Clarifying expression patterns by renal lesion using transcriptome analysis and vanin-1 as a potential novel biomarker for renal injury in chickens

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ABSTRACT Bird death is often caused by renal lesions induced by chemicals. The avian kidney has a renal portal system with significant blood flow that is sensitive to many chemicals. However, early avian biomarkers for kidney injury are yet to be identified. This study aimed to identify novel renal biomarkers. Acute kidney injury (**AKI**) can be divided into acute interstitial nephritis (**AIN**) and acute tubular necrosis (**ATN**). A chicken model of kidney damage was created by an injection of diclofenac or cisplatin, which caused either AIN or ATN, respectively. Microarray analysis was performed to profile the gene expression patterns in the chickens with nephropathy. A gene enrichment analysis suggested that the genes related to responses to external stimuli showed expression changes in both AIN and ATN. However, hierarchical clustering analyses suggested that gene expression patterns differed between AIN and ATN, and the number of biomarkers relating to renal damage was low. To identify early biomarkers for nephropathy, we focused on genes that were induced at various levels of renal damage. The gene, vanin-1 (***VNN1***) was highly induced in the early stages of renal damage. A quantitative real-time PCR analysis supported this finding. These results suggest VNN1 could be a useful early biomarker of kidney injury in avian species.

Key words: acute interstitial nephritis (AIN), acute tubular necrosis (ATN), bird, VNN1 (vanin-1), renal biomarker

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INTRODUCTION

Bird death is often caused by renal lesions induced by chemicals. For instance, diclofenac, a nonsteroidal anti-inflammatory drug (**NSAID**), has led to the death of many vultures in the Indian subcontinent after an accidental intake of diclofenac after consuming cattle carcasses and the subsequent development of severe kidney injury (Green et al., 2004; Swan et al., 2006). Other therapeutic agents can also cause renal damage in birds. The threshold levels of these chemicals are very low in birds compared to other animals.

One of the reasons for the low threshold is due to the structure of the avian kidney, which has a renal portal system that does not exist in mammalian species (Lierz, 2003). This unique portal system causes birds to have significant blood flow into the kidney (Shideman et al., 1981; Harr, 2002), making them sensitive to many chemicals. Moreover, this unique portal system encourages gene expression changes after chemical exposure which might differ from mammalian species. Primary avian kidney cells have been shown to be much more susceptible than mammalian cells to both diclofenac and meloxicam (Naidoo and Swan, 2009).

In mammals, kidney gene expression profiling has been performed and clinical information has been gathered (Yasuda et al., 2006). Moreover, biomarkers of

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kidney function have been identified in mammals. Blood urea nitrogen (**BUN**) and creatinine have been used as biomarkers of kidney injury in hospitals. Early biomarkers used for mammalian kidney injury has also been identified such as neutrophil gelatinase-associated lipocalin (**NGAL**) (Cruz et al., 2010), cystatin C (Dharnidharka et al., 2002), kidney injury molecule-1 (**KIM-1**) (Han et al., 2002), and microRNA (Ichii et al., 2012). However, avian species have no early biomarkers for renal damage. Uric acid (**UA**) levels are generally used to assess renal injury in birds. However, the levels of UA only vary when the kidney is severely damaged, and high levels of UA are typically linked with death within a few days. Although phosphorus levels are useful tools to assess kidney damage in some birds, they are generally uncommon for assessing avian species (Tully et al., 2009). Therefore, the discovery of novel biomarkers for kidney injury in birds is required. In a previous report, we created 2 chicken models for acute kidney injury (**AKI**) using cisplatin or diclofenac. One model was an acute interstitial nephritis (**AIN**) model involving chickens injected with diclofenac. The second model was an acute tubular necrosis (**ATN**) model involving chickens injected with cisplatin. We proposed that glycan levels could be a biomarker for AIN, but not for ATN (Ishii et al., 2018).

The current study builds on our previous research with the aim to discover and identify novel renal gene biomarkers of both AIN and ATN in avians. To identify candidate renal biomarkers, we used the same samples from our previous study and compared the gene expression levels in the kidney with the levels of renal damage already identified in the previous study.

