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Original Article

Cat-NPC2, a Newly Identified Allergen, With High Cross-Reactivity to Can f 7

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ABSTRACT

Purpose: Pet-derived allergens are the common indoor inhalant allergens. Among them, cat and dog allergens constitute more than 80% of animal allergic patients, which greatly affect the quality-of-life of patients and increase the burden of social health care. The aim of this study was to identify Cat-Niemann pick type C2 (NPC2) protein, a homologue of Can f 7, as a new allergen.
Methods: Cat-NPC2 complementary DNA (cDNA) was cloned and optimized for amplification and expression in *Escherichia coli*. Then, recombinant Cat-NPC2 (rCat-NPC2) was purified by Ni^{2+} affinity chromatography. The allergenicity was assessed by enzyme-linked immunosorbent assay (ELISA), western blot and basophil activation test (BAT). Based on the sequence similarity, the cross-reactivity between Cat-NPC2 and Can f 7 was investigated by inhibition ELISA. Circular dichroism spectroscopy and homology modeling were used to characterize the structure of Cat-NPC2.
Results: The cDNA sequence of Cat-NPC2 was cloned with a 450-bp open reading frame coding for 149 amino acids (GenBank MN_737596). The condon-optimized NPC2 gene was subcloned and expressed in *E. coli* with a molecular weight of 18.9 kDa. The native Cat-NPC2 was detected in cat dander extracts. The allergenicity determined by ELISA, western blot and BAT suggested at least 14.5% cat-allergic patients displayed high specific immunoglobulin E (IgE) recognition of Cat-NPC2. The predicted structure of Cat-NPC2 was found to consist of 7 β-strands arranged in 2 β-sheets. An ELISA based assay showed that rCat-NPC2 bound to cholesterol in a dose dependent manner. Based on the structure and sequence similarities, IgE cross-reactivity was demonstrated between Cat-NPC2 and Can f 7/Der f 2.
Conclusions: In the study, a novel cat allergen, belonging to the NPC2 protein family, was identified and characterized at both molecular and immunological levels. The study will offer a deeper understanding of cat allergens and improve a component-resolved diagnosis in pet allergy.

Keywords: Allergy; allergens; *Felis domesticus*; NPC2 protein; cross reactions

INTRODUCTION

Allergic diseases are critical worldwide health and medical problems, which can increase the risk of developing more complex diseases or disorders.[1] According to the World Allergy

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Disclosure

There are no financial or other issues that might lead to conflict of interest.

Organization report, more than 25% of the world population are suffering from allergic diseases, including allergic rhinitis (AR), asthma, conjunctivitis, eczema, food allergies and drug allergies, and the trends are still in the rise.[2] In China, a nationwide increased prevalence of AR, together with comorbid asthma, has been observed in both children and adults over the past decades.[3] Due to the high prevalence, allergic diseases have increased financial costs of affected households and created considerable burden on socialized health care system.[4,5] Allergic diseases characterized by inflammatory responses are usually immunoglobulin (Ig) E-mediated, with many types of cells and molecules involved, such as basophils, mast cells, cytokines (*e.g.* interleukin-4) and soluble mediators (*e.g.* histamine). Exposure to allergens, such as pollen, house dust mite (HDM), pet, cockroaches and mold, constitutes a relevant risk factor for the development of allergic diseases. Other risk factors, such as genetic predisposition, increased air pollution, changes in lifestyle and decreased infections, are also pointed out as adjuvant factors for allergic sensitization.[4,6]

Pet-derived allergens are the third leading cause of respiratory allergies, after mites and pollens.[3] Cat (*Felis domesticus*) is a significant source of pet allergens due to its frequent and intimate contact with humans. Particularly in China, 1-child policy has made pet ownership increased consequently to be an alternative companionship for single young children. The cat population had the largest growth by a compound annual growth rate of 33% between 2013 and 2017.[7] Allergic responses to other animals are thought to be similar.[8] In addition, cat allergens are considered ubiquitous because they can easily be carried by gases, vapors and particles for passive transfer. Numerous studies have demonstrated that cat allergens are found not only in pet homes, but also in other private and public places where cats have been never kept.[9-11] Therefore, avoidance of allergen exposure cannot be well achieved. Being allergic not only takes enjoyment out of pet ownership, but also causes serious health problems with reduced quality-of-life and performance at school and work. In clinical practice, allergen immunotherapy (AIT) with crude allergen extracts is a common therapeutic method. However, cat allergy is often underdiagnosed and undertreated, resulting from considerable variations in the quantity of active allergens comprised in commercial extract preparations.[12,13] Thus, it is crucial to clear the component allergens and their potency in order to further standardize extract preparations used for diagnosis and AIT.

So far, 8 cat component allergens have been recognized as Fel d 1 to Fel d 8 by WHO/IUIS (www.allergen.org). Among them, Fel d 1, also known as uteroglobin-homolog, is a dominant cat allergen accounting for up to 96% of cat sensitive patients.[14] Meanwhile, other potential allergen proteins have continuously been identified and defined. Of note, many patients usually simultaneously display sensitization to both cat and dog, indicating either molecular mechanisms for cross-reactivity or co-sensitization to different allergens.[15,16] Cross-reactivity could be explained by high-sequence homologies or structural similarities between cat and dog allergens, such as lipocalins (*e.g.* Fel d 4 and Can f 6) and albumin (*e.g.* Fel d 2 and Can f 3).[17] Interestingly, the study showed that cat-/dog-sensitive patients are 14 times more likely to be sensitized to other mammalian animals.[18] These findings suggest that IgE cross-reactivity is widely presented between cat and dog allergens. Can f 7, a recently identified allergen, has been classified as Niemann pick type C2 (NPC2) protein, an intracellular cholesterol transporter, with an apparent seroprevalence of 10% to 20%.[19] Interestingly, dog NPC2 proteins share more than 60% sequence similarity with group 2 allergens from 12 mites which are NPC2 proteins.[19] Among them, Der p 2 and Der f 2, the major mite allergens recognized in more than 90% mite-allergic individuals, share 23% identity with dog NPC2.[19] With respect to cat, NPC2 gene-encoded proteins have not yet been reported; thus, it is essential to explore its allergenicity and potency in cat allergy.

In our study, we presented NPC2 protein as a new allergen in cat allergy. The recombinant Cat-NPC2 (rCat-NPC2) was cloned, expressed in *Escherichia coli* and purified by Ni-NTA chromatography. The allergenicity was assessed on cat-allergic patients by enzyme-linked immunosorbent assay (ELISA), western blot and basophil activation test (BAT). The sequential and 3-dimensional (3D) structural comparison illustrated the homology between Cat-NPC2 and Can f 7, and their cross-reactivity was demonstrated by ELISA. The identification and characterization of Cat-NPC2 would help improve diagnostics of cat allergy.

MATERIALS AND METHODS

Patients and samples

A total of 110 cat-allergic patients were recruited in this study. Serum samples was collected from each participant under the approval by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (2015-SRFA-005). The clinical information about participants involved in the study is displayed in Table and **Supplementary Table S1**.

Cloning and sequencing of Cat-NPC2

First, complementary DNAs (cDNAs) were obtained by reverse transcription using RNA isolated from cat skin. The following primers were used to amplify a 594-bp gene fragment encoding Cat-NPC2: NPC2F, 5′-GCAGGTTTATCTTGTGACTGAGG-3′ (sense); and NPC2R, 5′-AGGTTAGACTCGATTTCTCCCG-3′ (antisense). The primers were designed in terms of the predicted genomic cat NPC2 sequence (NCBI Accession: XM_003987833.5). The polymerase chain reaction-amplified DNA was subsequently cloned into pCE2 TA/Blunt-zero vector (Vazyme, China), which was used to transform the *E. coli* JM109. Positive clones were selected on Luria-Bertani (LB) plates containing 100 μg/mL ampicillin and further confirmed by DNA sequencing. The sequences obtained were compared to the predicted sequences present in the Genbank using the BLAST program.

