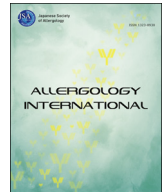




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Original Article

Human eosinophils constitutively express a unique serine protease, PRSS33

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Ab, antibody; ADAM, a disintegrin and metalloprotease; CCR, CC chemokine receptor; COL, collagen; DAPI, 4,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ECP, eosinophil cationic protein; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GM-CSF, granulocyte macrophage colony-stimulating factor; HSA, human serum albumin; IFN- γ , interferon- γ ; Ig, immunoglobulin; IL-, interleukin-; IMEM, Iscove's minimum essential medium; MMP, metalloproteinase; PAR-2, protease-activated receptor-2; PBMCs, peripheral blood mononuclear cells; PIC, protease inhibitor cocktail; PRSS33, serine protease 33; qPCR, quantitative polymerase chain reaction; sIgA, secretory IgA; TGF- β 1, transforming growth factor- β 1

ABSTRACT

Background: Eosinophils play important roles in asthma, especially airway remodeling, by producing various granule proteins, chemical mediators, cytokines, chemokines and proteases. However, protease production by eosinophils is not fully understood. In the present study, we investigated the production of eosinophil-specific proteases/proteinases by transcriptome analysis.

Methods: Human eosinophils and other cells were purified from peripheral blood by density gradient sedimentation and negative/positive selections using immunomagnetic beads. Protease/proteinase expression in eosinophils and release into the supernatant were evaluated by microarray analysis, qPCR, ELISA, flow cytometry and immunofluorescence staining before and after stimulation with eosinophil-activating cytokines and secretagogues. mRNAs for extracellular matrix proteins in human normal fibroblasts were measured by qPCR after exposure to recombinant protease serine 33 (PRSS33) protein (rPRSS33), created with a baculovirus system.

Results: Human eosinophils expressed relatively high levels of mRNA for metalloproteinase 25 (MMP25), a disintegrin and metalloprotease 8 (ADAM8), ADAM10, ADAM19 and PRSS33. Expression of PRSS33 was the highest and eosinophil-specific. PRSS33 mRNA expression was not affected by eosinophil-activating cytokines. Immunofluorescence staining showed that PRSS33 was co-localized with an eosinophil granule protein. PRSS33 was not detected in the culture supernatant of eosinophils even after stimulation with secretagogues, but its cell surface expression was increased. rPRSS33 stimulation of human fibroblasts increased expression of collagen and fibronectin mRNAs, at least in part via protease-activated receptor-2 activation.

Conclusions: Activated eosinophils may induce fibroblast extracellular matrix protein synthesis via cell surface expression of PRSS33, which would at least partly explain eosinophils' role(s) in airway remodeling.

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Introduction

The pathogenesis of asthma is characterized by repeated exacerbation of type 2 inflammation due to exposure to allergens, viral infection and so on. Recurrent and/or chronic type 2 inflammation reportedly induces structural changes in the lung (so-called airway remodeling), including goblet cell hyperplasia, basement membrane thickening, smooth muscle hypertrophy/hyperplasia, tissue fibrosis and hypervascularity.^{1–4} At least some components of this airway remodeling are steroid-insensitive,⁵ and airway remodeling causes early lung function decline. Prevention of airway remodeling is one of the major unmet needs in current asthma practice.⁶

Both leukocytes and tissue resident cells are involved in airway remodeling through complicated interactions among cytokines, chemokines, chemical mediators and proteases/proteinases. Eosinophils are known to play important roles in the pathogenesis of asthma, especially in airway remodeling.^{6–8} Eosinophils produce and release several growth factors, including vascular endothelial growth factor (VEGF),⁹ transforming growth factor- β 1 (TGF- β 1)¹⁰ and amphiregulin,¹¹ and some proteases.

Proteases/proteinases not only facilitate replacement of soft tissue extracellular matrix proteins (EMP) with hard EMP, but also directly activate protease-activated receptor-2 (PAR-2) to trigger proliferation of airway smooth muscle cells.^{12,13} To date, various proteases/proteinases have been reported, but only a few—such as matrix metalloproteinase-9 (MMP-9)¹⁴ and MMP17¹⁵—have been reported to be produced by human eosinophils. Recently, eosinophil-targeted intervention therapy for bronchial asthma using anti-IL-5 or anti-IL-5R mAbs was approved. In that context, there is a need for a better understanding of the proteases produced specifically by eosinophils.

In the present study, we investigated the mRNA expression profiles of all proteases/proteinases in human eosinophils and other leukocytes by transcriptome analysis.

Methods

Reagents

All culture reagents were purchased from Life Technologies (Grand Island, NY) unless otherwise noted. Recombinant human IL-5 was purchased from R&D Systems (Minneapolis, MN). Recombinant human granulocyte-colony stimulating factor (GM-CSF), IFN- γ and macrophage-colony stimulating factor (M-CSF) were purchased from PeproTech (Rocky Hill, NJ).

Recombinant human serine protease 33 (PRSS33) was synthesized at Sysmex Corporation (Kanagawa, Japan) using a baculovirus gene expression system based on the reference sequence (NM_152891.2).

Isolation of leukocytes

Each type of leukocyte was isolated from peripheral blood of both healthy and mildly allergic donors ($n = 10$) by density gradient sedimentation using Lymphocyte Separation Medium (Wako Pure Chemical Industries, Osaka, Japan) or Percoll (GE Healthcare, Piscataway, NJ), and also by positive and/or negative selection using immunomagnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany), as described previously.^{11,16} The purity of eosinophils based on light microscopic examination of cytocentrifuge preparations using Cytospin (Shandon, Pittsburgh, PA) and staining with Diff-Quik (American Scientific Products, McGraw Park, IL) always exceeded 98%. Eosinophil viability always exceeded 99% by trypan blue (Sigma) dye exclusion. The purity of other types of blood cells always exceeded 95%.

Culture of eosinophils

Purified eosinophils were suspended at a cell density of 1×10^6 cells/ml in Iscove's minimum essential medium (IMEM), supplemented with 10% heat-inactivated fetal calf serum (FCS; Equitech-Bio, Kerrville, TX), 5×10^{-5} M 2-mercaptoethanol and an antibiotics mixture (10 units/ml penicillin G and 10 μ g/ml streptomycin; Nacalai Tesque, Kyoto, Japan). The cells were cultured in PBS at 4 °C overnight in 24-well flat-bottom plastic plates (IWAKI, Tokyo, Japan) pre-coated with 1% heat-denatured human serum albumin (HSA; Sigma–Aldrich, St. Louis, MO) to reduce non-specific adherence of eosinophils to the plates.¹⁷ To examine the effects of various stimulants, the cells were cultured in the presence and absence of various concentrations of IL-5, 10 ng/ml GM-CSF or 10 ng/ml IFN- γ at 37 °C for 6 h. In some experiments, the cells were cultured at 4 °C overnight in 96-well flat-bottom plates (IWAKI) coated with 100 μ g/ml secretory IgA (slgA; ICN Biomedicals, Aurora, OH). After washing the wells, 0.2 ml of 1% heat-denatured HSA in PBS was added to each well, and the plates were incubated at 4 °C for at least 2 h before use.¹¹

Preparation of monocyte-derived macrophages

Monocyte-derived macrophages were obtained as described previously.¹⁸ Briefly, PBMCs were suspended at a density of 2×10^6 cells/ml in RPMI 1640 medium (Nacalai Tesque) supplemented with 10% FCS in T75 flasks (IWAKI) and incubated at 37 °C for 1 h. The adherent cells (mainly monocytes) were obtained after removal of non-adherent cells by gentle pipetting and washed once with PBS. To obtain macrophages, the adherent cells were then cultured in the presence of 10 ng/ml M-CSF in T75 flasks at 37 °C for 7 days.

Macrophage-like U-937 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The U-937 cells were subcultured twice per week in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 10 mM Hepes buffer, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 1.0 mM sodium pyruvate and the previously described antibiotics mixture at 37 °C in a 5% CO₂ incubator. For assay, these cells were treated with 160 nM phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich, St. Louis, MO) for 2 or 5 days, as described previously by others.¹⁹

Culture and stimulation of fibroblasts

Human nasal fibroblasts were obtained from normal mucosal membranes of the sphenoid sinus removed during surgery for pituitary adenoma as previously described,²⁰ with slight modification. In brief, extensively dissected nasal tissue pieces were cultured in Dulbecco's modified Eagle's medium/F-12 (DMEM/F12) medium supplemented with 10% FCS and the antibiotics mixture without digestive enzymes. Cultured cells were analyzed between the third and eighth passages. The fibroblasts (1×10^5 cells/ml) were cultured in DMEM/F12 supplemented with an antibiotics mixture, but without FCS, one day before planned stimulation. On the next day, the fibroblasts were cultured in the presence and absence of 25 ng/ml recombinant human PRSS33, a 0.1% protease inhibitor cocktail (PIC) (Sigma–Aldrich) or 10 μ M FSLRT-NH₂, a PAR-2 antagonist (Tocris, Ellisville, MO), at 37 °C for 24 h. The cells were then harvested, and the total RNA was extracted.

