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Research article

Analysis of serum proteomic in cats with polycystic kidney disease-1 gene mutation

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ABSTRACT

Feline autosomal dominant polycystic kidney disease (PKD) is common in Persian and Persian-cross cats. This study aims to investigate proteins that can be potential biomarkers for early disease diagnosis in cats with PKD1 heterozygous gene mutations and compare them with chronic kidney disease cats and normal wild-type cats. Thirty-three client-owned cats of variable breeds (ten PKD1 gene mutation cats, twelve wild-type cats with normal blood profiles, and eleven wild-type cats with chronic renal disease) were enrolled in this study. This study used serum-based proteomic profiling analysis in cats. Abdominal ultrasounds were examined in all cats. Proteomic analysis was conducted by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and liquid chromatography-tandem mass spectrometry (LC-MS/MS). One hundred and fifty-nine proteins were significantly differentially expressed between each group. The proteins identified in this study are known to regulate the apoptosis pathway. The apoptosis regulator Bcl-2 (BCL2) was overexpressed in the chronic kidney disease (CKD) group, while apoptotic peptidase activating factor 1 (APAF1) and BCL2-associated X apoptosis regulator (BAX) were only expressed in the PKD group. Ingenuity pathway analysis revealed that proteins uniquely expressed in the PKD group were linked to the Wnt signaling pathway and MAPK pathway. Asparagine synthetase domain-containing and secreted frizzled-related proteins were interesting proteins that should be studied further for the possibility of a candidate protein for disease detection. The proteomic profiles identified in this study could be used as potential novel biomarkers for the early detection of PKD in cats.

1. Introduction

Feline autosomal dominant polycystic kidney disease (ADPKD) is common in Persian and Persian-related breeds. The condition will cause a progressive development of cysts in the kidney and other organs, such as the liver, biliary tract, and pancreas [1]. The disease's

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progression depends on age, leading to kidney malfunction and finally developing into renal failure. Research has shown many affected breeds other than Persian, such as exotic shorthair, Himalayan, British short hair, American shorthair, Burmese, Ragdoll, and Maine Coon. The pathogenesis of this disease has been studied, and there is some association between the disease and the PKD1 gene [2]. The PKD1 gene is translated as a cell wall-associated protein called polycystin-1 [3], which is usually associated with cell-cell or cell-matrix interactions that induce ciliopathies and produce multiple cysts in the kidney or other organs [4].

Ultrasound is a reliable imaging test for diagnosing and monitoring disease progression. In Thailand, there was a study investigating the prevalence using ultrasonography, which had been reported at 16.7 % [5]. Unfortunately, many studies have shown that the number of cysts and size progressively correlate with age. Ultrasonography may not be an appropriate method for preventing breeding. The genetic evaluation is one of the easiest detections for the owner to test and discontinue disease transfer. PCR (polymerase chain reaction) was developed to identify and amplify the DNA fragment of interest, which is the PKD1 gene, and the most popular PCR is RFLP-PCR (restriction fragment length polymorphism-PCR) [6].

Recently, proteomics has been widely studied because it provides information about complex biological systems, especially in living organisms [7]. Mass spectrometry (MS) has become an important technique in molecular research because of its ability to analyze and identify proteins. Humans also have a common inherited disease called ADPKD, as in cats. The exact mechanism of disease progression is still not fully studied. Even though this disease is inherited and cannot be cured, there has been an effort to find biomarkers that might be useful for disease prevention and monitoring [8]. Many scientists have attempted to analyze the possible biomarkers linked to ADPKD by using ELISA (based on a protein database) in the urine and serum of a cohort of ADPKD patients [9]. The results showed that urinary epidermal growth factor (EGF) and serum apelin (APLN) levels were significantly downregulated in ADPKD patients.

On the other hand, serum vascular endothelial growth factor alpha (VEGFA) and urinary angiotensin (AGT) were significantly upregulated in ADPKD patients compared with healthy controls, and arginine vasopressin (AVP) was upregulated in ADPKD patients compared with CKD patients. Apart from the human model, feline autosomal dominant polycystic kidney disease still lacks information about proteomic study in serum and urine. Previous study showed that the urinary proteome can reflect the dynamic changes associated with growth and development in mice over a short time. Therefore, urine proteome markers may provide the impact of short-term development on proteinuria [10]. This study aimed to analyze serum proteomics specific to the PKD1 mutation gene in cats compared with cats with chronic kidney disease and normal blood profile cats by using a mass spectrometry technique including MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) and HPLC (reverse-phase high-performance liquid chromatography).

We hypothesized that cats with PKD1 mutations would have different protein expression levels. In addition, proteomics analysis distinguished the proteins between wild-type, PKD1 gene mutation, and CKD cats and identified potential biomarkers for PKD1 mutation. Therefore, this study aimed to investigate and analyze the serum proteomics of cats with PKD1 heterozygous gene mutations and compare them with cats with chronic kidney disease and normal wild-type cats using MALDI-TOF MS.

Table 1
Clinical parameters of all recruited cats. Data is presented as mean ± SEM for continuous data or as a percentage for categorical data.