Expression and purification of rCat-NPC2 protein in *E. coli*

The GenScript (Nanjing, China) rare codon analysis tool was applied to synthesis of the Cat-NPC2 gene with codon and sequences optimization to *E. coli*. The optimized the Cat-NPC2 gene was cloned into the pET28a vector at Sac I and Xho I sites, resulting in 6×His-tagged fusion gene construction. The recombinant pET28a-cat-npc2 plasmids were introduced into *E. coli* BL21(DE), and the positive recombinant strain was grown up based on kanamycin resistance. Subsequently, the cloned strain selected was cultured at 37°C in LB medium containing 50 μg/mL kanamycin. When the optical density (OD) value at $A_{600\,nm}$ reaches 0.5, a final concentration of 1 mM isopropyl-D-thiogalactopyranoside was added to induce expression. The cell extracts were collected and lysed via sonication as previously described.[20] The cell lysate was isolated and the rCat-NPC2 was expressed as inclusion bodies. Ni-NTA affinity chromatography (GenScript) was used for protein purification. Briefly, we solubilized the inclusion body with 8 M urea lysis buffer (100 mM NaH_2PO_4, 10 mM Tris and 8 M urea, pH 8.0). Then, the solution was loaded onto the Ni-NTA column pre-equilibrated with urea lysis buffer. Washing buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea, 10 mM Imidazole, pH 8.0) was used to remove low-affinity bound protein, followed by higher concentrations of imidazole (30–500 mM) to elute the protein. Purified rCat-NPC2 was characterized on 13% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel. Refolding was carried out by urea-gradient dialysis and redox refolding buffer (0.1 M Tris, 0.5 M L-arginine, 1 mM oxidized glutathione, 1 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM reduced

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glutathione, pH 8.5). The concentration of refolded rCat-NPC2 was measured by using the BCA kit with bovine serum albumin (BSA) as a standard.

Immunization protocol

A New Zealand White rabbit (female, 2–2.5 kg) subcutaneously received 1 mg of rCat-NPC2 in Freund's complete adjuvant (1:1, v/v) on day 0, followed by subcutaneous boosts of rCat-NPC2 (0.5 mg) in incomplete Freund's adjuvant (1:1) on days 14, 21 and 28. Blood was collected from the rabbit on day 35.

Cholesterol-binding activity of Cat-NPC2

According to the method of Reginald and Chew,[21] ELISA was used to characterize cholesterol-binding activity of Cat-NPC2. The microplate wells were pre-coated with cholesterol by ethanol evaporation overnight at 4°C. The wells were blocked with 1% BSA in phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBST) at 37°C for 1 hour prior to incubation with 100 µL of 0.5 µg/mL rCat-NPC2 for 1 hour. NPC2 protein binding was then detected using specific polyclonal anti-rabbit IgG. Horseradish peroxidase (HRP)-labelled anti-IgG were then added. After that 100 µL of tetramethylbenzidine (TMB) substrate solution was added for 30 minutes and terminated by 50 µL of 2 M H_2SO_4. The plate was read by using BioTek™ Eon™ microplate spectrophotometer (BioTek Instrument Inc, Winooski, VT, USA) at OD_{450}.

Detection of native Cat-NPC2 by immunoblot analysis

Western blot analysis was performed for native Cat-NPC2 characterization in commercial cat dander extracts (Macro-Union Pharmaceutical Limited Corporation, Beijing, China). Cat-NPC2- specific rabbit IgG polyclonal antibodies were used for native Cat-NPC2 determination.

Immunoreactivity of human serum with rCat-NPC2 by ELISA

The microplate wells were pre-coated overnight with 100 µL per well of 10 µg/mL rCat-NPC2 at 4°C. Then, the plate was blocked with 1% BSA in PBST at 37°C for 2 hours. Serum samples (1:10 diluted in PBS) were added onto the plate (100 µL/well) and incubated at 37°C for 2 hours. After washing 3 times with PBST, 100 µL of HRP-conjugated goat anti-human IgE (Serotec, Kidlington, UK) (1:2,500 diluted in PBST) was added for another 1 hour incubation. Subsequently, the color was developed using TMB substrate, and the absorbance was measured at 450 nm.

Immunoblot analysis of rCat-NPC2

Purified rCat-NPC2 (10 µg/lane) was separated by 15% SDS-PAGE and eletrotransferred onto polyvinylidene difluoride membranes. After blocking the membrane with 5% milk in PBST for 2 hours, patient serum samples (1:10 diluted in PBS) were used as the primary antibody to incubate the membranes overnight at 4°C. After intensive wash with PBST, the membranes were treated with HRP-conjugated goat anti-human IgE (1:5,000 diluted in PBST) for 1 hour at room temperature with shaking. IgE binding bands were visualized by Immobilon™ Western HRP substrate luminol reagent (Merck KGaA, Darmstadt, Germany) using a chemiluminescent imaging system.

Inhibition ELISA test

The microplate wells were pre-coated overnight with 100 µL per well of 10 µg/mL commercial cat crude extracts at 4°C. Then, each well was blocked with 200 µL of 1% BSA in PBST at 37°C for 2 hours. Pooled sera from 10 Cat-NPC2-allergic patients (1:20 diluted in PBS) was

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pre-incubated with increasing concentrations of the crude extracts and rCat-NPC2 at 37°C for 1 hour. After blocking and PBST washing, the pre-incubated sera were added onto the wells and incubated for another 1 hour at 37°C. Specific IgE was detected using HRP-conjugated goat anti-human IgE (1:2,500 diluted in PBST) and incubated at 37°C for 1 hour. The color reaction was developed using TMB substrate, and the absorbance was measured at 450 nm. The percentage inhibition of rCat-NPC2 was calculated using the following formula: (OD_{450} uninhibited − OD_{450} inhibited)/(OD_{450} uninhibited − OD_{450} control) × 100%.

BAT

BAT is used for the *in vitro* diagnosis of IgE-mediated allergies. C-C chemokine receptor (CCR) 3 and cluster of differentiation (CD) 63 expressions have been considered as an indicator of basophil activation for measuring specific IgE.[22,23] Basically, peripheral blood samples from cat-allergic volunteers were collected on EDTA within 24 hours. Leukocytes were isolated by means of red blood cell lysis and washed twice with PBS. Later, the cells were stimulated with 100 μL of activation buffer supplemented with either anti-IgE or rCat-NPC2 (final concentration, 10 μg/mL) at 37°C for 30 minutes. The stimulation was stopped by adding 900 μL of cold PBS containing 2.5 mM EDTA, followed by centrifugation at 4°C for 5 minutes. After removal of the supernatant, the pellets were resuspended with 100 μL of staining cocktail containing 5 μL of anti-human Alexa647-conjugated CD63 antibody (561983, BD Biosciences, Frankin Lakes, NJ, USA) and 5 μL of phycoerythrin-conjugated CCR3 antibody (184123, eBioscience Inc., San Diego, CA, USA) and thereafter incubated for 20 minutes on ice. Basophil expressions of CCR3 and CD63 were evaluated using a FACSCanto Plus and analyzed using the build-in Diva software (BD Biosciences).