Microarray analysis

Transcriptome analysis using a microarray system was performed as described previously.^{21,22} Briefly, total RNA from leukocytes, excluding eosinophils, was extracted and then digested using

RNeasy (Qiagen, Valencia, CA) and RNase-free DNase I (Qiagen), respectively, according to the manufacturer's instructions. Separately, eosinophils were first lysed in Isogen (Nippon Gene, Toyama, Japan) according to the same protocol as used for the other leukocytes. cRNA was prepared from 5 µg of the total RNA. To normalize the results and obtain 5 µg of total RNA for each cell type, equal amounts of total RNA from 3 to 8 separate donors were mixed. Using the cRNA, gene expression was examined with GeneChip Human Genome U133 plus 2.0 probe arrays (Affymetrix, Santa Clara, CA), which contain the oligonucleotide probe sets for 54,120 full-length genes and expressed sequence tags, according to the manufacturer's protocol. GeneSpring software version 7.2 (Silicon Genetics, Redwood City, CA) was used. To normalize the staining intensity variation among the chips, the average difference in values for all the genes on a given chip was divided by the median expression value for all the genes on the chip. To eliminate genes whose expression represented only background noise, genes were selected only if the raw data was <100, and if the gene expression was judged to be 'present' by GeneChip Analysis Suite 5.0 (Affymetrix).

Real-time quantitative PCR

The primer sets for PRSS33 (sense, 5'-CAGAGTCCAAGCCC-TAGGCA-3'; and antisense, 5'-CCAACGATCCGACTGGACA), CCR3 (sense, 5'-ATGCTGGTGACAGAGGTGAT-3'; and antisense, 5'-AGGT-GAGTGTGGAAGGCTTA-3'), IL-8 (sense, 5'-GTCTGCTAGCCAGGATC-CACAA-3'; and antisense, 5'-GAGAAACCAAGGCACAGTGGAA-3'), CD68 (sense, 5'-CGACAGAGCCAGACTGTCTCAA-3'; and antisense, 5'-CCTTCTCCGACTGCATTATCTC-3'), β -actin (sense, 5'-CCCAGC-CATGTACGTTGCTAT-3'; and antisense, 5'-TCACCGAGTCCATCAC-GAT-3'), RSP18 (sense, 5'-CATGTGGTGTGAGGAAAG-3'; and antisense, 5'-CTTGACTGGCGTGGATTC-3'), collagen 1a1 (COL1A1) (sense, 5'-GACCTGCGTGTACCCACTC-3'; and antisense, 5'-CCG-CCATACTCGAACTGGAAT-3'), COL8A1 (sense, 5'-TGGCAAA-GAGTATCCACACTACC-3'; and antisense, 5'-TTCCCTCGTAAAC-TGGCTAATG-3'), fibronectin (sense; 5'-CTTGAACCAACCTACGGAT-GACT-3'; and antisense; 5'-ATTTCGTTCCACTCATCTCAA-3'), transforming growth factor- β 1 (TGF- β 1: sense; 5'-ACTGCAAGTG-GACATCAACG-3'; and antisense; 5'-TGGCCATGAGAAGCAGGAAAG-3'), versican (sense; 5'-GCACCTGTGTGCCAGGATA-3'; and anti-sense; 5'-CAGGGATTAGAGTGACATTCATCA-3') and α smooth muscle actin (α SMA: sense; 5'-CTGTTCCAGCCATCCTTCAT-3'; and anti-sense; 5'-CCGTGATCTCCTTCTGCATT-3') were synthesized at FAS-MAC. Human universal reference (HUR) RNA (BD Biosciences, Palo Alto, CA) was used as a positive control. First-strand cDNA was synthesized from the isolated RNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). To determine the exact copy number of each target gene, standards were prepared for use in each experiment by serially diluting quantified concentrations of the purified PCR product of each gene. Aliquots of cDNA equivalent to 5 ng of the total RNA samples were used for each quantitative PCR (qPCR). The mRNA expression levels of the target genes were normalized to those for β -actin in each sample. Real-time quantitative PCR analyses were performed using the CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-Rad) and SYBR Green I PCR reagents (Toyobo, Osaka, Japan).

Flow cytometric analysis

Cells were incubated with 10 µl of 5 mg/ml human IgG in FACS buffer (Hanks' solution containing 2% FCS) for 20 min on ice. The cells were washed and then fixed by resuspending in 200 µl of 4% formaldehyde (Wako) in PBS and incubating at r.t. for 20 min. Then, for permeabilization, the cell pellet was incubated at r.t. for 10 min after addition of a 200-µl aliquot of 0.3% Tween 20 (Sigma–Aldrich)

in PBS. After washing, the cells were incubated in FACS buffer containing 10 µg/ml goat anti-human PRSS33 polyclonal Ab (Santa Cruz Biotechnology), at 4 °C overnight. After washing with FACS buffer, the cells were incubated with 2.5 µg/ml allophycocyanin (APC)-conjugated AffinPure F(ab')₂ fragment donkey anti-goat IgG (H + L) (Jackson ImmunoResearch, West Grove, PA) for 15 min on ice. After final washing with FACS buffer, the cells were analyzed using a FACSCanto II (Becton Dickinson, San Jose, CA) and FlowJo software (Tomy Digital Biology, Tokyo, Japan).

Immunofluorescence staining

Cells were placed on microscope slides by centrifugation with Cytospin (Shandon, Pittsburgh, PA) and fixed with 4% formaldehyde (Wako) in PBS for 20 min. In some experiments, cells were permeabilized with 0.3% Tween 20 (Sigma–Aldrich) in PBS for 10 min. The cells were then incubated with 4 µg/ml goat anti-human PRSS33 polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA) and 4 µg/ml rabbit anti-human ECP polyclonal Ab (Santa Cruz Biotechnology) in PBS at 4 °C overnight. After washing with PBS, the cells were incubated with 10 µg/ml Alexa Fluor 594-conjugated donkey anti-goat IgG (Invitrogen, Carlsbad, CA) at 4 °C for 2 h in the dark. After washing with PBS, the cells were incubated with 20 µg/ml Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) in 15 mg/ml goat serum at 4 °C for 2 h in the dark. After final washing with PBS, coverslips were mounted onto the slides using SlowFade Gold anti-fade reagent with 4,6-diamidino-2-phenylindole (DAPI, Invitrogen), and the slides were stored at 4 °C in the dark until examination. The cells were analyzed using a confocal laser-scanning microscope system (Olympus FV1200, Tokyo, Japan) with SlideBook software (Olympus).

ELISA

After designated culture periods, culture supernatants were centrifuged to eliminate contaminating cells. The cell pellets were freeze-thawed three times to obtain cell lysates. The concentrations of PRSS33 and eosinophil-derived neurotoxin (EDN) in the cell lysates or culture supernatants were measured using specific ELISA kits for human PRSS33 (detection limit > 0.312 ng/ml; USCN Life Science, Wuhan, China) and human EDN (detection limit > 0.164 ng/ml Immundiagnostik AG, Bensheim, Germany), respectively, according to each manufacturer's protocol.

Ethics and statistical analysis

The human nasal tissue samples and PBMCs used in this study were obtained from the Department of Otorhinolaryngology, Jikei University School of Medicine (Tokyo, Japan), and the National Center for Child Health and Development (NCCHD, Tokyo, Japan), respectively, after receiving written informed consent from each subject. This study was approved by the ethics boards of Jikei University School of Medicine and NCCHD.

All the data are presented as the median and 90th percentile unless otherwise indicated. Differences between the groups were analyzed using Mann–Whitney's *U* test after Kruskal–Wallis analysis, with Prism software (GraphPad Software, San Diego, CA). Differences were considered significant if *P* < 0.05.

Results

PRSS33 is expressed constitutively in human eosinophils

First, the expression levels of protease/proteinase mRNA in various types of leukocytes were comprehensively determined by

transcriptome analysis. As shown in Table 1, freshly-isolated peripheral blood eosinophils constitutively expressed mRNA for PRSS33, metalloproteinase 25 (MMP25), a disintegrin and metalloprotease 8 (ADAM8), ADAM10 and ADAM19 (raw expression levels > 1000). The complete list of all proteases and proteinases can be found in Supplementary Table 1. Among these genes, the expression level of PRSS33 mRNA was the highest and was more than 10-times higher than in any of the other cell types.

qPCR analyses confirmed the microarray data showing that freshly-isolated peripheral blood eosinophils, but not neutrophils, expressed a large amount of PRSS33 mRNA (Fig. 1A). In agreement with the mRNA expression, the lysate of freshly-isolated peripheral blood eosinophils contained approximately 48 ng of PRSS33 protein per 10^6 cells by ELISA, whereas neutrophils did not (Fig. 1B).