	Total (n = 33)	HET mutation (n = 10)	WT without CKD (n = 12)	WT with CKD (n = 11)	Reference
Male (n)	18 (54.55 %)	4/33 (12.12 %)	8/33 (24.25 %)	6/33 (18.18 %)	–
Female (n)	15 (45.45 %)	6/33 (18.18 %)	4/33 (12.12 %)	5/33(15.15 %)	–
Age (month)	57.56 ± 5.75	63.3 ± 9.74[a]	31.09 ± 7.02	78.8 ± 8.24[h]	–
BUN (mg%)	43.45 ± 6.80	49.7 ± 17.61	23.59 ± 1.56	58.12 ± 9.19	15–34
Creatinine (mg%)	3.08 ± 0.70	1.96 ± 0.43[c]	1.30 ± 0.09	3.94 ± 0.97[g]	<2.0
SDMA (ug/dL)	13.17 ± 1.09	16.30 ± 1.49[b]	8.50 ± 0.75	21.17 ± 2.69[i]	<18.0
PCV (%)	34.37 ± 1.57	33.5 ± 3.82	36.57 ± 2.04	32.32 ± 2.35	30–45
Albumin	2.87 ± 0.15	1.93 ± 0.23***	3.3 ± 0.17	3.00 ± 0.09[d]	2.6–4.2
Urinalysis					
Urine specific gravity	1.024 ± 0.002	1.027 ± 0.004[d]	1.036 ± 0.002	1.015 ± 0.001[i]	1.007–1.034
UPC ratio	0.52 ± 0.07	0.61 ± 0.05	0.24 ± 0.037	0.66 ± 0.14	<0.2
WBCs (cells/HPF)	1.22 ± 0.25	0.33 ± 0.21	0.55 ± 0.22[e]	2.2 ± 0.29[f]	<5/HPF

[a] $p < 0.05$.
[b] $p < 0.01$ when HET vs. WT.
[c] $p < 0.05$.
[d] $p < 0.01$, $\#\#p < 0.01$.
[e] $p < 0.001$ when HET vs. WT with CKD.
[f] $p < 0.05$.
[g] $p < 0.01$.
[h] $p < 0.001$.
[i] $p < 0.0001$, when WT without CKD vs. WT with CKD. HET: heterozygous mutation, WT: wild-type, CKD: chronic kidney disease, BUN: blood urea nitrogen, PCV: packed cell volume, SDMA: symmetric dimethylarginine, UPC: urine protein creatinine, WBCs: white blood cells.

2. Materials and methods

2.1. Animals

All cats were managed as clinical cases by an attending clinician following the standard of clinical care. Client consent for the contribution of images and medical records was obtained for all animals in the study. This follows the guidelines in the Guide for the Care and Use of Laboratory Animals, the Committee approved the protocol on the Ethics of Animal Experiments of Kasetsart University (ACKU-65-VET-077). A total of thirty-three client-owned cats were enrolled in this study at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Kasetsart University. This study used a cross-sectional methodology and collected data from convenience sampling between September 2022 and August 2023. To assess their general condition, all cats in the study underwent a complete physical examination. Blood was collected using a 23G needle from a jugular vein and placed in an EDTA-coated container. The sample was stored at 4 °C for further use. Thirty-three cat blood samples were sent to a laboratory at Kasetsart University. Cats were randomly divided into three groups: the PKD1 mutation gene group (defined as the PKD group), the wild-type gene with blood profiles within the reference range (defined as the control group), and the wild-type gene with CKD (defined as the CKD group) as shown in Table 1. Ten cats were diagnosed with polycystic kidney disease by ultrasound. Twelve cats had normal blood profiles with nonremarkable findings on ultrasound. Eleven cats in the chronic kidney disease (CKD) group were diagnosed based on BUN, creatinine, SDMA, and urinalysis tests according to IRIS staging [11] (Table 1).