IgE cross-reactivity among rCat-NPC2, rCan f 7 and Der f 2

Inhibition ELISA was performed to evaluate IgE cross-reactivity. First, rCat-NPC2 was immobilized onto the microplate wells overnight at 4°C. A serum pool from 10 patients with high rCat-NPC2 reactivity (1:20 diluted in PBS) were pre-incubated with increasing concentrations of either rCan f 7, rDer f 2 or rCat-NPC2 overnight at 4°C, whereby rCan f 7 and rDer f 2 were obtained as previously described for Cat-NPC2. The pre-incubated sera were used for the further ELISA processes as previously described in inhibition ELISA test section.

Circular dichroism analysis

Circular dichroism analysis of rCat-NPC2 and rCan f 7 was carried out on a Chirascan Circular dichroism spectrometer (Applied Photophysics, Leatherhead, UK) in the 200–260 nm wavelength range. Refolded rCat-NPC2 and rCan f 7 at 0.125 mg/mL were applied in 10-mm path-length quartz cuvettes at 1-nm bandwidth and 0.5 seconds per point. The final spectra were averaged from 10 consecutive scans. The results were presented as mean residue ellipticity in deg × cm^2 × $dmol^{-1}$ and analyzed using the K2D3 program.[24]

Homology modeling

The amino acid sequence alignment among Cat-NPC2, Can f 7 and Der f 2 were performed by CLUSTAL-W. The Cat-NPC2 protein sequence was searched for homology in the Protein Data Bank (PDB, http://www.rcsb.org/). PDB code 5kwy.1.C was used as the search model. The structural models of proteins were generated by SWISS-MODEL (http://swissmodel.expasy.org/interactive). Figures were displayed using Pymol. PROCHECH.[25] ERRAT[26] and VERIFY 3D[27] programs were applied to check the errors in 3D structures.

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Statistical analysis

Analyses were performed using GraphPad Prism version 7.0 software (GraphPad Software, Inc., San Diego, CA, USA). The data was presented as mean ± standard deviation. A *P* value of < 0.05 was considered significant.

RESULTS

Molecular cloning of Cat-NPC2

Cat-NPC2 cDNA fragments were amplified and identified by 1% agarose gel (594 bp, **Fig. 1A**). The DNA sequencing results demonstrated that the gene of Cat-NPC2 was identical as predicted and submitted to Genbank (accession: MN_737596). The open reading frames of the Cat-NPC2 gene had 450 base pairs and encoded 149 amino acids, including a 19-amino acid signal peptide. The putative protein has a molecular mass of 14.22 kDa and a theoretical pI of 8.04.

Expression and purification of rCat-NPC2

The codon-optimized Cat-NPC2 gene (**Fig. 2**) was cloned into the pET28a vector and expressed in *E. coli* BL21 (DE3). The expressed rCat-NPC2 was found almost presented as inclusion bodies in cell lysate by SDS-PAGE. The rCat-NPC2 was purified using Ni-NTA chromatography under denaturing conditions. The calculated molecular weight of rCat-NPC2 along with His-tag is approximately 18.9 kDa. The purified rCat-NPC2 migrated as a single band indicating a purity of 95% (**Fig. 1B**). Gradual removal of denaturant by dialysis with redox refolding buffer was used for protein refolding. We then ran SDS-PAGE under reducing and non-reducing condition and a higher mobility of refolded rCat-NPC2 was observed in non-reducing condition, indicating the presence of disulfide bond formation (**Fig. 1C**). The refolded protein was characterized by circular dichroism analysis (**Fig. 3**). Similar to rCan f 7, the spectra of rCat-NPC2 showed a properly folded protein with a predominant β-sheet feature and the minima was obtained at 212 nm. The secondary structure contents estimated by using the K2D3 program were 0.74% α-helix and 45.94% β-sheets in Cat-NPC2, and 1.43% α-helix

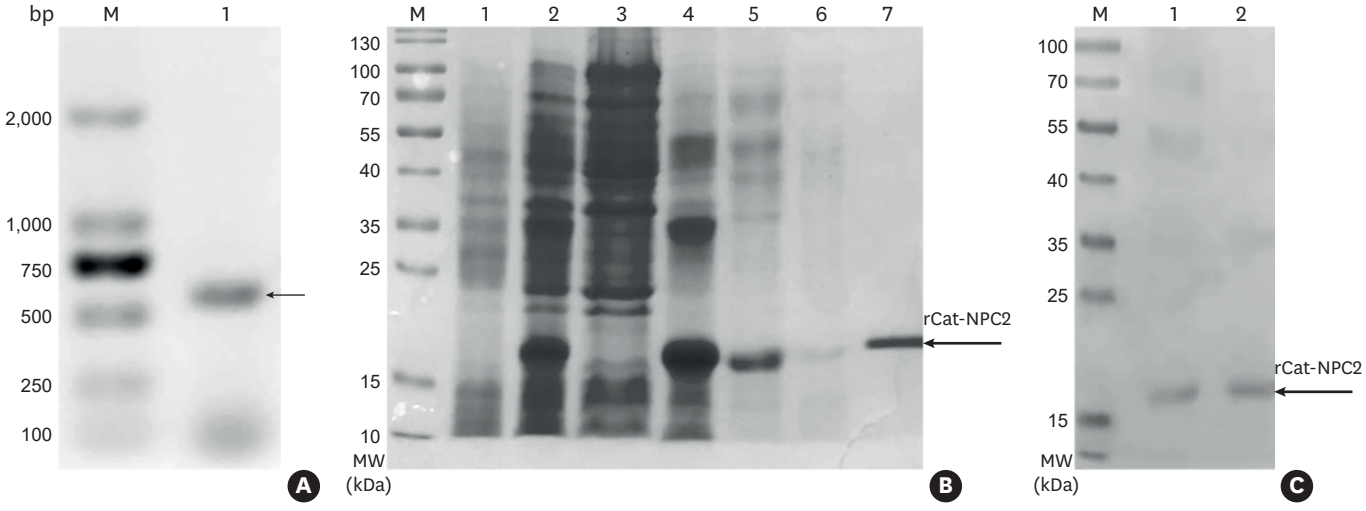

Fig. 1. Cloning, expression and purification of rCat-NPC2 in *E. coli*. (A) The PCR product was separated on 1% agarose gel. Lane M, DNA marker DL2000; lane 1, Cat-NPC PCR product (arrow). (B) The rCat-NPC2 resolved on 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel was visualized with Coomssie Blue G-250. Lane M, standard maker; lane 1, non-induced total cell extracts; lane 2, IPTG-induced total cell extracts; lane 3, soluble fraction of *E. coli* sonicates; lane 4, insoluble fraction of *E. coli* sonicates; lane 5, flow-through elution; lane 6, wash-down elution; and lane 7, refolded purified Cat-NPC2.
NPC2, Niemann pick type C2; rCat-NPC2, recombinant Cat-Niemann pick type C2; PCR, polymerase chain reaction.