MATERIALS AND METHODS

Experimental Design and Sample Collection

All samples used in this study were previously collected in our experiment to discover biomarkers using the glycemic approach (Ishii et al., 2018). All experimental protocols were approved by the Laboratory Animal Care and Use Committee of Hokkaido University (approval number: 14-0119), and the animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals of Hokkaido University, Faculty of Veterinary Medicine, which conforms to the Association for the Assessment and Accreditation of Laboratory Animal Care International (**AAALAC**). Briefly, male white leghorn chickens (*Gallus gallus domesticus*) ($n = 15$, at 10 wk of age) were housed under constant temperature (20 ± 2°C) and humidity (40 ± 10%) conditions, with a 12:12-h light: dark cycle. Plasma was collected 1 wk after the birds began acclimatizing to the experimental conditions, prior to the experiment which was initiated one week later. Birds were randomly assigned to one of 4 groups. Each bird in the diclofenac experiment received an injection of either 20% dimethyl sulfoxide (**DMSO**) (Nacalai Tesque, Kyoto, Japan) diluted in saline (control group) or diclofenac sodium (Tokyo Chemical Industry Co., Tokyo, Japan) diluted in 20% DMSO (diclofenac group) into the pectoral muscle once daily in the morning for 4 consecutive days. The birds in the cisplatin experiment received a single injection of cisplatin (Wako Pure Chemical Industries, Osaka, Japan) diluted in saline (cisplatin group) into the basilic vein. Therefore, the experimental design involved the following groups: control group ($n = 3$, IDs: Cont.-1, -2, -3) injected with 20% DMSO; the diclofenac sodium group A (1.5 mg/kg body weight; $n = 4$, IDs: A-1, -2, -3, -4); the diclofenac sodium group B (2.0 mg/kg body weight' $n = 4$, IDs: B-1, -2, -3, -4), and the cisplatin group (3.5 mg/kg body weight; $n = 4$, IDs: C-1, -2, -3, -4). The cisplatin-treated chickens were euthanized on the third day due to severe diarrhea. The diclofenac-treated chickens were euthanized on the fifth day, which is consistent with previously published reports (Naidoo et al., 2007; Jain et al., 2009; Mohan et al., 2012).

Blood samples were collected from all birds prior to the first injection. Prior to feeding each morning, blood samples were collected from the basilic vein from the second day after the initial injection (24, 48, 72, and 96 h after the first injection in the diclofenac and control groups. Blood samples were taken at 24 and 48 h after the injection in the cisplatin group. Whole blood was stored on ice after collection. Within 2 h of initial collection, the plasma was prepared by centrifugation at $1{,}630 \times g$ for 20 min at 4°C. Plasma specimens were immediately frozen in liquid nitrogen and stored at −80° C. After each bird was euthanized, the kidney was collected. The excised kidney was cut into small pieces and placed in RNAlater (Sigma-Aldrich) at −20°C and incubated overnight at 4°C in preparation for microarray analysis.

RNA Isolation and Microarray Analysis

Chickens were divided into 6 groups according to the level of renal damage by the histopathological analyses, the details have been published previously (Ishii et al. 2018, Table 1). Total RNA was extracted from the cranial division of the kidney using NucleoSpin RNA II (Takara Bio Inc., Tokyo, Japan). The RNA samples from each group were pooled (Table 1).

The microarray analysis was performed by the Hokkaido System Science Co., Ltd. (Hokkaido, Japan). RNA was quantified using a NanoDrop 1000 (Thermo Scientific, Wilmington, DE) and further qualified using the Agilent 2100 Bioanalyzer series II (Agilent Technologies, Palo Alto, CA). In brief, the total RNA was reverse-transcribed into cDNA, synthesized with cyanine 3 (Cy3)-labeled cRNA, and amplified using a Low Input Quick Amp Labeling kit (Agilent Technologies), RNeasy mini spin columns (Qiagen, Valencia, CA), and a Gene Expression Hybridization kit (Agilent Technologies). The Cy3-labeled cRNA probes were hybridized onto a 4×44 K Agilent custom chicken oligo

Table 1. Renal damage scores of all chickens using histopathological analysis (Ishii et al., 2018) and classification.

