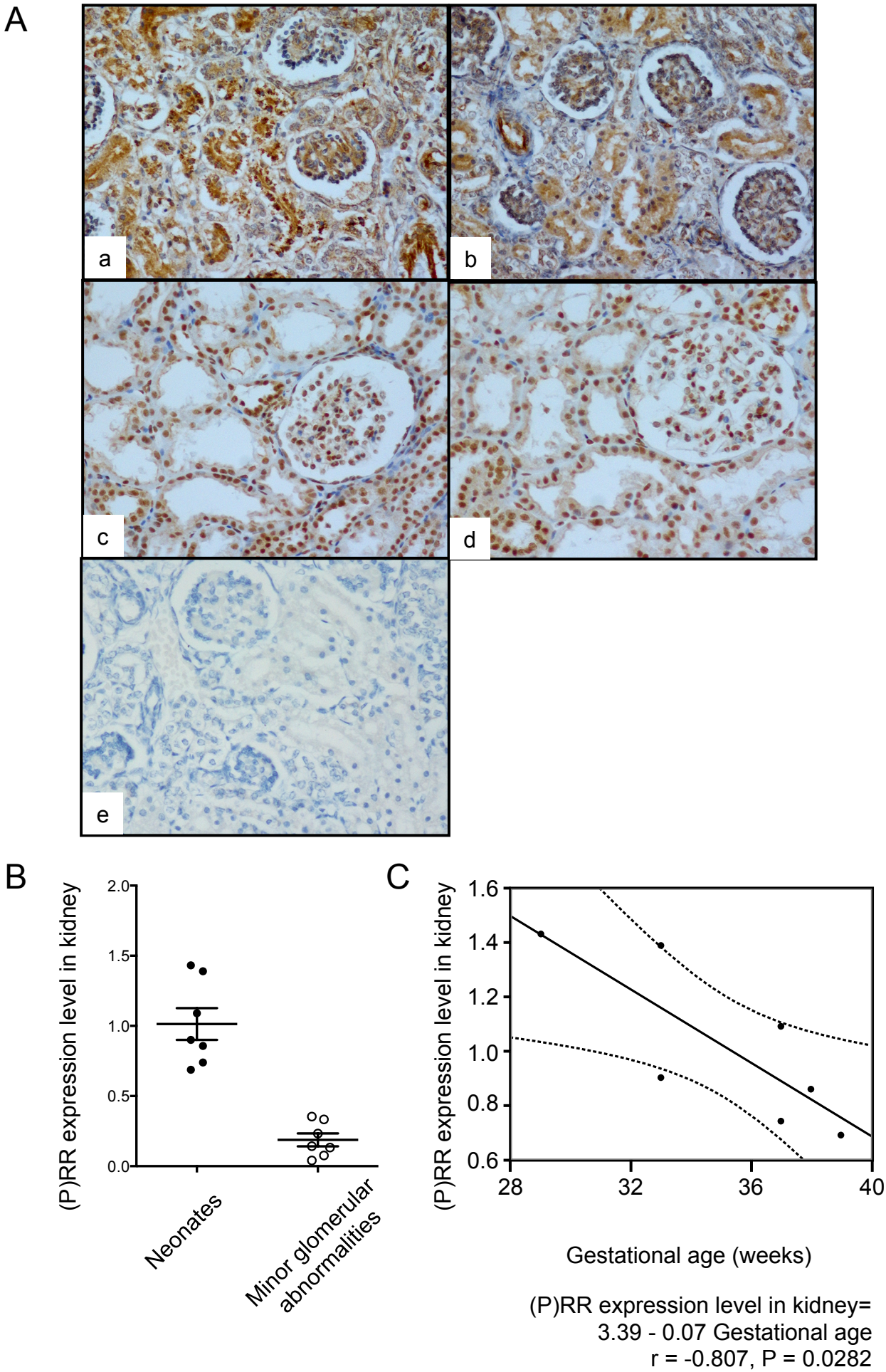
A
a
b
c
d
e
B
(P)RR expression level in kidney
2.0
1.5
1.0
0.5
0.0
Neonates
Minor glomerular abnormalities
C
(P)RR expression level in kidney
1.6
1.4
1.2
1.0
0.8
0.6
28
32
36
40
Gestational age (weeks)
(P)RR expression level in kidney=
3.39 - 0.07 Gestational age
r = -0.807, P = 0.0282

Figure 2