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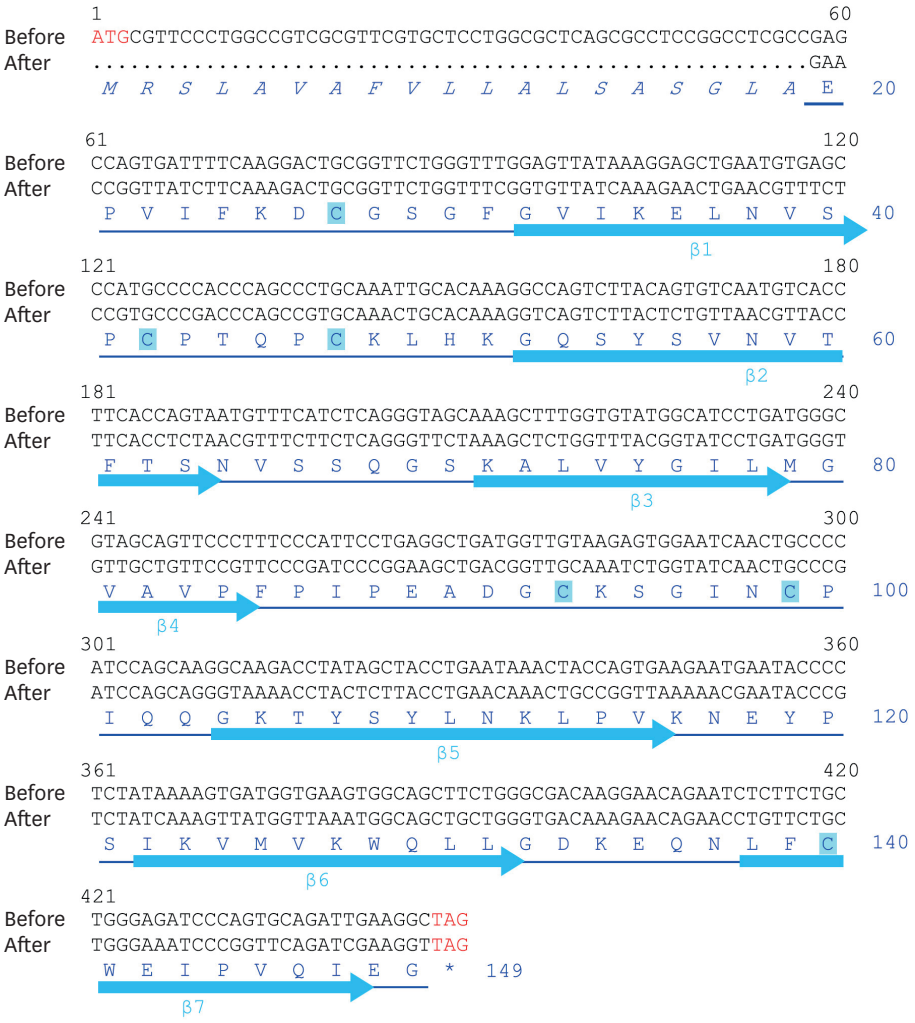

Fig. 2. Complementary DNA and deduced amino acid sequence of Cat-NPC2. Before: the native *Cat-NPC2* gene; After: the codon-optimized *Cat-NPC2* gene. The signal peptide is depicted in italic type. Seven β-strands were identified in *Cat-NPC2* by the Swiss-model. Six conserved cysteine residues were highlighted in blue. NPC2, Niemann pick type C2.

and 43.52% β-sheets in Can f 7. Additionally, the native NPC2 presented in commercial cat dander extracts was confirmed by immunoblot analysis (**Supplementary Fig. S1**).

Cholesterol-binding activity of Cat-NPC2

We assayed the binding of rCat-NPC2 to cholesterol based on ELISA results. The result showed rCat-NPC2 bound to cholesterol in a dose-dependent manner (**Fig. 4**).

IgE reactivity of rCat-NPC2

ELISA and western blot analysis were performed to determine the allergenicity of rCat-NPC2. As shown in data, 28 out of 110 cat-allergic serum samples showed a positive reaction to rCat-NPC2; among them, 16 displayed high positivity (**Fig. 5A**). IgE immunoblot analysis was carried out to quantify the IgE-binding capacity to rCat-NPC2 in a representative group of 17 patients and 3 negative controls (**Fig. 5B**). The binding results mostly complimented the previous results from ELISA. Pooled sera collected from 10 patients with high IgE-binding reactivity to rCat-NPC2 was used in inhibition ELISA test (**Fig. 5C**). The crude extract

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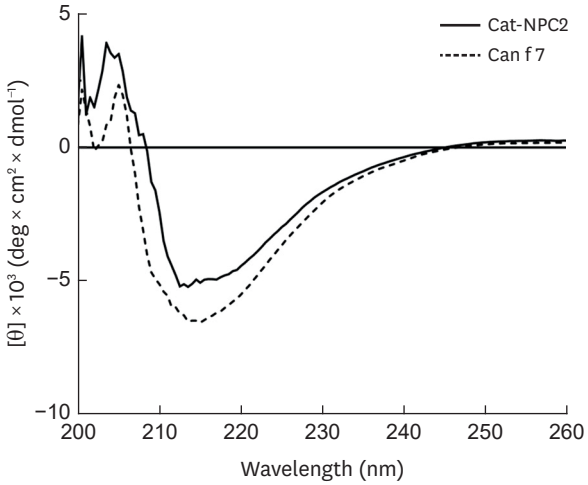

Fig. 3. Circular dichroism analysis of rCat-NPC2 and rCan f 7 expressed in *E. coli*. The far ultraviolet spectra showed that rCat-NPC2 and rCan f 7 were similar in β-sheet structure.
rCat-NPC2, recombinant Cat-Niemann pick type C2.

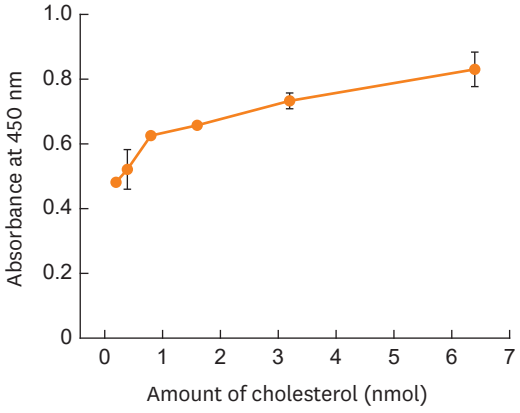

Fig. 4. Binding of rCat-NPC2 to cholesterol. Cholesterol was immobilized on the microplate wells in serial dilutions and incubated with 0.5 μg/mL rCat-NPC2. The bound rCat-NPC2 was probed with rabbit polyclonal IgG anti-Cat-NPC2, followed by anti-IgG linked to horseradish peroxidase. The color was developed using the tetramethylbenzidine substrate, and the absorbance was measured at 450 nm.
NPC2, Niemann pick type C2; rCat-NPC2, recombinant Cat-Niemann pick type C2; Ig, immunoglobulin.

inhibition was used as a positive control. About 20% inhibition rate was shown at a 10 μg/mL concentration of rCat-NPC2. The results indicated that rCat-NPC2 may be a crucial component of crude extracts.

BAT

Fig. 5E shows BATs performed in 3 cat-allergic patients. The proportion (%) of CD63-positive out of CCR3-positive cells was used to determine the basophil activation (**Supplementary Fig. S2**). Anti-IgE was used as a positive control, and buffer was used as a negative control. As shown, an activation of 15.4% to 37.5% was achieved at a concentration of 10 μg/mL rCat-NPC2, suggesting that rCat-NPC2 may have a strong basophil activating capacity.