ID	Treatment	Renal damage scores	Classification for microarray analysis
Cont.-1	Control	K0	Control
Cont.-2	Control	K0	Control
Cont.-3	Control	K0	Control
A-1	Diclofenac 1.5 mg/kg	K4	Dic-severe
A-2	Diclofenac 1.5 mg/kg	K0	Not used
A-3	Diclofenac 1.5 mg/kg	K0	Not used
A-4	Diclofenac 1.5 mg/kg	K0	Not used
B-1	Diclofenac 2.0 mg/kg	K5	Dic-severe
B-2	Diclofenac 2.0 mg/kg	K2	Dic-moderate
B-3	Diclofenac 2.0 mg/kg	K0	Not used
B-4	Diclofenac 2.0 mg/kg	K1	Dic-mild
C-1	Cisplatin 3.5 mg/kg	K5	Cis-severe
C-2	Cisplatin 3.5 mg/kg	K3	Cis-moderate
C-3	Cisplatin 3.5 mg/kg	K5	Cis-severe
C-4	Cisplatin 3.5 mg/kg	K4	Cis-moderate

microarray (design ID:026441). The arrays were scanned using an Agilent Technologies Microarray Scanner, extracted, and analyzed by Agilent Feature Extraction 12.0.3.1 (Agilent Technologies).

Microarray data were normalized using GeneSpring GX (Agilent Technologies) with a 75-percentile shift per chip. Data filtration was performed according to AFE quantification flags (gIsSaturated: 0, gIsFeatNonUnifOL: 0, gIsBGNonUnifOL: 0, gIsFeatPopnOL: 0, gIsBGPopnOL: 0, gIsPosAndSignif: 1, and gIsWellAboveBG: 1), resulting in 29,826 valid probes. To analyze expression pattern changes, we classified the difference criteria to be those greater than a 5-fold change or less than a 0.2-fold change in signal intensity in all the levels of renal damage (K1−K5) compared to the control (K0). Venn diagrams of differentially expressed genes were constructed using the VennDiagram (Chen and Boutros, 2011) package in R software (ver. 3.5.1) (R Core Team, 2018). For differentially expressed genes, gene ontology (**GO**) analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (**DAVID**) 6.7 (https://david.ncifcrf.gov/; Huang et al., 2009; Huang da W et al., 2009). We also performed a Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathway analysis (http://www.genome.jp/kegg/) (Ogata et al., 1999; Kanehisa et al., 2016). Hierarchical clustering analyses were performed using the group average method with the Euclidean metric for the ratio of differentially expressed genes in the samples from Dic-moderate or Cis-moderate in R software (ver. 3.5.1). The heat map was created based on the expression ratios transformed to log2 values.

To identify the biomarkers, genes with increased expression levels that were consistent with the degree of renal damage and possessing a greater than 5-fold change were analyzed with quantitative real-time PCR. The microarray data used in the present study are available at DDBJ with the accession number E-GEAD-449.

Quantitative Real-Time PCR

The total RNA was reverse-transcribed using ReverTra Ace (Toyobo, Osaka, Japan), according to the manufacturer's instructions. Gene-specific quantitative real-time PCR primers (Table 2) were obtained from Sigma-Aldrich (Tokyo, Japan). The efficiency of all the primers was 97 to 100%. Quantitative real-time PCR was performed using the Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA). The 10 μL reaction mixture consisted of the Fast SYBR Green Master Mix (Applied Biosystems), forward and reverse primers, and cDNA derived from the total RNA. Phosphoglycerate kinase 1 (***PGK1***) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (***YWHAZ***) genes were used as the reference genes (Olias et al., 2014). All samples were analyzed in duplicate using the following protocol: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. At the end of each PCR run, a melting curve analysis was performed in the range of 60 to 95°C. Gene measurements were performed in duplicate. The expression of each gene was normalized with respect to the average expression levels of *PGK1* and *YWHAZ* and calculated relative to the control levels using the comparative threshold cycle (**Ct**) method. The PCR products were confirmed as single fragments by electrophoresis and direct sequencing.