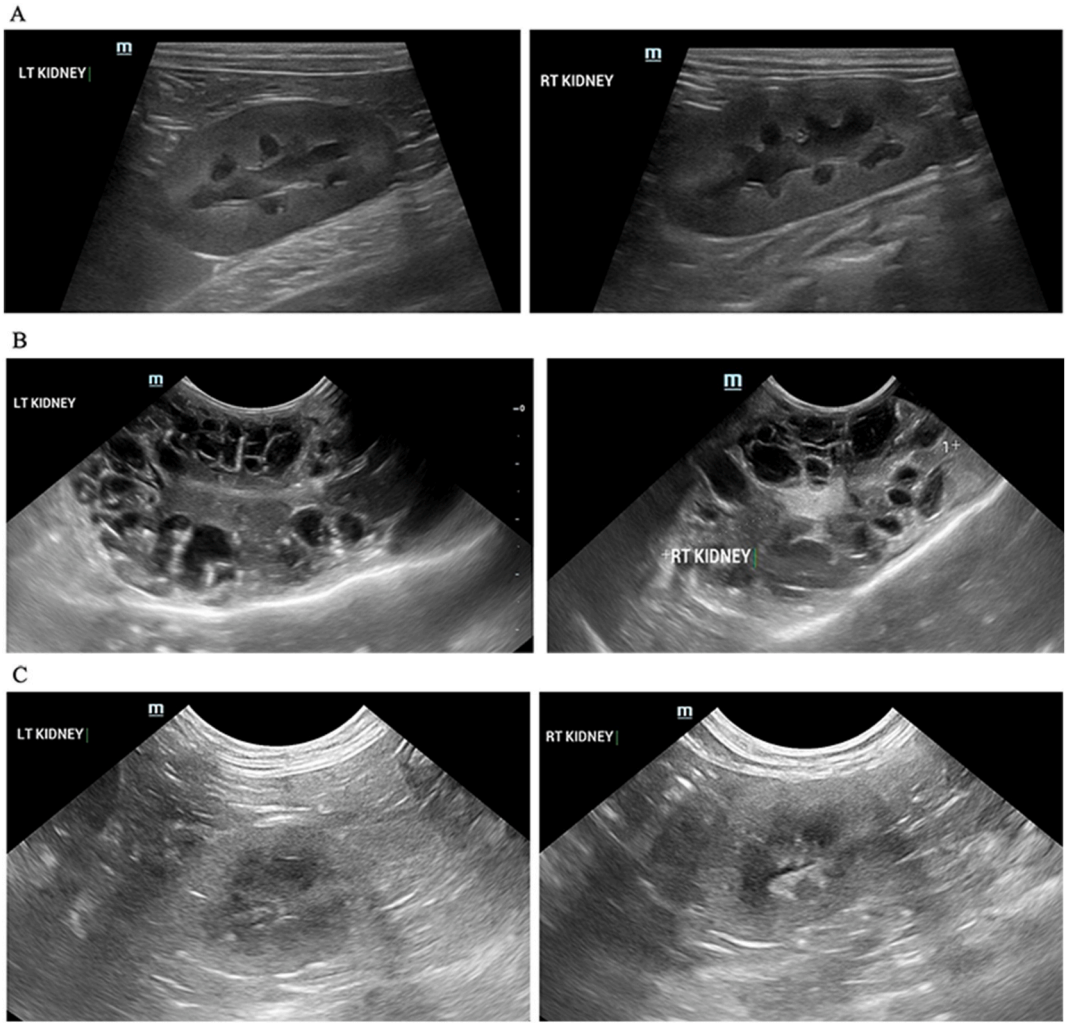

Fig. 1. Ultrasound image of kidneys with cysts. (A) normal kidney appearance without cystic formation. (B) Multiple cyst formations were observed in the renal parenchyma of the kidney in the PKD1 mutation cat, and (C) the kidney appearance was without cystic formation in the CKD cat. The ultrasonography images were cropped for the proper size.

2.2. Blood examination

Blood in an EDTA-coated container was used to measure the complete blood count (CBC) for evaluating the quantity and size of red blood cells, hemoglobin, white blood cells, and platelets, while serum was used to assess creatinine, blood urea nitrogen (BUN), total protein, and albumin afterward. The remaining sample was stored at −20 °C for further use in the molecular technique.

2.3. Urinalysis

Urine samples were collected by cystocentesis and analyzed immediately following collection using an automatic Veterinary Urine analyzer (Vet Scan UA®, Zoetis).

2.4. Ultrasonography

This study evaluated all cats for kidney ultrasonography using a previously reported procedure [5]. An ultrasound was performed using a Mindray real-time ultrasound machine with a linear transducer (frequency 7.5–12.0 MHz). The kidneys were examined in the longitudinal and transverse planes, and the images were recorded for each cat. Ultrasound images can be assessed using the renal crest as a landmark. The size of the kidney can be measured from the long-axis perspective. The glomerular length of the right and left kidneys was measured to determine the kidney's structure (Fig. 1A, B, and C).

2.5. Genotyping of PKD1 gene mutations

The DNA was extracted using a commercial blood DNA extraction kit (FavorPrep Blood Genomic DNA Extraction Mini Kit, Taiwan) following the manufacturer's instructions. DNA amplification with PCR was performed using a specific primer for PKD1 mutation following the previous study [6]. After that, the BT sequencing method was performed to detect a nucleotide of the PCR product. After that, the PKD1 polymorphism was detected by using the BioEdit program and ApE (a plasmid editor) program. This genetic detection confirmed that all polycystic kidney disease cats are from the PKD1 mutation gene, while other groups are wild-type genes.

2.6. Proteomics analysis of MALDI-TOF mass spectrometry

Each plasma sample was purified using a Ziptip C18 column (Millipore, Burlington, MA, USA) according to the manufacturer's protocol. Lowry's assay determined the total protein concentration using bovine serum albumin as a standard at 690 nm. The next step was to add 1 μl of the digested solution mixed with a prepared matrix solution composed of α-cyano-4-hydroxycinnamic acid, and 1 μl was spotted into the MALDI target plate (JEOL, Japan). MALDI-TOF MS spectra were collected using JMS-S3000 SpiralTOF-plus (JEOL, Japan) in a linear positive mode with a mass range of 2,000–6,000 Da. This machine generates laser light in the peptide-matrix mixed solution, which causes an ionization effect and separates proteins into many molecules. Each molecule will pass through the TOF tube (time-of-flight). The tiniest molecule will reach the detector, followed by other larger molecules. This detector will generate the difference peak graph called the peptide barcode. The result was determined using Metaboanalyst 5.0 (https://www.metaboanalyst.ca/).

2.7. Proteomics analysis of LC-tandem mass spectrometry

Peptide digestion was performed using 0.25 μg trypsin and 5 μg protein in 100 mM ammonium bicarbonate and incubated at 37°C for 4 hours. Then, a nano-LC-ESI MS/MS was used to analyze tryptic peptides. Peptides were separated in a 75 μm I.D. x 15 cm Acclaim PepMap nano-LC column, Thermo Scientific, UK). A gradient of 5–55% linear concentration of 80% ACN in 0.1% FA was used to elute peptides at a flow rate of 300 nL/min within 30 min. Then, the peptides were ionized into gas-phase ions and moved to a ZenoTOF 7600 mass spectrometer (SCIEX, Framingham, MA, USA). To minimize the effect of experimental variation, each sample was analyzed three times. Maxquant 2.2.0.0 was used to analyze data quantification based on MS signal intensities of individual analysis [12]. All data were searched within the UniProt database for protein identification with an Andromeda score of $p<0.05$. UniProtKB was used to detail the protein sequence and information. The correlation of candidate proteins was performed using the Stitch program version 5.0 [13].

2.8. Statistical analysis

Animal characteristic data are presented as the mean ± standard error of the mean (SEM). Shapiro-Wilk tests were used to confirm data normality distribution. Due to the non-normal data distribution, differentially regulated proteins between PKD, control, and CKD were analyzed with the Wilcoxon test, followed by the Bonferroni test to adjust the p-value. Correlations were determined using the Spearman rank test. Student t-tests were performed to compare age, weight, Renal biochemistry parameters, and protein validation data among the PKD1 mutation, wildtype, and CKD groups. GraphPad Prism Software (version 10.1.0) (264) was used for the above analyses. Statistical significance was defined as $p < 0.05$.