Next, we used qPCR to examine the PRSS33 mRNA expression level in eosinophils after exposure to various eosinophil-activating cytokines. The PRSS33 mRNA expression level was not affected by exposure of the eosinophils to IL-5, GM-CSF or IFN- γ (Fig. 1C).

No PRSS33 release from activated eosinophils

The mean concentration of EDN in the supernatants of eosinophils cultured on plates coated with sIgA was almost 60% of the total EDN amounts in the cell lysates at 24 h, and increased to nearly 80% at 72 h. In sharp contrast, PRSS33 protein was not detectable even after culture for up to 72 h on similar plates.

Cytoplasmic localization of PRSS33 in eosinophils

When we tried to stain eosinophils with anti-PRSS33 antibody without permeabilization of the cell membrane, almost no increase in the mean fluorescence intensity was observed in flow cytometry (Fig. 2A). However, obvious PRSS33 staining was observed after cell membrane permeabilization (Fig. 2B), suggesting that PRSS33 is expressed in the cytoplasm.

To further analyze the cytoplasmic localization of PRSS33 in eosinophils, immunofluorescence staining was performed after permeabilization of the cell membrane. Laser confocal microscope examination revealed that PRSS33 was co-localized with a granule protein, ECP (Fig. 2C), suggesting that PRSS33 is anchored to a granule membrane.

This observation was consistent with the fact that the putative amino acid sequence of PRSS33 contains a single highly hydrophobic domain, thus indicating that PRSS33 is likely a membrane-bound molecule (Supplementary Fig. 1).

Eosinophil surface expression of PRSS33 was increased after degranulation

When eosinophils degranulate, the inner membrane of the granule membrane fuses to the cell surface membrane. Thus, we

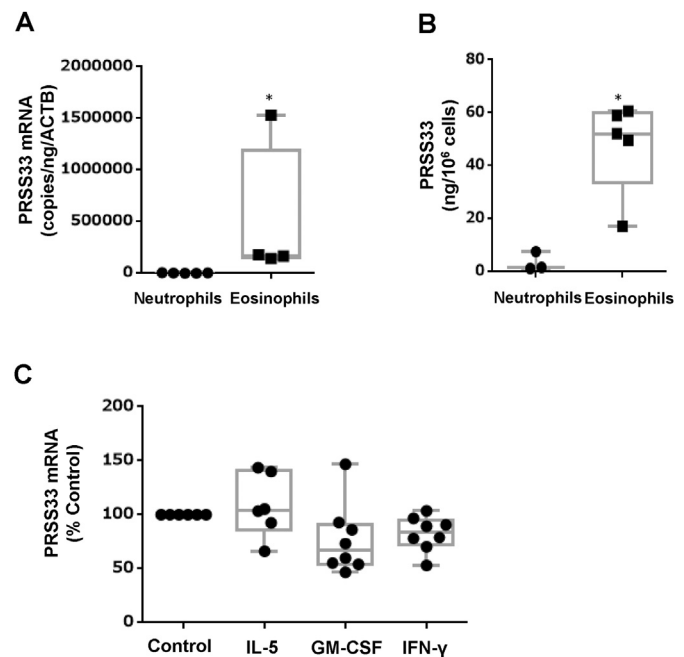


Fig. 1. Human eosinophils constitutively express PRSS33 mRNA and protein. (A) Expression levels of PRSS33 mRNA in freshly isolated human peripheral blood eosinophils and neutrophils were evaluated by qPCR (n = 8). (B) The amounts of PRSS33 protein in the whole cell lysates of eosinophils and neutrophils were measured by ELISA (n = 6). (C) Purified eosinophils were stimulated with IL-5, GM-CSF or IFN- γ for 6 h *in vitro*. Expression of PRSS33 mRNA was evaluated by qPCR. Data are shown as the median and 90th percentile (n = 7), *P < 0.05.

tested whether PRSS33 could be stained in un-permeabilized eosinophils after GM-CSF stimulation. After exposure to GM-CSF for 24 h, we stained for PRSS33 in eosinophils that had not been permeabilized (Fig. 3A), and confocal laser scanning microscopy indeed detected PRSS33 fluorescence in eosinophils after GM-CSF stimulation, without permeabilization (Fig. 3A and B). In addition, cell surface expression of PRSS33 was increased when eosinophils were stimulated with GM-CSF, suggesting that PRSS33 is a trans-membrane protein expressed on a granular vesicle membrane.

Effect of rhPRSS33 on human fibroblasts

When human fibroblasts from 7 separate donors were cultured with 20 ng/ml of recombinant human PRSS33, expression of mRNA for each of Col1A1, Col8A1, fibronectin and versican was significantly increased (Fig. 4). The increases in Col1A1, Col8A1 and fibronectin were significantly diminished when a PIC was added. Similarly, the increases in Col1A1, fibronectin and versican were almost completely abrogated when a PAR-2 antagonist was added.

Table 1

Proteases/proteinases mRNA with relatively high expression levels in human eosinophils and other cells in the peripheral blood.

Gene symbol	Eosinophil		Basophil		Neutrophil		CD4+ T cell		CD8+ T cell		B cell		Monocyte		Affymetrix ID	Genbank accession no.
	Raw	Fl	Raw	Fl	Raw	Fl	Raw	Fl	Raw	Fl	Raw	Fl	Raw	Fl		
PRSS33	5443.0	P	377.9	P	25.1	A	66.9	A	8.9	A	13.4	A	38.4	A	1552348_at	NM_152891
MMP25	1495.2	P	2272.6	P	3881.0	P	33.8	A	40.0	A	7.1	A	129.7	P	207890_s_at	NM_022718
ADAM8	3742.2	P	1136.6	P	1848.8	P	440.8	P	608.8	P	426.0	P	620.6	P	205180_s_at	NM_001109
ADAM10	1141.5	P	1884.4	P	2118.5	P	2757.0	P	2314.6	P	1914.8	P	3013.6	P	202603_at	N51370
ADAM19	1677.9	P	825.2	P	414.5	P	473.6	P	196.1	P	990.7	P	420.6	P	209765_at	Y13786

PRSS, protease serine; MMP, metalloproteinase; ADAM, a disintegrin and metalloprotease; Fl, flag.

Human eosinophils and other cells were purified from peripheral blood by density gradient sedimentation and negative/positive selections using immunomagnetic beads. mRNA expression of proteases/proteinases were evaluated by Affymetrix microarray system. The whole list of all proteases and proteinases can be found in Supplementary Table 1.