RESULTS

Scoring Renal Damage Levels

We calculated renal damage scores based on the histopathological analysis (Fig. S1), consistent with our previously published report. All cisplatin-treated chickens and 4 diclofenac-treated chickens showed kidney damage. In common, they both showed degenerative and necrotic lesions in the proximal and distal tubules and sometimes in the glomeruli, and the nuclear shape of the

Table 2. Primers used in this study.

Target gene[1]		Nucleotide sequence 5′ to 3′	Fragment length (bp)	Reference
VNN1	Forward	TTACGTACTGGGCGCATTTG	140	designed in this study
	Reverse	CATGGCAAACTTGGTCTGTG		
PGK1	Forward	AAAGTTCAGGATAAGATCCAGCTG	167	Philipp et al. 2014
	Reverse	GCCATCAGGTCCTTGACAAT		
YWHAZ	Forward	GTGGAGCAATCACAACAGGC	222-224	Philipp et al. 2014
	Reverse	GCGTGCGTCTTTGTATGACTC		

[1]Abbreviations: *PGK1*, Phosphoglycerate kinase 1; *VNN1*, vanin-1; *YWHAZ*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

proximal tubules became indistinct. On the other hand, differences were also observed: diclofenac-treated chickens showed the infiltration of leukocytes, including heterophils, in the interstitium, and the cisplatin group showed necrosis of the tubules and many protein casts in the tubular lumen (Ishii et al., 2018). Treatments and the scores are presented in Table 1. Briefly, renal lesions were given scores ranging from K0 (no lesions) to K5 (most severe), according to the ratio of the outer/lumen areas at the cross-section of the tubules. No remarkable histopathological changes were observed in other tissues. For the microarray analysis, kidneys were pooled (5 groups) according to the drugs and level of damage as follows: Controls (Cont.-1, -2, -3), Diclofenac-mild (B-4), Diclofenac-moderate (B-2), Diclofenac-severe (A-1, B-1), Cisplatin-moderate (C-2, C-4), and Cisplatin-severe (C-1, C-3).

Microarray Analysis

The comparison of the gene expression in the kidney among the samples of each renal damage level was performed by microarray analysis. In Diclofenac-mild, 135 genes showed a greater than 5-fold increase, and 35 genes were decreased by less than 0.2-fold. In Diclofenac-moderate, 199 genes showed a greater than 5-fold increase, and 30 genes had less than a 0.2-fold decrease. In Diclofenac-severe, 824 genes showed a greater than 5-fold increase, and 392 genes had less than a 0.2-fold decrease. In Cisplatin-moderate, 788 genes showed greater than 5-fold increase and 565 genes had less than a 0.2-fold decrease. In Cisplatin-severe, 904 genes showed a greater than 5-fold increase, and 773 genes had less than a 0.2-fold decrease. Of the differentially expressed genes, 101 genes showed a greater than 5-fold increase and 6 genes had a less than 0.2-fold decrease in expression with all moderate and severe levels of renal injury (K2−K5) (Figures 1 and 2). Of the 107 differentially expressed genes from all moderate and severe levels of renal injury, 72 were classified by DAVID. The functional annotation clustering of differentially expressed genes between the control and damaged groups indicated that many genes responded to the cell cycle (Table S2). In pathway analysis, some differential expression genes were contained in "p53 signaling pathway" or "Cell cycle" in KEGG pathway maps (Fig. S2).

Hierarchical clustering analyses of differentially expressed genes in Diclofenac-severe or Cisplatin-severe revealed that gene expression changes were clustered into 2 groups: K2 or lower, and greater than K3 (Figure 3). The highly damaged cluster was further divided into 2 groups: diclofenac-injected and cisplatin-injected. The heat map indicated that most of the genes showed reversed changes between the 2 clusters. Only a few genes showed the same tendency (increase or decrease, regardless of injection type).

To control the chemical effects, genes that showed increased expression levels that were consistent with the level of renal injury were selected for further analysis. The microarray analysis showed that the expression levels of 4 genes were proportional to the level of renal injury, with a 5-fold change in expression when compared to their control response (K0) (Table 3).