IgE from Cat-NPC2-sensitized subjects cross-reacts with Can f 7

IgE cross-reactivity was assessed by inhibition ELISA test using rCan f 7 and rDer f 2 due to their sequence similarities (shown in **Supplementary Fig. S3**). Purified rCan f 7 was

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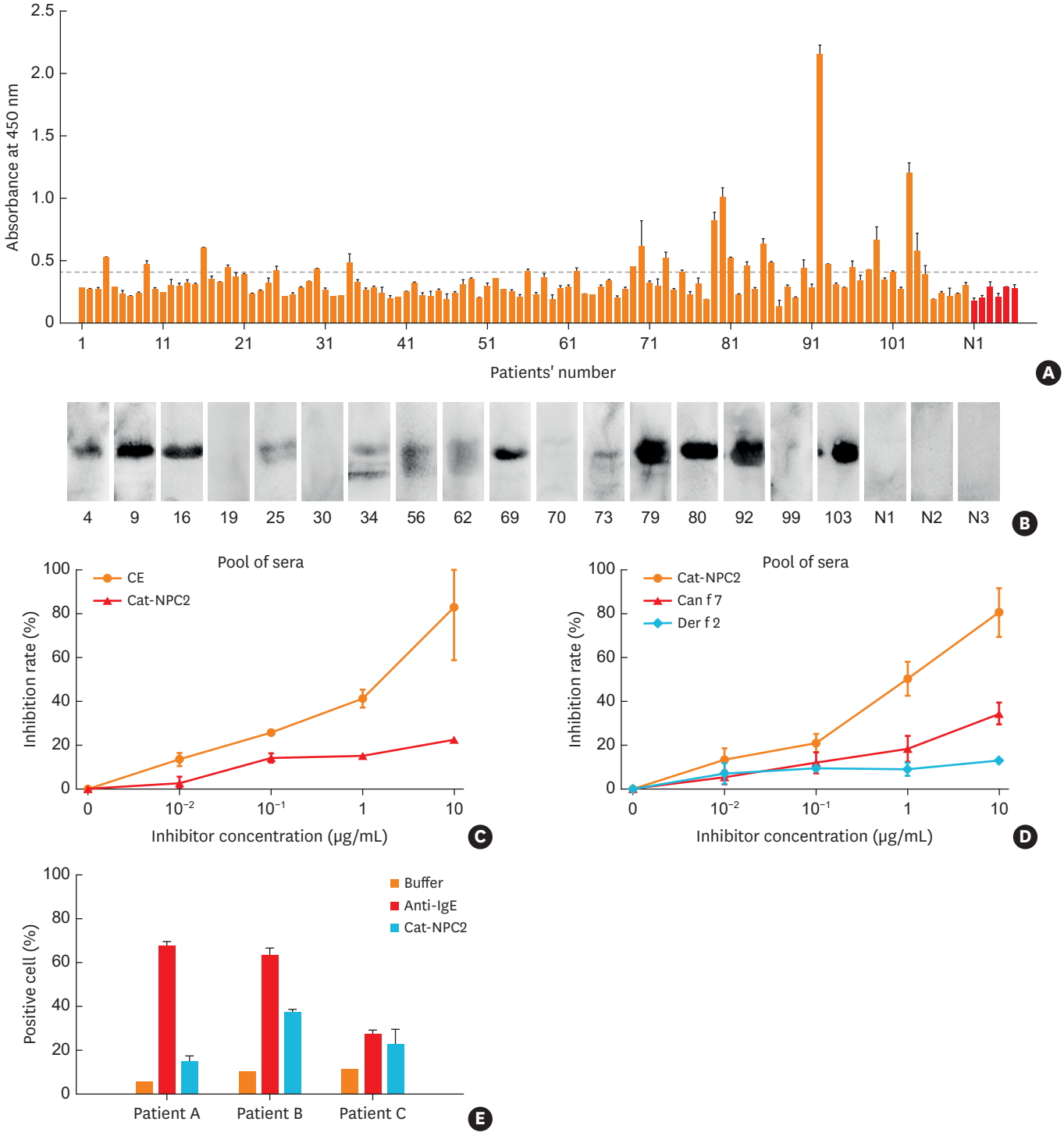

Fig. 5. IgE reactivity of rCat-NPC2. (A) Immuno-reactivity of rCat-NPC2 to IgE was assessed by direct ELISA in 6 non-allergic individuals and 110 allergic patients. The cutoff values are presented as mean OD value ± 3 SDs of negative controls. The data are presented as mean ± SD (n = 3). (B) IgE-binding activity determined by western blot analysis. The rCat-NPC2 was incubated with serum samples of 17 cat-allergic patients and 3 non-allergic individuals. (C) Inhibition ELISA test. Pooled sera from 10 rCat-NPC2 allergic patients (No. 4/9/16/34/69/70/79/80/92/103) was used to conduct the inhibition test. Inhibitor concentrations ranged from 0 to 10 μg/mL. The data are presented as mean ± SD (n = 3). (D) Assessment of NPC2 cross-reactivity by ELISA. Inhibition assay was performed by pre-incubating pool of sera from Cat-NPC2 allergic patients with rCan f 7 or Der f 2, and rCat-NPC2 were used as a positive control. The data are presented as mean ± SD (n = 3). (E) Basophil activation test. Allergen-specific basophil degranulation was assessed by monitoring the basophil activation markers C-C chemokine receptor 3 and CD63. CE, commercial cat dander extracts; NPC2, Niemann pick type C2; Ig, immunoglobulin; rCat-NPC2, recombinant Cat-Niemann pick type C2; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; OD, optical density; CD, cluster of differentiation.

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obtained as previously described.[20] The expression and purification of rDer f 2 are shown in **Supplementary Fig. S4**. Our assay showed that the reactivity to rCat-NPC2 reduced when IgE was pre-inhibited with increasing concentrations of rCan f 7, which clearly demonstrated a dose-dependent cross-reactivity (up to 34%) between rCat-NPC2 and rCan f 7. Although Cat-NPC2 shares only 25.74% sequence identity with HDM allergen Der f 2, the cross-reactivity between these 2 molecules was observed up to 13%.

Homology modeling and validation

A BLAST search against the PDB identified the Epididymal secretory protein E1 from Human (PDB ID 5kwy.1.C) as the closest known molecule to Cat-NPC2 in terms of amino acid sequence (with 81.25% identity). Cat-NPC2 was thus modeled with the known 3D crystal structure as a template by the Swiss-model program and displayed by Pymol. The predicted overall fold of Cat-NPC2 is presented in **Fig. 6**, in comparison with the structure of Can f 7 and Der f 2. The overall structure of Cat-NPC2 was found to consist of 7 β-strands arranged in 2 β-sheets, almost identical to the modeled structure of Can f 7 and similar to the crystal