The result from mass spectrometry was performed by using ordinary one-way ANOVA statistical analysis followed by the Tukey post hoc test to identify the significantly varying peptides among sample groups.

3. Results

3.1. Animals

The blood serum from ten cats in the PKD1 gene mutations group was used for analysis in the proteomic study, which consisted of 4 male and six female cats aged between 12 and 96 months (mean 63.3 months). Twelve wild-type cats with normal blood profiles were involved in this study: seven male and four female cats with an age range between 7 months and 66 months (mean 31.09 months). Moreover, 11 wild-type cats with chronic renal disease, including six male and five female cats with an age range between 48 months and 96 months (mean 78.82 months), were also enrolled in this study. Among the CKD group, there were 4 cats with CKD stage 2, 5 cats with CKD stage 3, and 2 cats with CKD stage 4. One of CKD stage 3 cats was confirmed of protein-losing nephropathy sign. Moreover, in the PKD1 mutation cat group, 3 cats had azotemia blood profile. The blood profile status is shown in Table 1.

3.2. Proteomics analysis using MALDI-TOF and LC-MS/MS

The LC-ESI MS/MS results showed an 18404 (peaks (mz/rt)) data matrix. After analyzing data with MetaboAnalyst program version 5.0, sample groups seem to have different patterns when performing the PLS score plot, as shown in Fig. 2 and showing the overlaps and unique protein by Venn diagram (Fig. 3). From all protein results, 987 proteins were seen only in the PKD groups. The UniProt program resulted in 380 proteins that provide information on biological processes, molecular functions, and cellular components, as demonstrated in the supplementary materials. The STITCH database program version 5.0 explored possible pathways in uniquely expressed proteins in the PKD group. A Wnt signaling pathway composed of WNT2, WNT2B, and WNT16 from the PKD unique expression group in Fig. 4A and the MAPK pathway, are shown in Fig. 4B. The list of proteins associated with these pathways is shown in Table 2. A group of G-protein coupled receptors also expressed in PKD without enriched network association is reported in Table 2.

The maturation and processing of PC1 and PC2 in tubular epithelial cells are represented in Fig. 5, which shows mutations in PKD1 due to a defect in the development of PC1 in tubular epithelial cells. N-linked glycosylation is a quality-control process for the maturation, folding, and trafficking of membrane or secreted proteins in the endoplasmic reticulum. In PKD1 mutation, intracellular signaling in tubular cells on the cilium results in a reduction of cytosolic calcium concentrations or an increase in intracellular cAMP. Intracellular calcium converts ATP to cAMP and stimulates the receptors for vasopressin and somatostatin, which results in increased fluid secretion and the activation of several proliferative pathways, including the MAPK, mTOR, β-catenin, and Wnt pathways.

Moreover, the results showed that 9193 proteins joined all sample groups. A total of 159 proteins were significantly different. Fifteen proteins were significantly overexpressed in the PKD groups, among others. Among these proteins, two proteins were significantly different (p-value <0.05) only between the PKD1 mutation groups and the control group One of these proteins is the asparagine synthetase domain containing 1 (ASNSD1) (p value $= 0.0014041$). Six other proteins were significantly different between the PKD and CKD groups, 16 proteins were significantly different only between the CKD and control groups, and two proteins were different among the CKD, PKD, and control groups.

Furthermore, 70 proteins were found to differ between the CKD and control groups and were also significantly different between the CKD and PKD groups. Finally, 14 proteins were found to be differentially expressed between PKD and CKD, with a difference

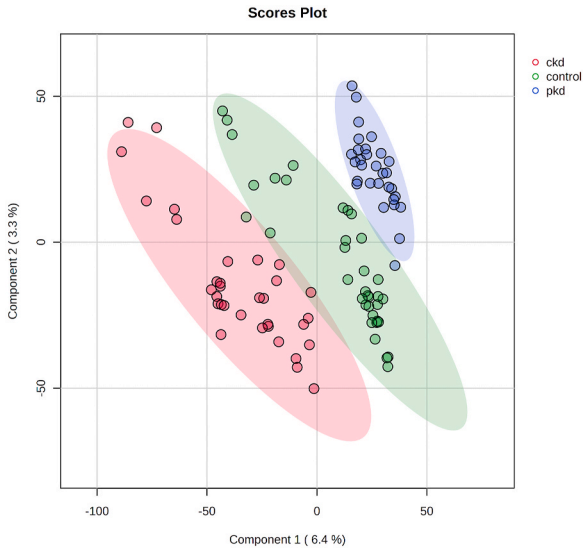

Fig. 2. Two-dimensional principal component analysis (2D PCA) score plot of significant differentially expressed proteins identified between the 3-sample group composed of PKD (blue color), CKD (red color), and control wild type group (green color).

Fig. 3. Venn diagram showing the unique and overlapping proteins among the 3 sample groups: wild-type gene with a normal blood profile group (control), the PKD1 mutation gene group (PKD), wild-type gene with CKD group (CKD).