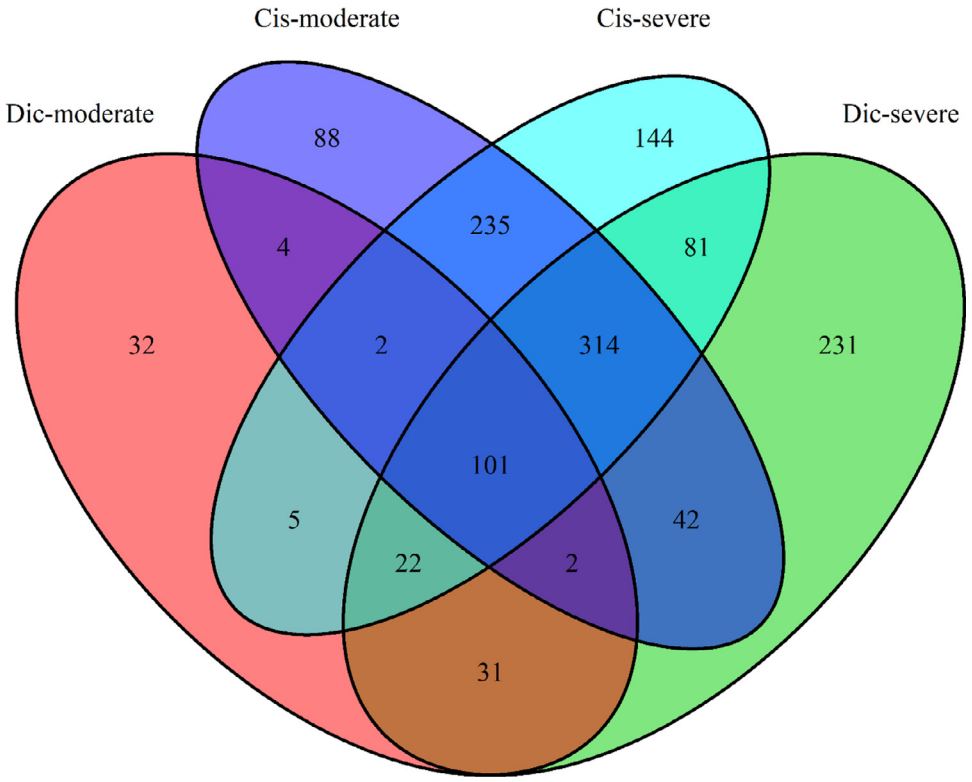

Figure 1. The number of genes that were induced more than 5-fold by moderate or severe kidney injury. One hundred one genes were induced in all moderately and severely injured chickens.