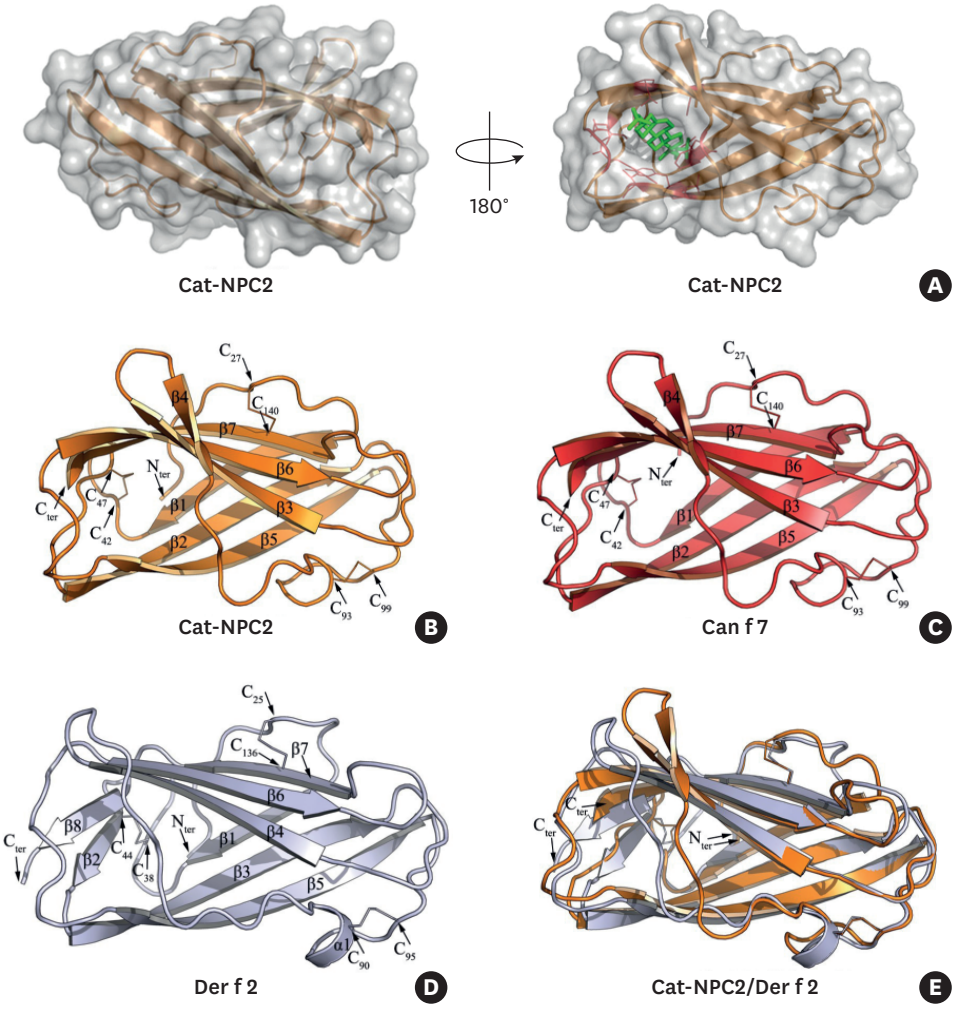

Fig. 6. Homology-based 3D structures of Cat-NPC2, Can f 7 and Der f 2. (A) Structural prediction of a Cat-NPC2 homology model. Residues that form the hydrophobic cavity are in red and relevant ligands are in green. (B) Predicted 3D structure of Cat-NPC2. (C) Predicted 3D structure of Can f 7. (D) 3D structure of Der f 2 (Protein Data Bank ID: 2f08). (E) Superposition of Cat-NPC2 and Der f 2.
3D, 3-dimensional; NPC2, Niemann pick type C2.

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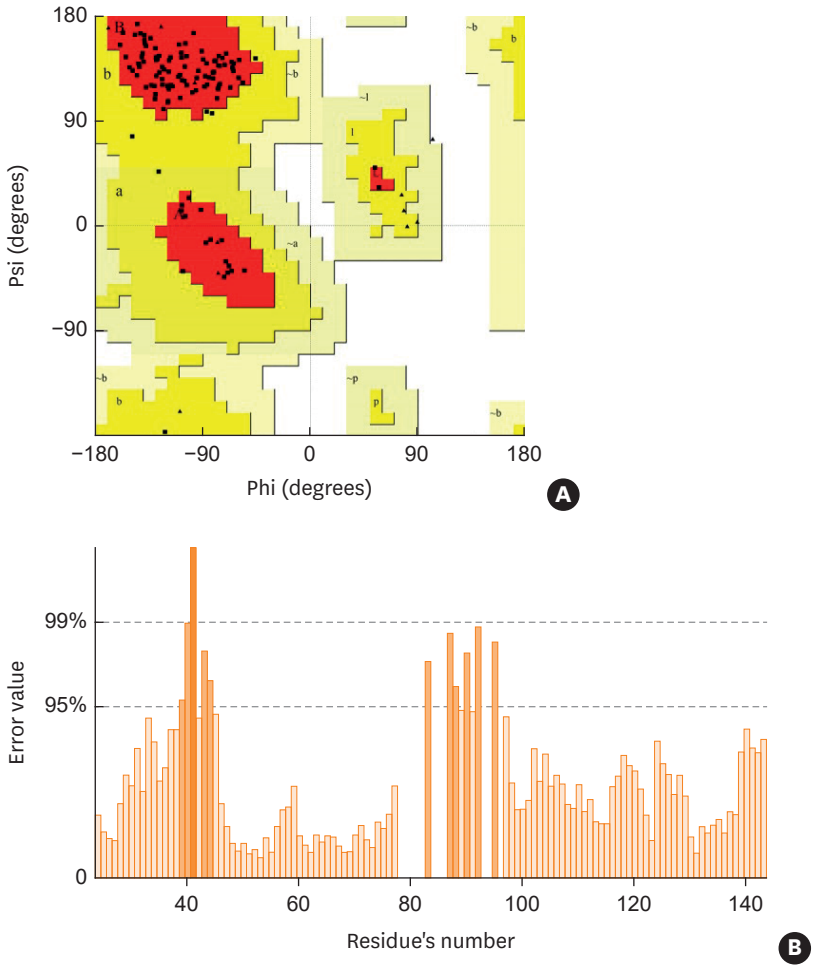

Fig. 7. Validation of the modeled protein structure of Cat-NPC2. (A) Ramachandran plot of a Cat-NPC2 homology model. Residues in favorable regions (red); residues in allowed regions (yellow); residues in generally allowed regions (light yellow); residues in disallowed regions (white). (B) Validation of a Cat-NPC2 homology model by the ERRAT program.
NPC2, Niemann pick type C2; rCat-NPC2, recombinant Cat-Niemann pick type C2.

structure of Der f 2 (PDB ID: 2f08). In addition, the Cat-NPC2 protein has 3 conserved disulfide bonds connecting the residues Cys-27-Cys-140, Cys-42-Cys-47 and Cys-93-Cys-96. As indicated by the Ramachandran plot shown in **Fig. 7A**, 95.2% of the residues in the model structure were within the most favored regions, 4.8% of the residues were in the additional allowed region, and none of the residues were in the generously allowed regions and the disallowed region. The Global Model Quality Estimation and Qualitative Model Energy Analysis values estimated by Swiss-model were 0.80 and 0.83, respectively. Moreover, the overall quality factor was 89.91 by the ERRAT program (shown in **Fig. 7B**) and 95.31% of the residues had an average 3D (atomic model)-1D (amino acid sequence) score ≥ 0.2 by the VERIFY_3D program, indicating that the modeled structure of Cat-NPC2 was favorable and had a relatively high resolution. The above validations illustrated that the generated homology model could be adopted for the study.

Clinical characteristics of the study subjects

Of 110 cat-allergic patients, the majority (80.91%) were diagnosed with rhinitis. In addition, 39.09% had asthma, 16.36% had conjunctivitis and 4.55% had conjunctivitis (**Table**). For

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Table. Clinical characteristics of 110 cat-allergic participants in the study

Clinical characteristics	Allergic patient (n = 110)
Age (yr)	
Mean ± SD (range)	24.81 ± 11.85 (1–52)
0–12	27 (24.54)
13–18	19 (17.27)
19–39	54 (49.09)
> 39	10 (11.00)
Sex	
Males	53 (48.18)
Females	57 (51.82)
Serum IgE level (kU/L)	
Median (min–max)	8.35 (0.76–100)
Allergic condition	
Rhinitis	89 (80.91)
Asthma	43 (39.09)
Dermatitis	18 (16.36)
Conjunctivitis	5 (4.55)
Single allergic condition	69 (62.73)
Multiple allergic condition	41 (37.27)

Allergic conditions after contact with cat were recorded according to patient history. Specific IgE levels were measured by commercial ImmunoCAP for *Felis domesticus*. Specific IgE values ≥ 0.35 KU/L were considered positive. Allergic conditions consist of asthma, rhinitis, dermatitis and conjunctivitis.
Ig, immunoglobulin.