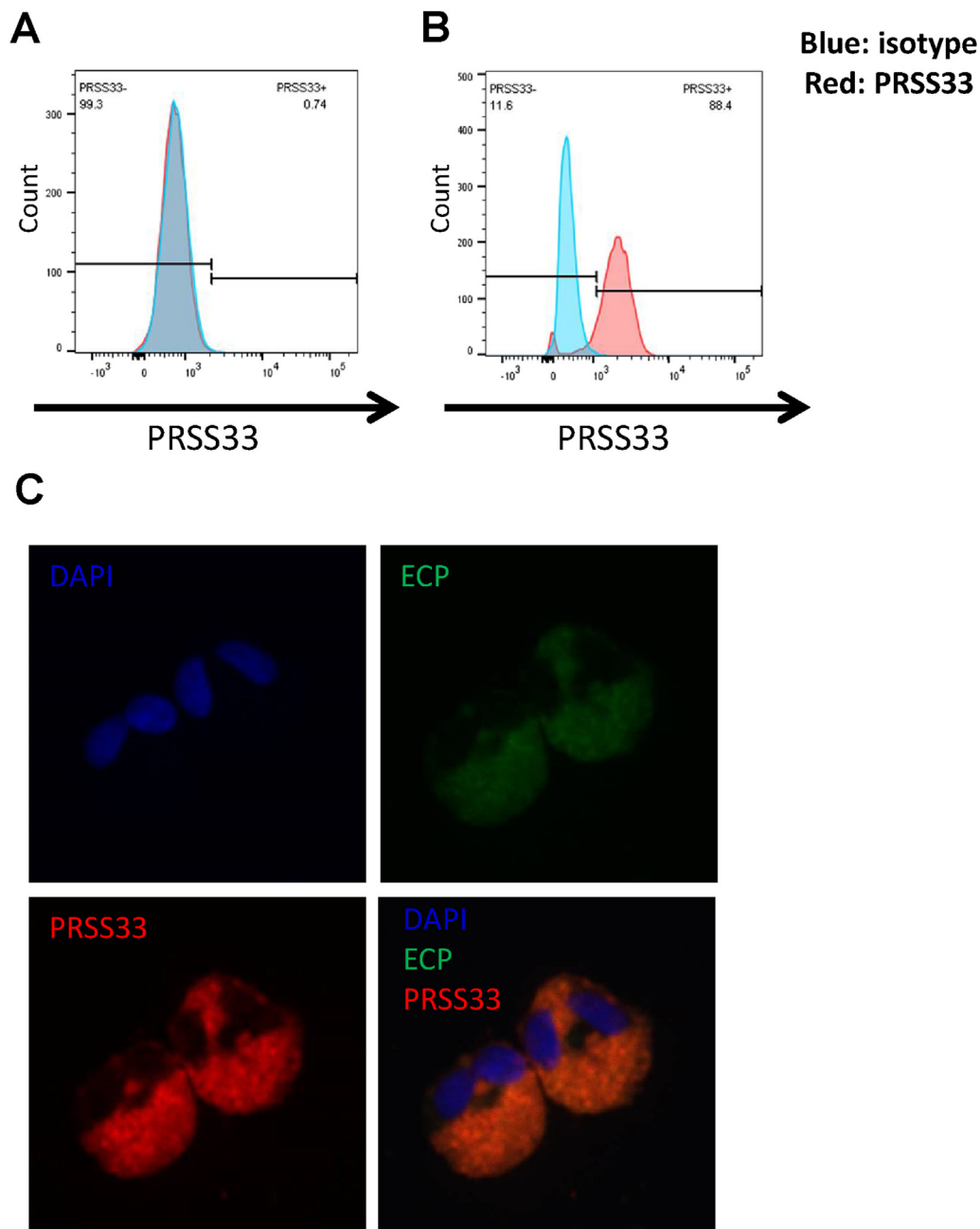


Fig. 2. PRSS33 is expressed not on the cell surface but in the granules of freshly isolated human eosinophils. Flow cytometric analysis of freshly isolated eosinophils stained with control goat serum (Blue) or goat anti-human PRSS33 polyclonal Ab (Red) before (**A**) and after (**B**) cell membrane permeabilization with Tween 20. Figures are representative of 3 separate experiments using peripheral blood eosinophils from 3 donors. (**C**) Confocal laser-scanning microscopic analysis of freshly isolated eosinophils permeabilized with Tween 20 and then stained with anti-PRSS33 polyclonal Ab (Red), rabbit anti-human ECP polyclonal Ab (Green) and DAPI (Blue). Figures are representative of 3 separate experiments using peripheral blood eosinophils from 3 donors.

Discussion

In order to elucidate eosinophil-specific proteases/proteinases, which are potential targets for preventing airway remodeling in asthma, we determined the comprehensive mRNA expression profiles of leukocytes. We found relatively high expression of five proteases: MMP25, ADAM8, ADAM10, ADAM19 and PRSS33 (Table 1). (The complete list of all proteases and proteinases can be found in Supplementary Table 1). Below, we describe some background knowledge and our present findings for each of those five proteases.

MMP25, a glycosyl-phosphatidyl inositol (GPI)-anchored protease, reportedly degrades ECM proteins (type IV collagen, gelatin, fibronectin and fibrin) and facilitates embryonic growth and development, uterine involution, ovulation and wound healing.²³ A nutrigenomic analysis found increased mRNA expression for MMP25 in induced sputum from asthmatic patients.²⁴ However, we found that the mRNA level of MMP25 in neutrophils (raw data: 3881.0) was much higher than that in eosinophils (raw data: 1495.2), showing that MMP25 is not eosinophil-specific.

ADAM8, a cell surface protease, is reportedly involved in remodeling of the extracellular matrix, cell migration and

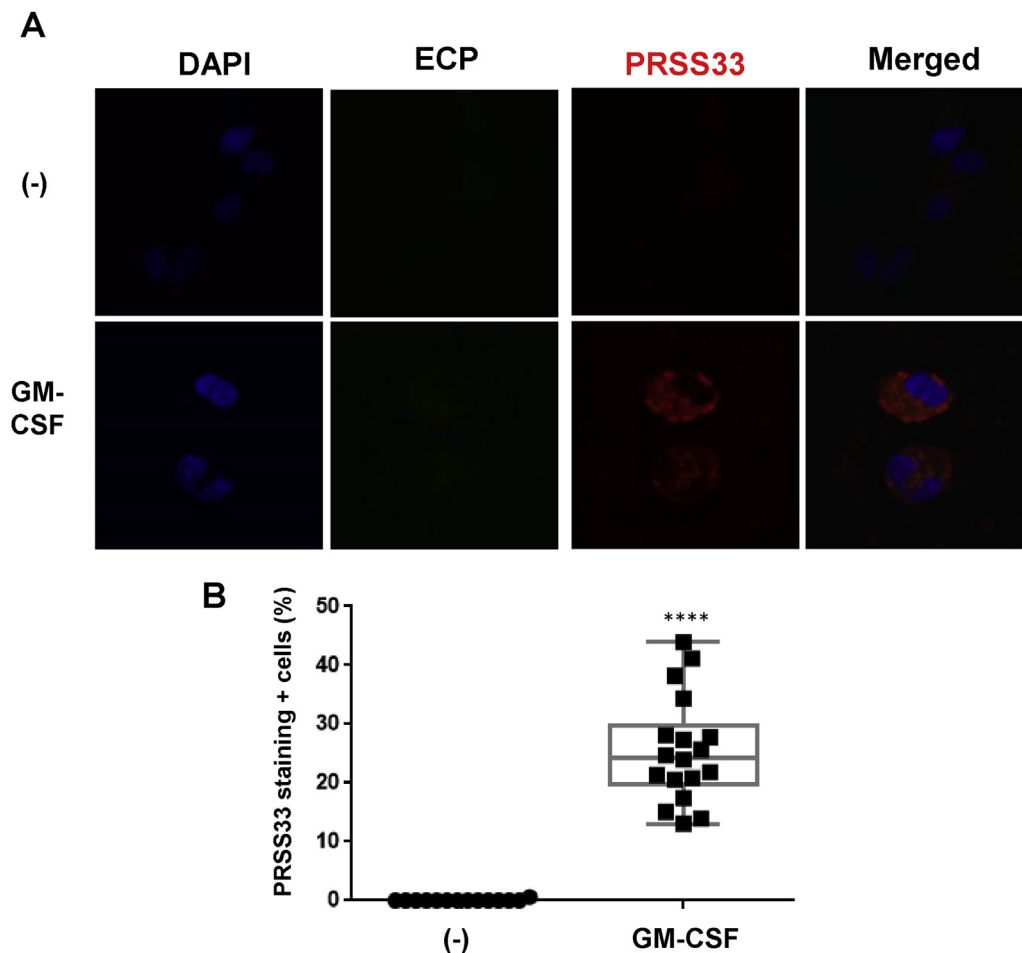


Fig. 3. Expression of PRSS33 and ECP before and after degranulation of human eosinophils without membrane permeabilization. **(A)** Confocal laser-scanning microscopic analysis of freshly isolated eosinophils stained with anti-PRSS33 polyclonal Ab (Red), rabbit anti-human ECP polyclonal Ab (Green) and DAPI (Blue) without permeabilization of the membranes before (upper panels) and after (lower panels) exposure to GM-CSF (10 ng/ml) for 24 h. Figures are representative of 3 separate experiments using peripheral blood eosinophils from 3 donors. **(B)** The percentages of eosinophils staining positive for PRSS33 in a low-power field before and after exposure to GM-CSF (10 ng/ml) for 24 h are shown. Data are shown as the median and 90th percentile, **** $P < 0.0001$.

processing of membrane-bound signaling molecules.²⁵ ADAM8 also reportedly plays critical roles in experimental asthma through induction of type 2 inflammation and bronchial hyper-responsiveness.^{26,27} Our data showed that eosinophils had the highest level of mRNA for ADAM8 (raw data: 3742.2) among all blood cells tested. However, all blood cells, including lymphocytes, expressed ADAM8 (raw data: 126.0–1818.8), showing that ADAM8 is also not eosinophil-specific.

ADAM10 possesses alpha-secretase activity that cleaves TNF- α , ephrin-A2, amyloid precursor protein, CD23 and others, and it plays critical roles in late-onset Alzheimer disease²⁸ and experimental asthma.²⁹ However, we found that all leukocytes expressed ADAM10 (raw data: 1884.4–3013.6), and the expression level was lowest in eosinophils (raw data: 1141.5); thus, ADAM10 is also not eosinophil-specific.

ADAM19 is highly homologous with ADAM12. Previous genome-wide association studies indicated that ADAM19 is likely involved in the development of airflow obstruction, especially chronic obstructive pulmonary disease.³⁰ Our data showed that eosinophils had the highest level of mRNA for ADAM19 (raw data: 1677.9) among all leukocytes tested. However, all other blood cells also expressed ADAM19 (raw data: 196.1–990.7), showing that ADAM19 is also not eosinophil-specific.