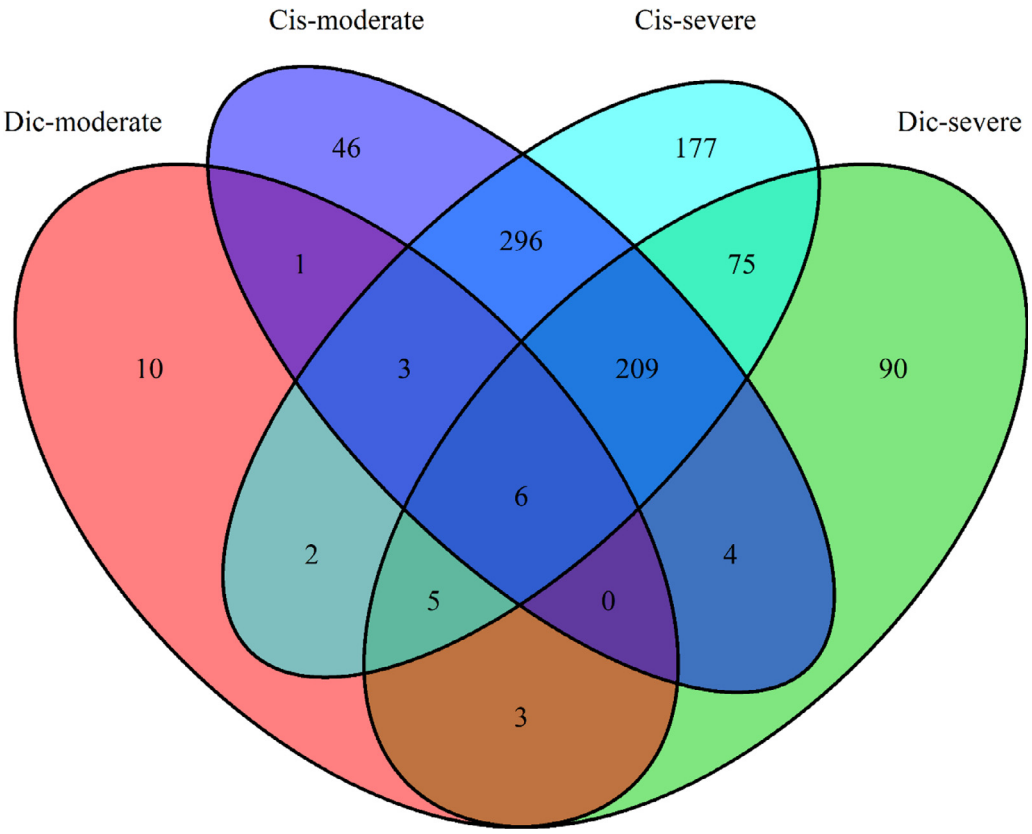

Figure 2. The number of genes that were reduced less than 0.2-fold by moderate or severe kidney injury. Six genes were reduced in all the moderately and severely injured chickens.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed to identify candidate biomarkers for bird species. The microarray analysis indicated that the vanin-1 (***VNN1***) gene is a promising avian renal biomarker. VNN1 is an ectoenzyme with pantetheinase activity (Aurrand-Lions et al., 1996). *VNN1* has also been reported as a marker of kidney damage in a rat model of type 1 diabetic nephropathy (Fugmann et al., 2011). Therefore, the expression levels of *VNN1* in all chickens were quantified by quantitative real-time PCR. A comparative Ct analysis showed that *VNN1* levels increased proportionally with the level of renal damage in both diclofenac- and cisplatin-treated chickens. Moreover, *VNN1* levels were even altered in slightly damaged kidneys, in the kidneys of chickens from the treatment groups of A-2, A-3, A-4, and B-3, which had renal damage scores of K0 (Figure 4).

DISCUSSION

Microarray analysis was performed to profile differentially expressed genes with renal lesions. GO functional annotation clustering analysis suggested that genes related to cell cycle responses were differentially expressed in moderately and severely damaged kidneys (K2−K5). Pathway analysis also indicated that the genes related to the cell cycle and p53 signaling pathway were induced (Fig. S2). In humans, AKI is known to activate the pathways of cell death and cell proliferation (Price et al., 2009). Therefore, the differentially expressed genes in kidney-injured chickens would reflect the same mechanisms as those in humans. The Venn diagram showed that the number of differentially expressed genes in Diclofenac-moderate were less than those in Cisplatin-moderate chickens (Figures 1 and 2). The renal damage level of Diclofenac-moderate was K2 and Cisplatin-moderate scored K3. Therefore, there appears to be a boundary between K2 and K3 that divided the gene expression patterns. The hierarchical clustering analyses of the differentially expressed genes in all the moderately or severely injured chickens also indicated that differentially expressed genes were clustered by damage level: K1−K2 and K3−K5. However, in the K3-K5 cluster, the effects of diclofenac and cisplatin differed (Figure 3). These findings suggest that most differentially expressed genes are affected by chemicals, with a few also dependent on the extent of the renal damage.

In this study, the expression of 4 genes—*VNN1*, anillin actin-binding protein (***ANLN***), gastrin-releasing peptide (***GRP***), and mini-chromosome maintenance complex component 10 (***MCM10***)—are dependent on the extent of the renal damage and increases more than 5-fold in mildly damaged kidneys. Mutations in ANLN cause focal segmental glomerulosclerosis (**FSGS**) (Gbadegesin et al., 2014). GRP is a growth factor for