IgE reactivity to Cat-NPC2, clinical characteristics across ages and levels of specific IgE were studied. There were no statistically significant differences between age groups and levels of specific IgE. However, IgE recognition of Cat-NPC2 was more frequently observed in females in all age groups, indicating a gender difference in Cat-NPC2-associated allergy (shown in **Fig. 8**).

DISCUSSION

The prevalence of allergy has been dramatically increasing over the past decade. Pet-derived allergens, in particular cat and dog, affect 7% to 25% of the allergic patients.[28] A deep understanding of cat allergens is crucial for standardizing allergen products for clinical diagnosis and therapy. In this study, we identified and characterized a new cat allergen NPC2 protein, a homolog of dog NPC2 protein which has been identified as Can f 7 allergen by WHO/IUIS and human NPC2 protein. Of note, in the Allergome database, the NPC2 proteins are specific on the list, because they constitute the most important HDM allergen group group 2 allergens from 12 mite species which are 14-kDa non-glycosylated members of the NPC2 family.[29]

The Cat-NPC2 gene has previously been predicted by automated computational analysis, but has not confirmed yet. In the present study, we amplified Cat-NPC2 cDNA and established its amino acid sequence for the first time. The mature Cat-NPC2 protein contains 130 amino acids residues with a theoretical molecular weight of 14.4 kDa. Then, the codon-optimized Cat-NPC2 gene was cloned and over-expressed as inclusion bodies in *E. coli*. About 95% purity of rCat-NPC2 obtained through Ni-NTA chromatography was assessed by SDS-PAGE. Gradual removal of urea by dialysis and the GSH/GSSG redox system were used for disulfide bond formation. Disulfide bonds have a high impact on the structural conformation and therefore the IgE-binding reactivity of the protein. Reduced and oxidized glutathione (GSH, GSSG) we used were widely applied for correct formation of disulfide bond.[20,30,31] Moreover, slight difference in the refolded rCat-NPC2 mobility was observed under the non-reducing

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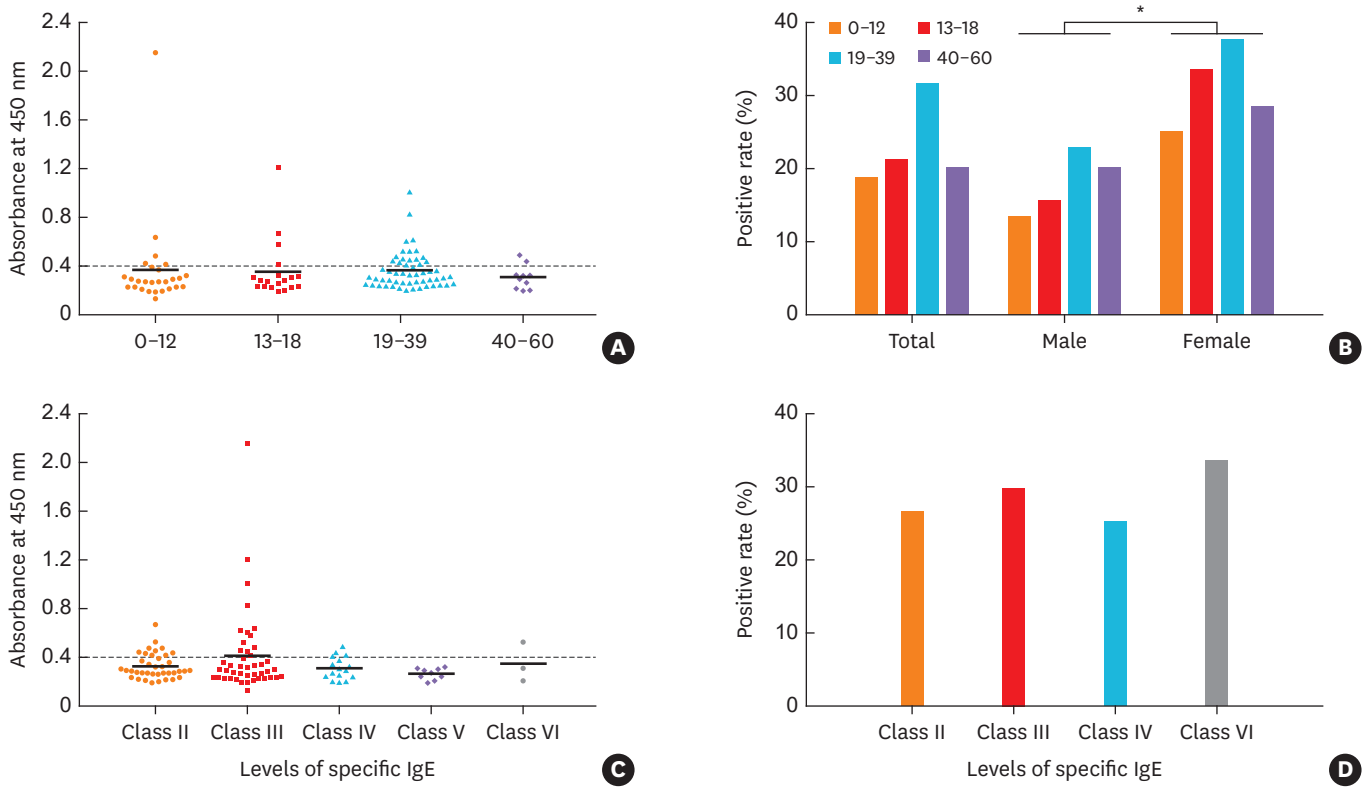

Fig. 8. Clinical analysis of 110 cat-allergic patients in the study. (A) IgE reactivity to Cat-NPC2 in different age groups. (B) Frequency of sensitized Cat-NPC2 and sex distribution in different age groups. (C) IgE reactivity to Cat-NPC2 and levels of specific IgE. (D) Frequency of sensitized Cat-NPC and levels of specific IgE. The mean value is shown as a red bar; the cutoff value was shown as a dotted bar.
OD, optical density; Ig, immunoglobulin; NPC2, Niemann pick type C2.
*$P < 0.05$.

condition compared to the reducing one, due to the presence of disulfide bonds causing more compact structures and higher mobility. Finally, NPC2 is a lipid transfer protein. The lipid-binding assay showed direct evidence that rCat-NPC2 could bind to cholesterol in a dose-dependent manner, further verifying the successful construction of rCat-NPC2. As Cat-NPC2 and Can f 7 are similar in sequence, a similar β-sheet fold was observed by far-UV circular dichroism spectra. The presence of the native NPC2 in commercial cat dander extracts was determined using WB with specific polyclonal anti-rabbit IgG. Additionally, the inhibition ELISA results showed that the IgE-binding capacity to crude extracts was inhibited by rCat-NPC2, which further confirming the native NPC2 protein in cat extracts.