PRSS33 is a novel serine protease first reported by Chen *et al.*, in 2003.¹⁹ Here, we found that, in eosinophils, the highest level of mRNA expression among the five proteases was that for PRSS33 (raw data: 5443.0). The only other type of leukocyte to express it was basophils (raw data: 377.9), at a level that was less than 1/10th that of eosinophils. Therefore, we subsequently focused on analysis of PRSS33 in this study.

Confirming the microarray data, qPCR and ELISA showed that fresh human eosinophils constitutively expressed PRSS33 at approximately 2.9×10^5 copies/ng/ACTB mRNA and 549 ng/ 10^6 cells, respectively (Fig. 1A, B).

PRSS33 was first identified in macrophages and PMA-stimulated U-937 cells.¹⁹ qPCR found that the respective mRNA expression levels of PRSS33 in peripheral monocyte-derived macrophages (CD68-positive) and PMA-stimulated U-937 cells were only 1/20000 (average 148 copies/ng total RNA) and 1/150 (average 18899 copies/ng total RNA) of the level found in fresh human eosinophils. Similarly, ELISA found that the respective protein levels of PRSS33 in those same macrophages and PMA-stimulated U-937 cells were only 1/246 (average 2.2 ng/ 10^6 cells) and 1/45 (average 12.1 ng/ 10^6 cells) of the level found in fresh human eosinophils. Those data suggest that eosinophils may be the major source of this protease in the blood. We found virtually no differences in mRNA or protein

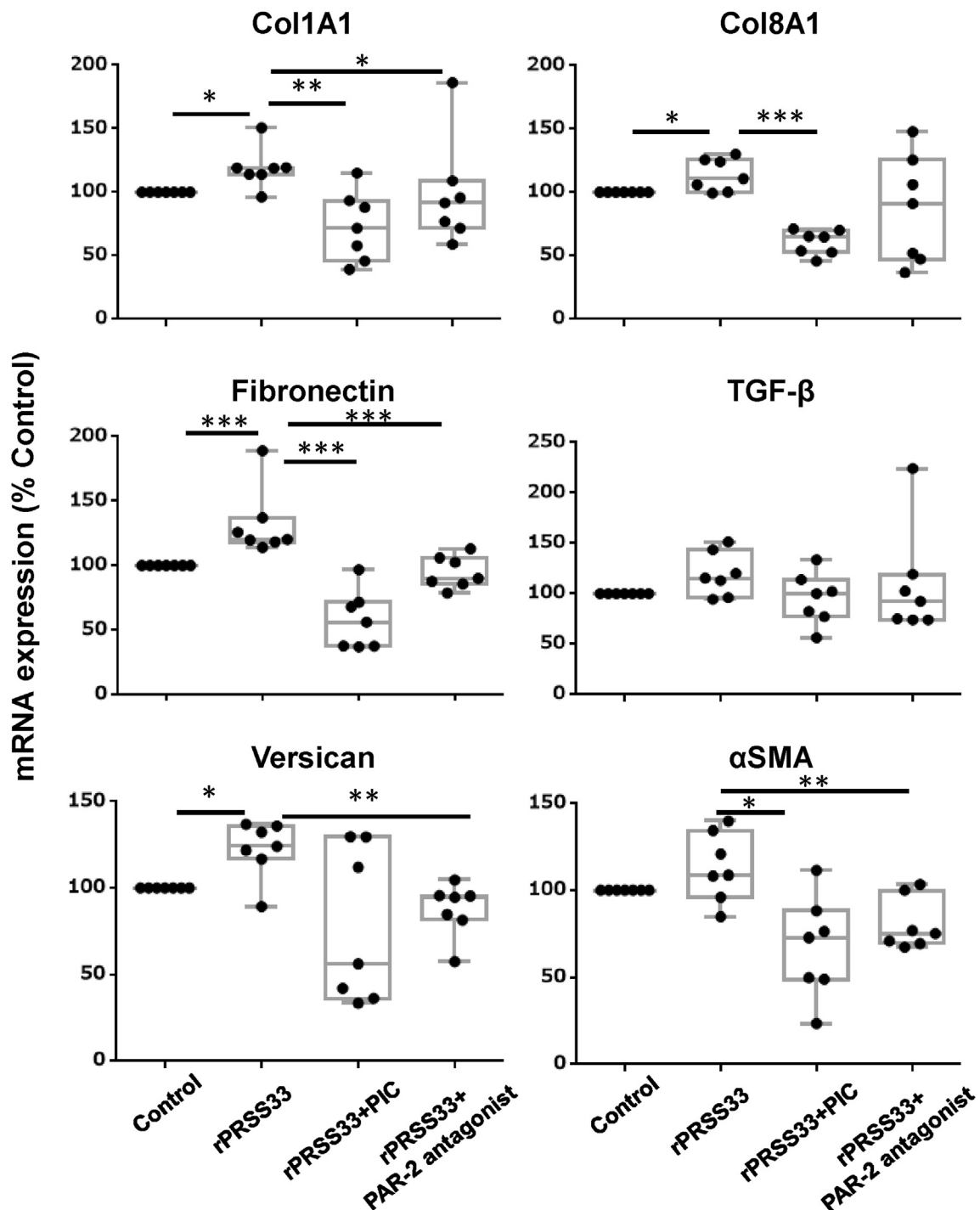


Fig. 4. Expression levels of extracellular matrix protein mRNA in human fibroblasts after stimulation with recombinant human PRSS33. Fibroblasts were stimulated with recombinant PRSS33 (rPRSS33) in the presence and absence of a protease inhibitor cocktail (PIC) or protease-activated receptor-2 (PAR-2) antagonist for 24 h *in vitro*. Expression levels of collagen 1A1 (COL1A1), COL8A1, fibronectin, transforming growth factor-β (TGF-β), versican and α-smooth muscle (α-SMA) mRNA were evaluated by qPCR. Data are shown as the median and 90th percentile of 7 experiments using 7 fibroblasts from 7 donors, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

levels of PRSS33 in eosinophils between healthy and allergic donors (data not shown).

When eosinophils were exposed to activating cytokines (IL-5, GM-CSF or IFN-γ), the PRSS33 mRNA levels were not altered (Fig. 1C), suggesting that this protease is pre-stored in fresh eosinophils. Flow cytometric analysis showed that eosinophils express no PRSS33 on their cell surface, but express it at the same location as a major granule protein, ECP (Fig. 2A–C). When

eosinophils were cultured on IgA-coated plates, up to 80% of eosinophil-specific granule EDN was released into the supernatant, but no PRSS33 was detected in the supernatant.

On the other hand, when eosinophils were exposed to GM-CSF, intracellular ECP staining was decreased (data not shown). PRSS33 staining was not detected in non-activated eosinophils when the cell membrane was not permeabilized (Fig. 2A, 3A upper panels). In contrast, when eosinophils were exposed to GM-CSF, PRSS33

staining was increased even without permeabilization (Fig. 3A lower panel). In addition, positively stained cells were increased (Fig. 3B). These findings suggest that PRSS33 is expressed only in the granule vesicle, and that degranulation (i.e., fusion of the granule membrane to the cell surface membrane) results in PRSS33's presence on the cell surface. Lending credence to such cell-surface expression, the amino-acid sequence of PRSS33 contains a single hydrophobic domain—a putative transmembrane domain (Supplementary Fig. 1).

PRSS33 is widely conserved throughout mammals (Supplementary Fig. 2), suggesting that it plays some critical role(s) in the survival of all mammals. However, no functional data have been reported. A homology search using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) showed that two conserved domains in PRSS33 (NM_152891.2) share 73% (130/170) and 72% (102/142) nucleic acid identity with human tryptase gamma 1 (TPSG1/PRSS31; NP_036599.3), which is known to be expressed by mast cells.³¹ Because mast cell tryptases in general are known to activate fibroblasts to produce type 1 collagen³² via PAR-2 activation,³³ we tested whether human PRSS33 induces collagen production in human fibroblasts. qPCR assays revealed that 25 ng/ml hrPRSS33 significantly induced mRNA expression for Col1A1, Col8A1, fibronectin and versican, at least in part through protease activity and PAR-2 (Fig. 4).

In summary, PRSS33 is a single hydrophobic-domain transmembrane protein constitutively expressed in human eosinophils, specifically in the granule membrane. Cell surface expression, but not release of PRSS33 into the supernatant, was induced upon activation with IgA and GM-CSF. PRSS33 is thought to activate PAR-2 in fibroblasts through direct contact with eosinophils and may be involved in the airway remodeling seen in type 2 inflammation with eosinophil infiltration.

Our results suggest that activated eosinophils may induce fibroblast extracellular matrix protein synthesis through cell-surface expression of PRSS33, which would at least partly explain eosinophils' role(s) in airway remodeling.