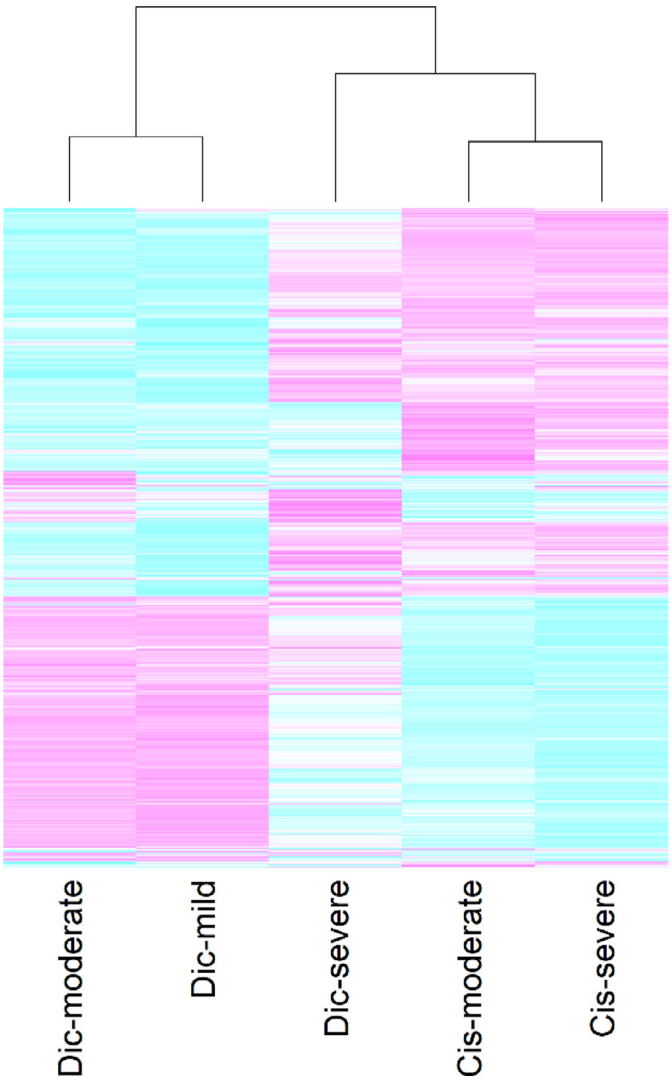

Figure 3. Heatmap of differentially expressed genes in chickens with renal damage scores of K0 and K1 − K5. The differentially expressed genes in chickens with Diclofenac-moderate or Cisplatin-moderate were clustered hierarchically. The gene expression ratio was transformed by log 2 prior to the comparison with the control group. The log fold-changes clustered into two groups: one containing Diclofenac mild (K1) and Diclofenac moderate (K2) and the other containing Diclofenac severe (K4, K5), Cisplatin moderate (K3, K4), and Cisplatin severe (K5). The red color indicates gene induction when compared to the control. The blue color indicates gene reduction when compared to the control. Abbreviations: Cis: cisplatin group; Dic: diclofenac group.

renal cell carcinoma (**RCC**) (Heuser et al., 2005). *MCM* genes are overexpressed in renal cancers (Rodins et al., 2002; Ha et al., 2004; Taran et al., 2011).

To identify biomarkers for kidney injury, the expression of the *VNN1* gene increased proportionally to the extent of renal damage and was induced more than 15-fold in mildly damaged kidneys. The quantitative real-time PCR of *VNN1* showed it has promise as a marker of renal damage, including slight renal failure, in both diclofenac- and cisplatin-treated chickens. Four chickens (A-2, A-3, A-4, and B-3) had normal UA levels with no histopathological changes in their kidneys (Table S1). However, their *VNN1* levels were increased compared to the control. Therefore, *VNN1* could serve as an earlier biomarker of kidney injury than UA, as it can be detected in conditions of mild renal damage.

VNN1 breaks down pantetheine into cysteamine and pantothenic acid (vitamin B5). VNN1 is known to have several functions related to these 2 metabolites. First, VNN1's primary physiological function is the recycling of pantothenic acid, an important precursor in the biosynthesis of coenzyme A (**CoA**). Second, cysteamine and cystamine (the oxidized form of cysteamine) are important sensors of oxidative stress, and many studies have elucidated the role of VNN1 with oxidative stress and inflammation (Bartucci et al., 2019). The same research found *VNN1*-KO mice were better protected against tissue inflammation in response to systemic oxidative stress, suggesting that VNN1 may promote inflammation.