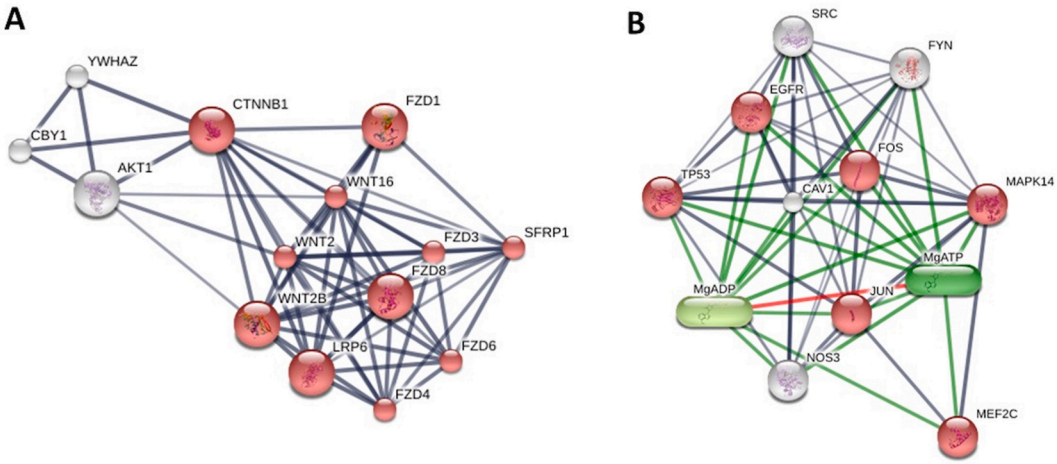

Fig. 4. The interaction network of differentially expressed proteins between the different groups. A Wnt signaling pathway composed of WNT2, WNT2B, and WNT16 from the PKD unique expression group. B Show MAPK pathway, MAPK14 is the only protein from PKD with a unique expression that links to this pathway.

between PKD and controls. The result is demonstrated in the supplementary materials. After that, all results were submitted to UniProt (UniProt: the Universal Protein Knowledgebase in 2023) and the PANTER classification system to identify protein names and investigate the association of these proteins with molecular function and biological processes. Among all results, 15 proteins were significantly overexpressed in the PKD1 gene mutation group compared to the others. At the same time, only one protein was underexpressed in PKD1 gene mutation compared to the other groups (Table 3). The apoptosis pathway was found by comparing proteins among the three groups (Fig. 6). The apoptosis regulator Bcl-2 (BCL2) was overexpressed in the CKD group, while apoptotic peptidase activating factor 1 (APAF1) and BCL2-associated X apoptosis regulator (BAX) were only expressed in the PKD1 gene mutation group.

Thirty-three serum samples from cats were analyzed with MALDI-TOF and LC-MS/MS. The results of MALDI-TOF mass spectrometry revealed the different mass peaks of peptide mass fingerprints (PMFs) from 3 groups of wild-type genes with a normal blood profile (Fig. 7A), wild-type genes with CKD (Fig. 7B), and the PKD1 mutation gene group (Fig. 7C). Moreover, serum protein expression in cats with the PKD1 mutation gene group was elucidated by LC-MS/MS. It was revealed that 9193 proteins were differentially expressed among the groups.

Table 2
PKD protein association with the Wnt signaling and MAPK pathways based on biological processes.

ID	Protein names	Gene Names	Gene Ontology (biological process)
Unique PKD protein expression in Wnt signaling pathway			
A0A337SFH8	Protein Wnt	WNT2B	Canonical Wnt signaling pathway, Chondrocyte differentiation, Hematopoietic stem cell proliferation, Mesenchymal-epithelial cell signaling, Positive regulation of branching involved in ureteric bud morphogenesis
A0A337SLB1	Protein Wnt	WNT16	Wnt signaling pathway
A0A337SW33	Protein Wnt	WNT2	Wnt signaling pathway
M3 WV93	Chibby family member 1, beta catenin antagonist	CBY1	canonical Wnt signaling pathway, cardiac muscle cell differentiation. cilium assembly, fat cell differentiation, negative regulation of canonical Wnt signaling pathway
Unique PKD protein expression in MAPKs signaling pathway			
M3XAW7	Mitogen-activated protein kinase 14 (Mitogen-activated protein kinase p38 alpha)	M4	Negative regulation of canonical Wnt signaling pathway Negative regulation of hippo signaling p38MAPK cascade positive Regulation of interleukin-12 production transmembrane Receptor protein serine/threonine kinase signaling pathway Vascular endothelial growth factor receptor signaling pathway
G protein-coupled receptor in unique PKD protein expression			
M3 W9Y9	Olfactory receptor family 10 subfamily K member 2	OR10K2	Detection of chemical stimulus involved in sensory perception of smell
M3 WGI5	Neuromedin U receptor 2	NMUR2	Calcium ion transport, Central nervous system development G protein-coupled receptor signaling pathway, Inositol phosphate-mediated signaling phospholipase C-activating G protein-coupled receptor signaling pathway
A0A337SI48	G-protein coupled receptors family 1 profile domain-containing protein	–	G1/S transition of mitotic cell cycle; negative regulation of G2/M transition of mitotic cell cycle
M3 WSP3	G protein-coupled receptor 35	GPR35	Cytoskeleton organization, Negative regulation of voltage-gated calcium channel activity Positive regulation of cytosolic calcium ion concentration involved in Phospholipase C-activating G protein-coupled signaling pathway
M3 W2Q8	Somatostatin receptor type 2	SSTR2	Adenylate cyclase-inhibiting G protein-coupled receptor signaling pathway. Cellular response to estradiol stimulus Cellular response to glucocorticoid stimulus neuropeptide signaling pathway
M3X84	Olfactory receptor	OR2A25	Detection of chemical stimulus involved in sensory perception of smell