However, our study has some limitations. We examined the mRNA expression profiles of “resting” human blood cells, but did not examine activated blood cells. It is possible that some cells may express high levels of PRSS33 after activation. Also, PRSS33 staining on the eosinophil surface was increased after exposure to GM-CSF, but it remains to be clarified whether the catalytic domains of PRSS33 are expressed on the eosinophil surface or not. In addition, our functional assay of PRSS33 used only recombinant protein. One reason for that is that knockdown of PRSS33 in human eosinophils could not be done because siRNA cannot be incorporated into the cells. In addition, since several proteases are expressed in human eosinophils, using a recombinant protein was the only feasible method, at least for now. We found that the function of PRSS33 was inhibited by a PIC, but did not test whether a serine protease-specific inhibitor shows a similar inhibitory effect. The *in vivo* role of PRSS33 in remodeling should be further tested using knockout/transgenic mouse models. It would also be good to examine the role of PRSS33 in a cigarette smoke-induced COPD model or dextran sodium sulfate-induced colitis model in which tryptase gamma 1 plays a role.³¹

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.alit.2017.01.001>.

Conflicts of interest

The authors have no conflicts of interest to declare.

Authors' contributions

Experiments and analysis, ST, NO and AM; significant advice, NO, AM, HM, HS, TF, SN and HK; drafting the manuscript, ST and KM.

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Supplementary Table 1 Proteases/proteinases mRNA levels in human eosinophils and other cells in the peripheral blood

Gene	Eosinophil		Basophil		Neutrophil		CD4+T cell		CD8+T cell		B cell		Monocyte		Affymetrix	Genbank
Symbol	Raw	Fl	Raw	Fl	Raw	Fl	Raw	Fl	Raw	Fl	Raw	Fl	Raw	Fl	ID	accession No.
PRSS1	21.1	A	2.5	A	3.0	A	21.1	A	35.1	A	39.1	A	30.4	A	205869_at	NM_002769
PRSS2	42.6	A	93.5	A	9.3	A	11.8	A	33.8	A	42.8	A	28.8	A	205402_x_at	NM_002770
PRSS3	90.9	A	256.9	P	98.1	P	75.9	P	87.9	P	84.5	A	93.0	A	213421_x_at	AW007273
PRSS7	119.6	A	151.4	A	67.1	P	88.6	P	108.0	P	170.7	A	103.0	P	217269_s_at	AP001672
PRSS8	20.1	A	10.8	A	18.8	A	10.6	A	15.3	A	30.9	A	41.6	A	202525_at	NM_002773
PRSS12	27.5	A	15.6	A	21.4	A	32.9	A	9.7	A	18.9	A	22.5	A	205515_at	NM_003619
PRSS15	98.3	A	168.8	A	50.4	A	431.9	P	416.1	P	352.1	P	351.3	P	209017_s_at	U02389
PRSS16	5.1	A	5.8	A	5.1	A	12.1	A	6.4	A	49.0	A	19.1	A	208165_s_at	NM_005865
PRSS21	528.7	P	291.1	P	12.5	A	20.0	A	91.8	A	87.0	A	108.2	A	220051_at	NM_006799
PRSS22	76.6	M	102.8	P	69.9	P	106.4	P	117.2	P	96.6	P	57.6	A	205847_at	NM_022119
PRSS23	38.1	A	31.9	A	43.5	A	53.5	A	586.2	P	296.4	P	46.5	A	226279_at	AW471145
PRSS27	5.0	A	4.1	A	2.0	A	6.5	A	3.7	A	10.1	A	4.8	A	232074_at	AW170323
PRSS33	5443.0	P	377.9	P	25.1	A	66.9	A	8.9	A	13.4	A	38.4	A	1552348_at	NM_152891
PRSS35	17.7	P	22.5	P	31.2	P	19.8	P	42.5	P	23.5	A	74.5	P	235874_at	AL574912
PRSS36	65.2	A	15.7	A	18.0	A	19.0	A	4.1	A	17.1	A	23.7	A	1552555_at	NM_173502
PRSS37	32.5	A	68.5	A	6.4	A	10.5	A	46.3	A	49.9	A	37.7	A	231572_at	NM_001008270
TMPRSS2	24.9	A	19.9	A	19.0	A	54.2	A	48.5	A	26.1	A	41.1	A	205102_at	NM_005656
TMPRSS3	39.9	P	33.2	A	23.7	A	43.3	A	29.1	P	25.6	A	23.8	A	223949_at	AB038160
TMPRSS4	8.2	A	9.7	A	4.8	A	9.5	A	5.7	A	13.2	A	13.2	A	218960_at	NM_016425
TMPRSS5	23.0	A	29.1	A	18.0	A	23.4	A	36.6	A	30.7	A	33.3	A	221032_s_at	NM_030770

TMPRSS6	52.2	A	78.6	A	77.5	A	72.2	A	62.9	A	74.2	A	98.4	A	214955_at	AI912086
TMPRSS11B	23.5	A	29.0	P	34.9	A	37.4	A	10.5	A	29.4	A	37.2	A	1560712_at	AL833167
TMPRSS11D	14.7	A	24.8	A	59.6	A	42.2	A	51.9	A	58.5	A	21.9	A	207602_at	NM_004262
TMPRSS11E	7.8	A	10.1	A	15.0	A	9.4	A	7.2	A	8.9	A	8.3	A	220431_at	NM_014058
TMPRSS12	30.7	A	43.2	P	14.5	A	23.9	A	24.6	P	44.4	A	47.8	P	1569176_at	BC035123
TMPRSS13	16.4	A	14.0	A	17.4	A	11.8	A	10.3	A	12.7	A	24.5	A	223956_at	AB048796
TMPRSS15	103.0	P	170.7	A	108.0	P	119.6	A	67.1	P	151.4	A	88.6	P	217269_s_at	NM_002772
MMP1	17.6	A	2.0	A	0.6	A	3.0	A	0.8	A	1.0	A	1.5	A	204475_at	NM_002421
MMP2	79.4	A	34.5	A	80.6	A	96.6	A	78.4	A	67.8	A	86.5	A	1566678_at	AK057680
MMP3	5.6	A	33.5	A	5.3	A	21.3	A	11.8	A	37.9	A	19.9	A	205828_at	NM_002422
MMP7	60.4	A	87.5	A	81.6	A	91.3	A	69.2	A	118.6	A	108.5	A	204259_at	NM_002423
MMP8	76.5	A	197.4	P	43.1	A	63.5	A	10.7	A	56.1	A	54.3	A	207329_at	NM_002424
MMP9	85.6	P	148.2	P	1542.4	P	192.1	P	90.2	P	102.0	P	141.7	P	203936_s_at	NM_004994
MMP10	31.3	A	29.4	A	43.7	A	40.6	A	30.5	A	39.1	A	42.2	A	205680_at	NM_002425
MMP11	12.4	A	7.5	A	7.2	A	5.9	A	11.7	A	14.7	A	12.0	A	235908_at	AW511464
MMP12	14.5	A	19.6	A	15.6	A	43.7	A	18.8	A	33.4	A	28.7	A	204580_at	NM_002426
MMP13	2.3	A	3.9	A	9.2	A	20.7	A	3.9	A	6.3	A	3.4	A	205959_at	NM_002427
MMP14	248.9	M	293.4	P	193.5	A	229.9	P	127.5	M	132.4	P	234.1	P	160020_at	Z48481
MMP15	9.3	A	38.0	A	39.7	A	8.4	A	9.6	A	16.5	A	24.5	A	243883_at	AI951673
MMP16	48.7	A	90.1	P	69.1	P	92.9	M	106.4	A	98.8	P	105.3	P	207013_s_at	AB009303
MMP17	3.5	A	52.1	A	17.3	A	16.8	A	39.2	A	63.2	A	75.3	A	206234_s_at	NM_016155
MMP19	83.6	P	63.4	A	54.5	A	24.7	A	26.4	A	80.3	A	63.9	A	204574_s_at	NM_002429
MMP20	9.0	A	1.4	A	2.0	A	5.1	A	1.1	A	5.2	A	2.7	A	207599_at	NM_004771