The mRNA expression of *VNN1* is mainly high in the kidney, intestine, and liver of mice. In both humans and rats, VNN1 in blood and urine is a potential biomarker of renal injury (Fugmann et al., 2011; Hosohata et al., 2011, 2012, 2018; Washino et al., 2019). The current study showed that *VNN1* levels were also altered in chickens and were consistent with renal damage scores. Our study showed that VNN1 could be a candidate biomarker for renal injury in birds. In birds, both urine and feces come together in a tubular cavity called the cloaca. Urinary system differs between birds and mammals. However, a high concentration of VNN1 in bird excrement could indicate renal diseases. Further analysis of VNN1 in blood and excrement would verify the usefulness of VNN1 as a renal biomarker.

Although further studies are needed, VNN1 may serve as an early biomarker of renal damage in avians. If kidney injury is detected at an early stage, it can be treated earlier, allowing chickens to recover. Moreover, many medicines are nephrotoxic and require effective monitoring of kidney function. In addition, renal biomarkers can be used to monitor chemical pollution that causes kidney injury, such as exposure to metals (e.g., Pb, Cd, Hg) and medicines (e.g., diclofenac).

Table 3. Gene induction using microarray analysis.

	Induction ratio compared to K0					
Gene name[1]	K0	K1	K2	K3-4	K4-5	K5
VNN1	1	15.74424	27.57141	51.04239	111.5134	119.5326
ANLN	1	7.449375	13.32174	16.19249	26.72836	26.8043
GRP	1	6.583954	10.6525	12.98904	17.54708	24.90333
MCM10	1	5.340534	5.514153	5.77615	7.039732	7.132706

[1]*ANLN*, anillin actin-binding protein; *GRP*, gastrin-releasing peptide; *MCM10*, mini-chromosome maintenance complex component 10; *VNN1*, vanin-1.

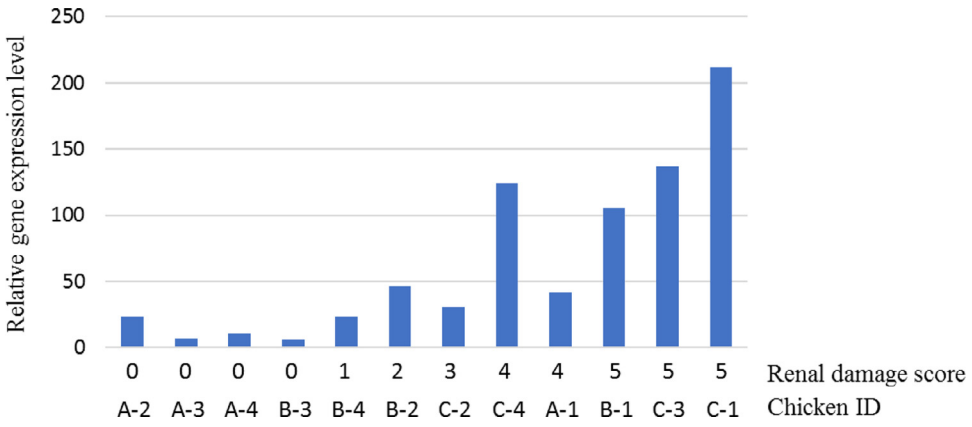

Figure 4. Gene expression levels of VNN1 by quantitative real-time PCR (Comparative Ct). Abbreviation: VNN1, vanin-1.

CONCLUSIONS

The transcriptome analysis suggests that AIN and ATN affect gene expression via different mechanisms. However, the expression levels of the *VNN1* gene are dependent on the severity of the renal damage and can even be detected with mild damage. Therefore, the *VNN1* gene may be a useful biomarker for early-stage renal lesions in birds. Early renal biomarkers in birds may contribute to the conservation of avian species.

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DISCLOSURES

Authors have disclosed any potential conflict of interest, financial or personal, or declared that there are none to disclose, in relation to the publishing of the research.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2022.102011.

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