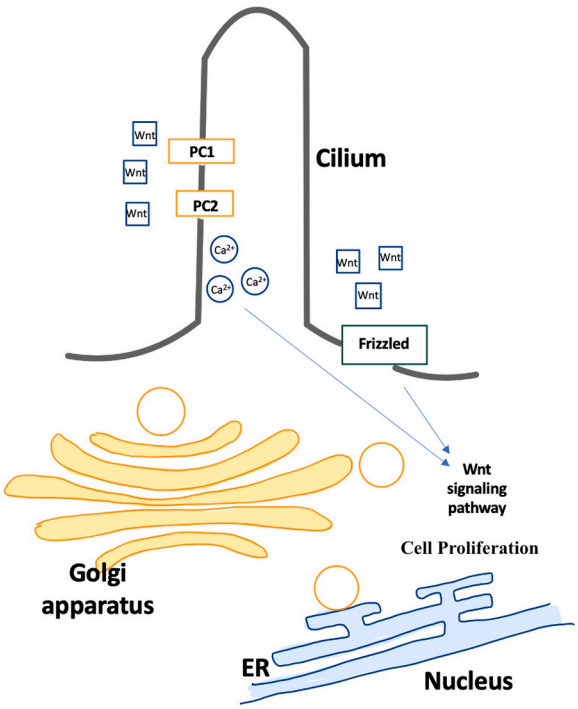

Fig. 5. Schematic overview of the genetic interaction of cystogenesis in PKD1 mutation.

Table 3
Over- and underexpressed proteins in PKD that were significantly different among the other sample groups.

ID	Gene Names	Cellular component	Gene Ontology (molecular function)
Overexpressed protein in PKD group			
A0A2I2UKK7	TMEM25	Membrane	Unknown
A0A337S0H6	SFRP2	Extracellular space	Endopeptidase activator activity Receptor ligand activity; Wnt-protein binding
A0A2I2UBC0	YTHDC1	Nucleoplasm Plasma membrane	mRNA binding
A0A5F5XN07	SLCO2A1	Plasma membrane	Transmembrane transporter activity
A0A2I2UGH1		Membrane	Unknown
M3 WU29	GPR15	Endosome, Plasma membrane	Coreceptor activity, G protein-coupled receptor activity, virus receptor activity
A0A5F5XQA4		Membrane	Unknown
A0A5F5Y3Y7		Z disc	Actin filament binding
A0A2I2U7G4	AP2A2	AP-2 adaptor complex	Clathrin adaptor activity
M3VY85	VILL	Actin cytoskeleton Cytoplasm	Actin filament binding, Phosphatidylinositol-4,5-bisphosphate binding
M3 WYL2	ASNSD1	Unknown	Asparagine synthase (glutamine-hydrolyzing) activity
M3 WBF5	RAVER1	Unknown	RNA binding
A0A5F5XZ68	H6PD	Unknown	6-phosphogluconolactonase activity
A0A5F5XL21	MUTYH	Unknown	Metal ion binding, Purine-specific mismatch base pair DNA N-glycosylase activity
Under expressed protein in PKD group			
M3 WMJ3	MYADML2	Cytoplasm membrane	Unknown

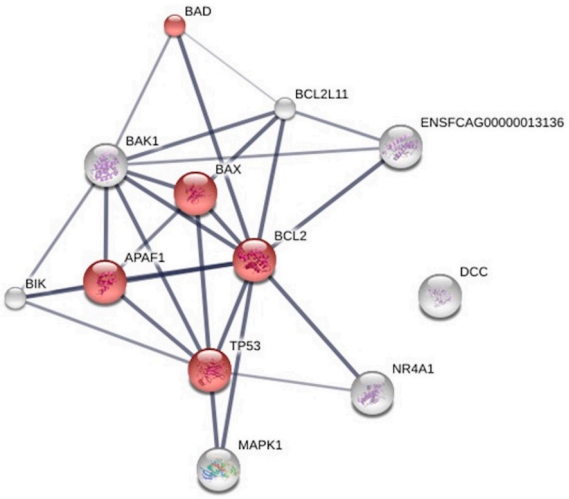

Fig. 6. Proteins from PKD unique expression that link to apoptosis pathway. Red nodes indicate the network of the apoptosis pathway.

4. Discussion

In the presented study, 33 cats were enrolled in this study composed of 10 PKD1 gene mutation cats, 11 control cats, and 12 CKD cats. According to the blood profile, the PKD group has the lowest albumin level compared with the control and CKD group. This can be explained by most of the PKD cats that enrolled in this study starting to develop the clinical signs of kidney disease even the acute kidney in juries of chronic renal failure. An assessment of fasting blood creatinine was performed on at least two occasions in the stable patient to identify and staging of chronic kidney disease in the CKD group. However, our study used a cross-sectional and convenience data sampling between September 2022 and August 2023. Therefore, blood profile data in the PKD1 mutation group were not able to determine the stage of CKD, which is one of the limitations of this current study.

Feline polycystic kidney disease is an inherited disease caused by the mutation of the PKD1 gene, which is mainly associated with a protein called polycystin 1. This study investigates the difference in serum proteomics among the PKD1 mutation gene, the wild-type gene with chronic kidney disease, and the wild-type gene with a normal blood profile using an MS-based approach. This may lead to candidate proteins for disease detection or monitoring. MALDI-TOF is a mass spectrometer that combines two techniques, matrix-assisted laser desorption ionization and time-of-flight (TOF), to make it more affordable for research laboratories [14]. Even if MALDI-TOF can provide qualitative information on proteins, there is still a lack of quantitative and protein sequencing information, which needs to be evaluated by other mass spectrometry methods. This study selected HPLC (high-performance liquid chromatography) for protein and peptide separation. Two interesting pathways were found in PKD's unique protein expression: the Wnt signaling pathway and the MAPK pathway. WNT2B, WNT16, and WNT2 are part of the Wnt signaling pathway, as shown in Fig. 4. The Wnt