MMP21	40.9	A	9.7	A	2.3	A	2.3	A	2.4	A	4.3	A	20.4	A	1552592_at	NM_147191
MMP24	43.0	A	18.3	A	41.2	A	4.4	A	36.8	A	37.7	A	75.7	A	208387_s_at	NM_006690
MMP25	1495.2	P	2272.6	P	3881.0	P	33.8	A	40.0	A	7.1	A	129.7	P	207890_s_at	NM_022718
MMP26	29.8	A	16.2	A	31.4	A	45.9	A	2.6	A	32.4	A	48.2	A	220541_at	NM_021801
MMP27	48.1	A	46.5	A	44.0	A	64.1	A	21.2	A	53.7	A	52.2	A	220783_at	NM_022122
MMP28	10.2	A	16.8	A	4.9	A	7.6	A	31.9	A	6.9	A	7.8	A	219909_at	NM_024302
MMPL1	117.7	P	164.3	A	243.4	P	128.3	P	132.0	M	112.3	M	286.5	M	207289_at	NM_004142
ADAM1A	58.9	P	70.0	P	26.2	A	91.9	P	119.0	P	86.2	P	70.9	P	228925_at	NR_036636.1
ADAM2	35.3	A	36.3	A	22.3	A	35.9	A	23.5	A	38.0	A	48.0	A	207664_at	NM_001464
ADAM3A	48.7	P	52.6	A	57.5	M	44.9	P	43.5	A	37.0	A	80.0	P	216276_s_at	X89657
ADAM5	65.0	A	56.0	A	66.0	A	88.2	A	21.6	A	52.8	A	72.1	A	216998_s_at	AJ132821
ADAM6	76.4	A	36.5	A	32.3	A	27.6	A	29.2	A	36.4	A	20.6	A	237909_at	AI024595
ADAM7	9.0	A	3.8	A	22.5	A	3.2	A	16.7	A	5.1	A	3.7	A	211238_at	AF215824
ADAM8	3742.2	P	1136.6	P	1848.8	P	440.8	P	608.8	P	426.0	P	620.6	P	205180_s_at	NM_001109
ADAM9	159.5	P	102.6	P	123.1	P	60.4	P	67.0	P	87.7	P	547.6	P	202381_at	NM_003816
ADAM10	1141.5	P	1884.4	P	2118.5	P	2757.0	P	2314.6	P	1914.8	P	3013.6	P	202603_at	N51370
ADAM11	15.1	A	7.0	A	9.2	A	5.5	A	3.5	A	13.3	A	4.4	A	207880_at	NM_002390
ADAM12	111.3	P	65.3	A	155.2	A	223.2	A	51.5	A	150.9	A	57.6	A	204943_at	NM_021641
ADAM15	104.7	A	61.4	A	144.4	A	194.8	A	105.4	A	83.3	A	362.6	A	1555896_a_at	BM973999
ADAM17	339.2	P	417.9	P	339.3	P	284.5	P	266.5	P	382.5	P	569.7	P	205745_x_at	NM_003183
ADAM18	46.7	A	78.3	P	37.0	A	45.8	P	12.3	A	73.6	A	20.0	A	207597_at	NM_014237
ADAM19	1677.9	P	825.2	P	414.5	P	473.6	P	196.1	P	990.7	P	420.6	P	209765_at	Y13786
ADAM20	6.3	A	3.4	A	6.4	A	9.9	A	7.3	A	7.8	A	14.0	A	207422_at	NM_003814

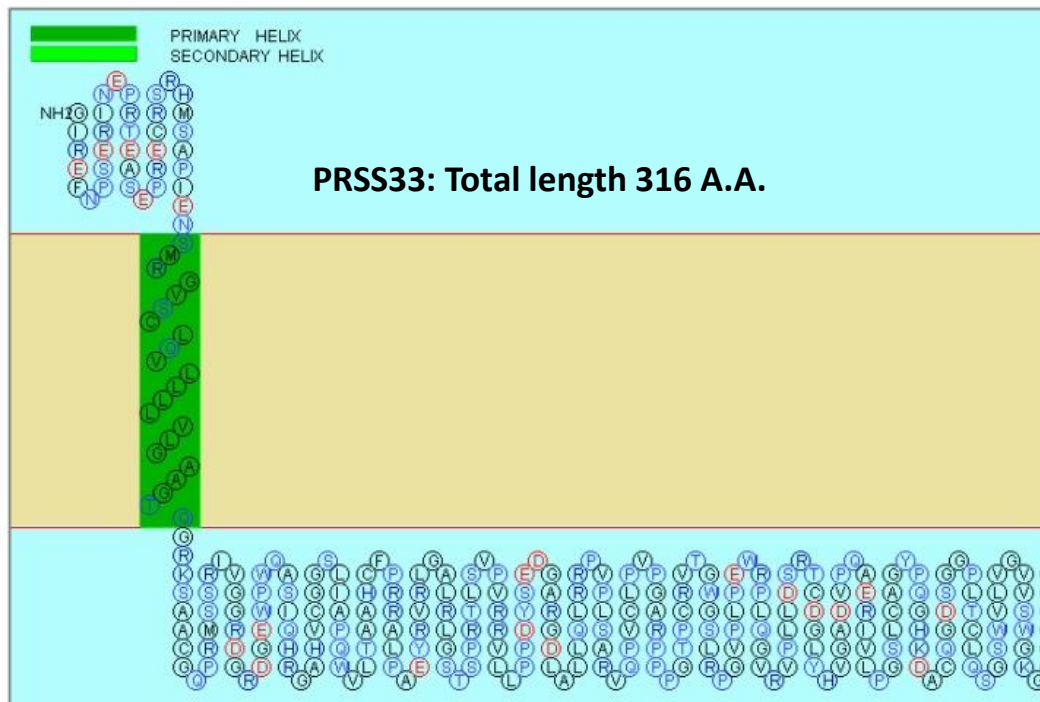
ADAM21	66.5	A	88.4	P	64.4	A	78.3	A	76.2	A	103.9	P	101.7	A	1552579_a_at	NM_003813
ADAM22	13.7	A	11.5	A	10.7	A	15.8	A	14.8	A	10.7	A	15.1	A	206616_s_at	AF155382
ADAM23	24.3	A	17.0	A	38.4	A	80.9	P	8.4	A	43.6	A	33.4	A	206046_at	NM_003812
ADAM28	607.2	P	634.4	P	266.4	P	7.6	A	24.7	A	1848.3	P	128.8	P	205997_at	NM_021778
ADAM29	13.8	A	21.4	A	9.1	A	16.4	A	12.3	A	38.6	A	37.3	A	221337_s_at	NM_021780
ADAM30	29.5	A	8.2	A	5.6	A	21.8	A	8.5	A	12.9	A	11.6	A	221446_at	NM_021794
ADAM32	20.7	A	20.2	A	60.4	A	21.3	A	64.4	A	17.6	A	45.0	A	1552266_at	NM_145004
ADAM33	233.3	A	202.0	A	226.4	A	125.2	A	80.9	A	134.6	A	202.5	A	233868_x_at	AL117415
ADAMDEC1	53.1	P	20.9	P	23.2	P	46.4	P	26.1	M	67.3	P	63.9	P	206134_at	NM_014479
ADAMTS1	5.2	A	3.6	A	3.2	A	5.4	A	4.5	A	6.2	A	9.6	A	222162_s_at	AK023795
ADAMTS2	49.8	A	78.9	A	61.3	A	65.0	A	56.3	A	39.7	A	64.1	A	214535_s_at	NM_021599
ADAMTS3	3.7	A	46.7	P	12.9	A	21.2	P	10.7	A	23.5	A	1.6	A	214913_at	AB002364
ADAMTS4	35.1	A	75.7	A	51.7	A	7.3	A	18.3	A	34.3	A	69.7	A	1555380_at	NM_005099
ADAMTS5	36.6	P	13.6	A	14.2	A	5.9	A	6.5	A	31.5	A	120.2	P	219935_at	NM_007038
ADAMTS6	29.6	A	42.1	A	45.8	A	71.2	A	53.4	A	58.5	A	55.7	A	220866_at	NM_014273
ADAMTS7	42.5	A	58.7	A	82.7	A	79.6	A	89.1	A	116.2	A	58.0	A	220705_s_at	NM_014272
ADAMTS8	14.5	A	3.9	A	5.3	A	29.6	A	52.3	A	62.5	A	18.1	A	220677_s_at	NM_007037
ADAMTS9	52.3	P	50.1	P	67.9	P	120.1	P	81.2	P	88.0	P	68.3	P	220287_at	NM_020249
ADAMTS10	40.8	A	17.6	A	7.3	A	106.8	A	87.2	A	64.7	A	33.6	A	230341_x_at	NM_001282352
ADAMTS11	36.6	P	13.6	A	14.2	A	5.9	A	6.5	A	31.5	A	120.2	P	219935_at	NM_007038
ADAMTS12	22.4	A	30.5	A	75.1	A	65.2	A	15.0	A	17.4	A	59.0	A	221421_s_at	NM_030955
ADAMTS13	56.2	A	209.8	A	65.1	A	60.9	A	94.7	A	70.0	A	109.9	A	220208_at	NM_139025
ADAMTS14	59.1	A	80.6	A	113.5	A	72.7	A	114.5	P	125.4	A	136.8	A	230167_at	NM_080722