ELISA analysis revealed that rCat-NPC2 has capacity to bind specific IgE in at least 14.5% (16/110) of cat allergic patients. Accordingly, the number of individuals with IgE-binding to each component is variable. It is generally accepted that Fel d 1 is the most important allergen with about 96% IgE binding,[14] whereas Fel d 4 is the second one with up to 60% of the cat-allergic individuals.[32] As for other listed allergens, IgE-binding reactivity ranged from 10% to 38%.[33] Additionally, as a homologue of Cat-NPC2, Can f 7 has recently been identified as a dog allergen component, with an apparent seroprevalence of 10% to 20%.[19] The findings suggested that Cat-NPC2 protein may play an essential role in cat allergy. Meanwhile, western blot analysis further confirmed the positive IgE-binding activity of Cat-NPC2. The differences in IgE-binding intensity between ELISA and western blot results indicated that sequential epitopes could play an important role in the degree of IgE recognition of Cat-NPC2. The

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allergenicity of rCat-NPC2 indicated the identification of native Cat-NPC2 as an allergen. The inhibition ELISA test suggested that rCat-NPC2 can abolish up to 20% of specific IgE-binding capacity to crude extracts. However, the inhibition rate varies among crude extracts. Moreover, the commercially available cat dander extracts used in the test have not been standardized and may contain inhibitory preservatives, resulting in unstable serum IgE-binding to the crude extracts preparation. In addition, BAT was performed as a functional *in vitro* assay to quantitatively evaluate basophilic degranulation responding to allergen stimulation.[34] CCR3 expression is highly and constitutionally expressed on the surface of basophils, which is used to identify basophils.[35] Once challenge with specific allergens, the up-regulated expression of CD63 is found to quantify basophil activation. Compared to the negative control, the increased proportion of $CCR3^{+}CD63^{+}$ revealed that Cat-NPC2 has ability to activate basophils, leading to allergic symptoms. Although the glycosylation of Can f 7 was confirmed in *Pichia pastoris*, a previous study reported that the non-glycosylated form of NPC2 expressed in *E. coli* had equal ability to bind specific IgE.[19] Thus, as a homologue of Can f 7, the glycosylation of Cat-NPC2 was not considered in allergy activity. However, we cannot absolutely eliminate the influence of glycosylation on IgE reactivity. Therefore, more studies on the comprehensive structure and function of native Cat-NPC2 are warranted.

In our study, 80.91% of the tested patients were diagnosed with rhinitis. According to a recent study, the most frequent allergic disease entities of cat-allergic patients are rhinitis (80.0%), followed by conjunctivitis (73.3%) and cutaneous symptoms (33.3%).[36] Interestingly, in all tested patients, there was a gender difference between Cat-NPC2-associated allergic disease entities. In fact, striking sex-specific biases have been observed in IgE-mediated allergic diseases such as respiratory allergies,[37] food allergy[38] and anaphylaxis.[39] Sexual hormones are thought to have the most significant effect.[40] Further studies on the relationships between sexes and allergic diseases are warranted. Moreover, in spite of the investigation into the IgE recognition of Cat-NPC2 in Chinese clinical samples, more information is required across broader regions in the future, considering the increased population of pet cat around the world.

Since Can f 7 shares 78% sequence identity and has a similar fold structure with Cat-NPC2, it clearly indicates possible cross-reactivity between the 2 proteins. In the ELISA-inhibition assay, the result showed that IgE reactivity to Cat-NPC2 was almost reduced by pre-inhibition of Can f 7. The fact that cat-allergic patients without dog allergy showed IgE antibody responses to Can f 7 suggested Cat-NPC2 highly cross-reacted with Can f 7. The cross-reactivity observed between Der f 2 and Cat-NPC2 also indicated a possible association between sensitizations to cat, dog and HDM.

To better understand the structure and function, we analyzed the 3D structures of Cat-NPC2. Homology modeling was applied to investigate Cat-NPC2 and found that the structure of 5kwy.1.C was the most appropriate template with highest identity. The build model structure is feasible by Ramachandran plot analysis, ERRAT program and VERIFY_3D program analysis, which showed that the modeled structure was adopted. The homology modelling and validation methods have been widely applied in other allergen studies of Der f 25,[41] Pla a 3,[42] Ole 12,[43] and Per a 9.[44]

NPC2 protein was first characterized in the human epididymis as a major secretory protein containing 132 amino acid residues.[45] In mammalian NPC2 proteins, the primary sequences of bovine, murine, macaque, porcine and canine proteins has been well described with

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6 conserved cysteine residues, 3 putative glycosylation sites and a proline-rich region.[46] A recent study reported that the hydrophobicity of knob domain is conserved among mammalian NPC2 proteins.[47] Based on the primary sequence study of Can f 7 and Der f 2, the Cat-NPC2 protein contains 6 conserved cysteine residues that form 3 disulfide bonds connecting the residues Cys-27-Cys-140, Cys-42-Cys-47 and Cys-90-Cys-93. As predicted, the hydrophobic cavity in Cat-NPC2 is formed primarily by the side chain of the residues V39, Y55, F85, L113, V115, Y119, P120, L124, I143 and I147, which bound to CHOLEST-5-EN-3-YL HYDROGEN SULFATE (C3S). As the lack of conserved glycosylation sites, the primary sequence of cat NPC2 has 25.74% identity and 48% similarity to Der f 2. Based on the initial findings, the NPC2 protein is also known to play an important role in cholesterol binding.[48,49] The data available indicated that NPC2 mutants may cause high accumulation of cholesterol in lysosomes, leading to Niemann-Pick disease type C2 which is a fatal hereditary disease.[49] However, there are still major gaps in our knowledge of the precise function of NPC2 protein.

In conclusion, the study identified Cat-NPC2 protein as a novel allergen associated with cat-allergic patients. We confirmed the presence of native NPC2 in cat dander extracts as well as cloned expressed and characterized the rCat-NPC2. The allergenicity of Cat-NPC2 has been proven by immunological analysis. Based on sequence homology, Cat-NPC2 was found to cross-react with Can f 7 and Der f 2 as well. The identification of Cat-NPC2 and the appearance of the recombinant form of Cat-NPC2 would be a useful tool for the treatment of cat and dog allergy.

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SUPPLEMENTARY MATERIALS

Supplementary Table S1

The clinical characteristics of 110 cat-allergic participants in the study

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Supplementary Fig. S1

Detection of native Cat-NPC2 in commercial cat dander extracts. The binding of the native NPC2 in cat dander extracts around 18.9 kDa was observed by WB with specific polyclonal anti-rabbit immunoglobulin G, which produced in house. The New Zealand White Rabbit was subcutaneously immunized with 1 mg of rCat-NPC2 in Freund's complete adjuvant, followed by subcutaneous boosts of rCat-NPC2 0.5 mg in incomplete Freund's adjuvant. Lane M, standard maker; lane 1, native Cat-NPC2.

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Supplementary Fig. S2

Basophils activation test. The basophil population was determined by positivity to CCR. Basophil activation was identified by the dual positivity to CD63 and CCR3. (A) Basophil gating strategy. (B) Representative flow cytometry plots of 3 cat-allergic patients' basphils stimulated with either buffer, anti-IgE or rCat-NPC2.

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Supplementary Fig. S3

Multiple sequence alignment of Cat-NPC2, Can f 7 and Der f 2. The identical and conserved sequences were highlighted by red and white blocks, respectively. The sequence identity was 78.5% with Can f 7 and 25.74% with Der f 2 at the amino acid level.

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Supplementary Fig. S4

Expression and purification of rDer f 2 in *E. coli*. The rDer f 2 resolved on a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel was visualized with Coomssie Blue G-250. (A) Expression of crude rDer f 2. Lane M, standard maker; lane 1, non-induced total cell extracts; lane 2, IPTG-induced total cell extracts; lane 3, soluble fraction of *E. coli* sonicates; and lane 4, insoluble fraction of *E. coli* sonicates. (B) Purified rDer f 2 from Ni-NTA column. Lane M, standard maker; lane 1, flow-through elution; lane 2, washing with 10 mM imidazole; lane 2, washing with 100 mM imidazole; and lane 3, washing with 250 mM imidazole. The rDer f 2 was denoted with an arrow.

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