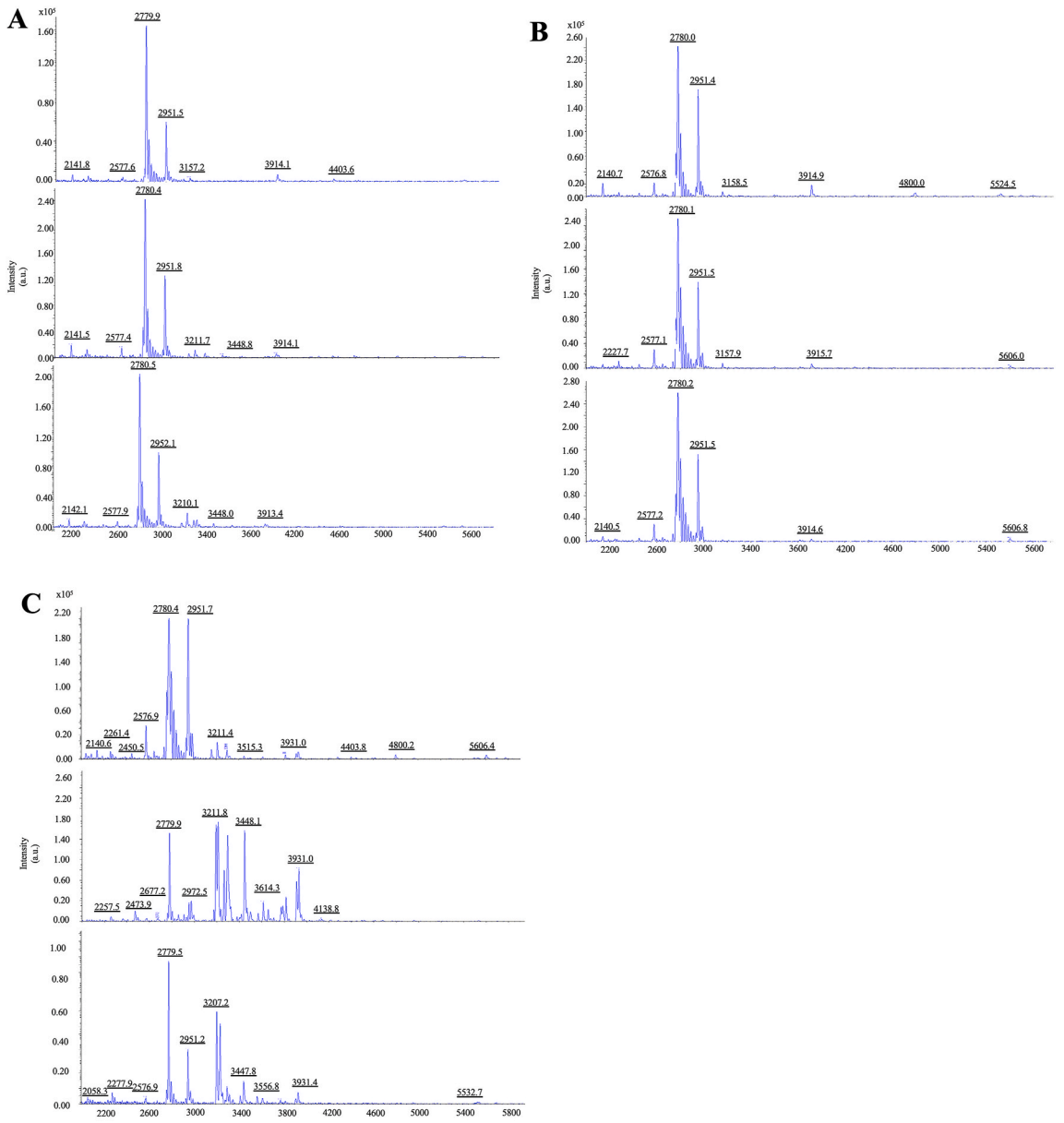

Fig. 7. Peptide mass fingerprints (PMFs) from MALDI-TOF mass spectrometry with (A) wild-type gene with a normal blood profile, (B) wild-type gene with CKD, and (C) the PKD1 mutation gene group.

signaling pathway is a signal transduction pathway in which extracellular Wnt glycoproteins signal via activation of cell surface frizzled receptors. In previous studies, Wnt signaling has played an essential role in cell migration, proliferation, apoptosis, and organ development [15]. Activation of the canonical Wnt pathway may affect polycystin-1 and result in severe polycystic kidney disease [16]. According to a previous study, the Wnt protein found in this study might be an interesting protein for PKD pathway information. It could be one of the candidate proteomes for disease detection. Since noncanonical Wnt signaling has also been demonstrated to play a role in cystogenesis [17], it is unclear whether Wnt/β-catenin signaling in the kidney is primarily responsible for cyst development and needs more studies to be proven.

Mitogen-activated protein kinase 14 (MAPK14) is a member of the MAP kinase family, which is involved in multiple cellular processes and plays an important role in the MAPK cascade. MAPKs can affect gene transcription profiles, leading to aberrant expression of key proteins, and can contribute to the loss of epithelial cells [18]. A similar result was observed in a study of mRNA and microRNA microarray profiles in human ADPKD, which found that MAPK signaling was significantly upregulated in ADPKD [19]. However, there was no significant reported pathway-direct association of MAPK14 with PKD, and another MAPK family was also found to upregulate the CKD group, MAP3K4. Further study needs to be performed to confirm the association of the MAPK pathway and PKD

pathogenesis.