ADAMTS15	30.8	A	37.5	A	8.6	A	10.2	A	12.0	A	10.7	A	9.4	A	1553427_at	NM_139055
ADAMTS16	3.6	A	3.7	A	3.5	A	3.7	A	3.0	A	5.6	A	4.0	A	238125_at	NM_139056
ADAMTS17	29.4	P	21.9	A	41.1	P	14.5	A	50.3	P	29.6	P	47.4	P	1552725_s_at	NM_139057
ADAMTS18	74.0	A	56.3	A	82.6	A	109.6	A	66.3	A	47.3	A	63.7	A	1553234_at	NM_139054
ADAMTS19	13.3	A	2.4	A	4.6	A	2.2	A	9.1	A	30.5	A	9.6	A	1553180_at	NM_133638
ADAMTS20	36.1	P	19.1	A	37.3	A	27.7	P	11.6	A	15.6	A	40.4	A	220717_at	NM_025003
ADAMTSL1	17.4	A	70.8	A	38.3	A	52.8	A	12.2	A	72.3	A	67.8	A	239909_at	NM_001040272
ADAMTSL2	1.9	A	2.7	A	2.4	A	2.4	A	2.3	A	3.7	A	4.0	A	206629_at	NM_001145320
ADAMTSL3	22.3	A	24.3	A	16.1	A	6.2	A	17.8	A	9.3	A	22.1	A	1559748_at	AF237652
ADAMTSL4	152.1	P	202.1	P	370.2	P	115.4	P	71.2	P	131.9	P	868.6	P	226071_at	NM_001288607
ADAMTSL5	6.6	A	18.1	A	13.1	A	18.4	A	32.4	A	64.5	A	20.1	A	242200_at	NM_213604
ADAMTSL6	100.9	A	70.7	A	69.1	A	81.2	A	36.8	A	91.0	A	99.9	A	226506_a	NM_001286429
KLK1	51.0	A	66.9	A	50.4	A	74.6	A	61.0	A	78.7	A	87.1	A	216699_s_at	L10038
KLK2	45.1	A	21.6	A	23.9	A	16.6	A	7.8	A	48.3	P	37.9	A	1555545_at	NM_001002231
KLK3	354.3	A	198.4	A	251.8	A	391.8	P	198.3	P	276.1	M	340.2	P	231629_x_at	NM_001030047
KLK4	24.6	A	8.7	A	9.7	A	32.4	A	8.8	A	9.9	A	11.0	A	224062_x_at	AF113140
KLK5	36.4	A	25.4	A	19.8	A	38.5	A	31.9	A	17.0	A	9.0	A	222242_s_at	AF243527
KLK6	55.9	A	54.7	A	74.6	A	80.3	A	52.1	A	46.0	A	78.4	A	204733_at	NM_001012964
KLK7	70.2	P	98.2	P	102.9	P	99.8	P	32.5	P	46.8	P	130.8	P	239381_at	NM_001207053
KLK8	53.7	A	46.2	A	64.2	M	62.7	A	27.4	A	76.8	A	81.9	A	206125_s_at	NM_001281431
KLK10	4.5	A	23.2	A	54.0	A	51.8	A	7.8	A	17.3	A	27.9	A	215808_at	NM_001077500
KLK11	2.9	A	6.1	A	3.6	A	3.9	A	4.7	A	4.4	A	4.2	A	205470_s_at	NM_001136032
KLK12	122.5	A	97.7	A	85.1	A	97.3	A	113.7	A	108.9	A	72.8	A	220782_x_at	NM_019598

KLK13	82.1	A	89.3	A	49.9	A	56.2	A	69.2	A	46.9	A	63.2	A	216670_at	NM_015596
KLK14	8.4	A	5.7	A	5.6	A	9.9	A	6.9	A	14.3	A	10.9	A	220573_at	NM_022046
KLK15	13.4	A	72.4	A	25.9	A	14.6	A	37.0	A	37.4	A	31.2	A	233477_at	AF242195

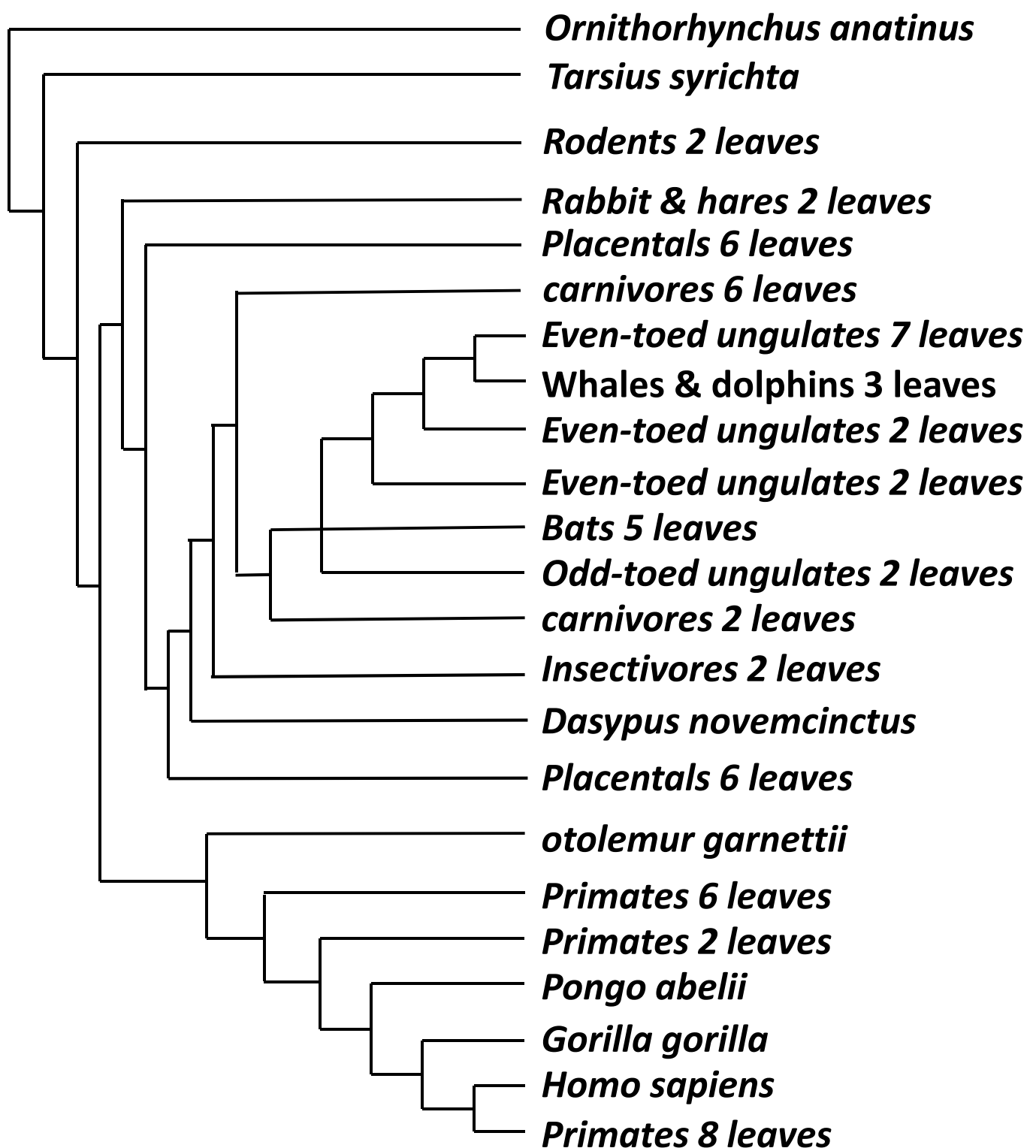
PRSS, protease serine; TMPRSS, transmembrane protease serine MMP, metalloproteinase; ADAM, a disintegrin and metalloprotease; ADAMDEC1, a disintegrin and metalloproteinase domain-like protein decysin-1; ADAMTS, metalloproteinase with thrombospondin type 1 motif; ADAMTSL, ADAMTS-like protein; KLK, kallikrein; Fl, Flag;

Human eosinophils and other cells were purified from peripheral blood by density gradient sedimentation and negative/positive selections using immunomagnetic beads. mRNA expression of proteases/proteinases was evaluated by Affymetrix microarray system.



Supplementary Fig. 1 The amino acid sequence of PRSS33 is a MEMBRANE PROTEIN that has one transmembrane helix.

Analysis of the amino acid sequence of the transmembrane region of PRSS33 (NP_690851) was performed using SOSUI software engine ver. 1.11. (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html). The amino acid sequence of PRSS33 is a MEMBRANE PROTEIN that has one transmembrane helix, MRGVSLQVLLLLVLGAAGTQ". Mitaku S, Hirokawa T, and Tsuji T. Amphiphilicity index of polar amino acids as an aid in the characterization of amino acid preference at membrane-water interfaces. *Bioinformatics* 2002;18;608-16.



Supplementary Fig. 2 PRSS33 orthologue in mammals.

The search for the amino acid sequence of PRSS33 (NM_152891.2) orthologue was performed using BLAST at the NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).