The study of asparagine synthesis was associated with ADPKD in humans [20]. The asparagine synthetase process uses the amide group from glutamine to synthesize asparagine from aspartate; this process is overexpressed in cancer cells [21]. Another study in Pkd1 mouse embryonic fibroblasts found that these cells depend on glutamine and show increased asparagine synthetase activity [22]. This supports the result of our study that ASNSD1 might be an interesting candidate protein for detecting PKD in cats. However, this hypothesis still needs more studied evidence to be confirmed because there was no significant difference between the PKD and CKD groups.

Another interesting protein is secreted frizzled-related protein 2 (SFRP2), which is one of the proteins that is significantly overexpressed in the PKD group among other groups, and its function as a Wnt-protein binding for the canonical Wnt signaling pathway, which regulates a range of cellular processes. The canonical Wnt signaling pathway involves the activation and nuclear translocation of β-catenin. According to ADPKD studies, polycystin-1 interacts with β-catenin so polycystin-1 may have a regulatory role in Wnt signaling [23]. Regardless, there is still no significantly enriched pathway to describe the direct correlation between SFRP2 and PKD-1 mutations, so further studies need to prove whether SFRP2 might be a candidate protein from PKD detection when compared with other proteins in this pathway, such as the Wnt proteins mentioned above.

All overexpressed proteins among the three samples were distinguished into different protein classes, including RNA metabolism proteins, cytoskeletal proteins, defense/immunity proteins, membrane traffic proteins, transmembrane proteins, and other uncharacterized proteins that provide different biological processes. There was no significantly enriched pathway for describing the association of over- and underexpressed proteins in the PKD1 mutation group on the STITCH program. This may be due to a less published study of the feline PKD pathway. Moreover, most of the significantly different proteins between groups were shown to be overexpressed in both the PKD group and the CKD group, including soluble frizzled-related proteins 2 (SFRP2), which have cellular functions such as endopeptidase activator activity, receptor-ligand activity, and Wnt-protein binding. The G-protein-coupled receptor 15 (GPR15) is a coreceptor activity, G protein-coupled receptor activity, and virus receptor activity. Adaptor-Related Protein Complex 2 Subunit Alpha 2 (AP2A2) that function as Clathrin adaptor activity.

These overexpressed proteins comprise complement and coagulation cascade pathways, cytokine receptor interaction apoptosis, and cancer pathways. This can be explained by some PKD1 gene mutation samples also having an azotemia blood profile, which may lead to similar protein expression with CKD samples.

Apoptosis is an essential biological process that contributes to tissue homeostasis. Aberrant apoptosis leads to many disease disorders, including ADPKD in humans [24]. Usually, cyst formation in the kidney may be caused by dysregulation of cell proliferation and other unclear molecular mechanisms. The Bcl-2 family plays a vital role in the extrinsic apoptotic pathway. BAX is a member of the Bcl-2 family and is only expressed in the PKD group. The expression of BAX might contradict further studies that suggest that Bcl-2-mediated apoptosis is not involved in cystogenesis in mice with Pkd1 deficiency [25]. Other apoptosis mechanism studies in Feline polycystic kidney disease should be further investigated. In this study, BCL2 seems to be significantly overexpressed in the CKD group compared with the control group, which might indicate that the increase in BCL2 levels may be associated with the progression of chronic kidney disease, but further study must prove this hypothesis.

The limitation of our study includes a small sample size in each group. Moreover, another limitation of this study is that the retroviral status of all recruited cats needs to be clarified, especially for the feline immunodeficiency virus and feline leukemia virus, since they are critical infectious diseases in Thailand [26]. Retrovirus requires the process of reverse transcription, which is error-prone because it lacks proof-reading function and may lead to mutation of a gene that regulates protein synthesis [27]. This may affect the proteomics results, so viral status investigation needs to be performed in further studies.

5. Conclusion

The present study used mass spectrometry to perform proteomics analysis of Feline polycystic kidney disease compared to normal wild-type genes and chronic kidney disease wild-type genes. The results show significantly different protein characteristics between each group. Moreover, the results provide a possible pathway in PKD: the Wnt signaling and MAPK pathways, also associated with human ADPKD. To the best of our knowledge, this study is the first to use a proteomic approach to study the proteomic profiles of feline polycystic kidney gene mutation (PKD), normal wild-type genes (wildtype), and chronic kidney disease wild-type genes (CKD) using matrix-assisted laser desorption/ionization-time of flight (MALDITOF) mass spectrometry and bioinformatics analysis to identify potentially active proteins that could act as potential biomarkers for diagnostic or disease monitoring tools in clinical use.

Data availability statement

The mass spectrometry proteomics data have been analyzed via the MetaboAnalyst (https://omicsforum.ca). The data reported in this paper have been deposited in Google Drive:

https://drive.google.com/file/d/1KSoeXxwFv_9lqv_jADAdsoEaX5Xz0VG/view?usp = sharing, https://docs.google.com/spreadsheets/d/1Wa6h74tPEAxB42eETg6SMlHjuN3KQc3k/edit?usp=sharing&ouid=112067745770592524071&rtpof=true&sd=true, https://drive.google.com/file/d/1ua-QGGq-HZVdnnfvJCs2g6NAWA1b87w1/view?usp=sharing.

Ethics approval and consent to participate

This study was reviewed and approved by the Ethics Committee of Kasetsart University, with the approval number: ACKU-65-VET-

077. All experimental procedures were carried out and reported in compliance with ARRIVE guidelines.

Ethics declarations

This study was reviewed and approved by the Ethics Committee of Kasetsart University, with the approval number: ACKU-65-VET-077. All experimental procedures were carried out and reported in compliance with ARRIVE guidelines.

CRediT authorship contribution statement

Palin Jiwaganont: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Kotchapol Jaturanratsamee:** Writing – review & editing, Methodology, Investigation. **Siriwan Thaisakun:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis. **Sittiruk Roytrakul:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis. **Soontaree Petchdee:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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