·Basic Research·

The involvement of proline-rich protein *Mus musculus* predicted gene 4736 in ocular surface functions

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Abstract

• AIM: To research the two homologous predicted proline-rich protein genes, *Mus musculus* predicted gene 4736 (MP4) and proline-rich protein BstNI subfamily 1 (Prb1) which were significantly upregulated in cultured corneal organs when encountering fungal pathogen preparations. This study was to confirm the expression and potential functions of these two genes in ocular surface.

• METHODS: A *Pseudomonas aeruginosa* keratitis model was established in Balb/c mice. One day post infection, mRNA level of MP4 was measured using real-time polymerase chain reaction (PCR), and MP4 protein detected by immunohistochemistry (IHC) or Western blot using a customized polyclonal anti-MP4 antibody preparation. Lacrimal glands from normal mice were also subjected to IHC staining for MP4. An online bioinformatics program, BioGPS, was utilized to screen public data to determine other potential locations of MP4.

• RESULTS: One day after keratitis induction, MP4 was upregulated in the corneas at both mRNA level as measured using real-time PCR and protein levels as measured using Western blot and IHC. BioGPS analysis of public data suggested that the MP4 gene was most abundantly expressed in the lacrimal glands, and IHC revealed that normal murine lacrimal glands were positive for MP4 staining.

• CONCLUSION: MP4 and Prb1 are closely related with the physiology and pathological processes of the ocular surface. Considering the significance of ocular surface abnormalities like dry eye, we propose that MP4 and Prb1 contribute to homeostasis of ocular surface, and deserve more extensive functional and disease correlation studies.

• KEYWORDS: proline-rich protein; *Mus musculus* predicted gene 4736; ocular surface; *Pseudomonas aeruginosa* keratitis
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INTRODUCTION

Proline-rich proteins (PRPs) are a family of proteins rich in proline, glycine, and glutamic acid/glutamine. These three types of amino acid may respectively contribute 25%-40%, 16%-22%, and 15%-28% of all amino acid residues in such proteins. A variety of PRPs have been identified in various animals, and the tissues reported to produce highest amount of PRPs are the salivary glands and/or parotid glands. The respiratory tract and pancreas are also known to express PRPs at moderate levels. The functional spectrum of PRPs is relatively narrow; specifically, they are restricted to binding to various chemical substance like tannic acid [1] or pathogens, thus potentially preventing the attachment and colonization of pathogens in affected tissues [2-6]. An isolated early report suggested that certain PRPs might be acute-phase reactants in humans[7].

In a previous study about the interaction between corneas and pathogens, we cultured mouse corneal buttons overnight with heat-inactivated *Aspergillus fumigatus* spores *in vitro* and detected the gene expression pattern using the microarray technique. Among all 61 probes that were upregulated over twofold were two probes corresponding to NM_053251 and BF536212. They were upregulated 6.30- and 3.37-fold, ranking 3rd and 16th, respectively, in terms of fold change [8]. Genbank research revealed that the entry for NM_053251, namely *Mus musculus* predicted gene 4736

Figure 1 Homology of MP4 (NP_444481) and Prb1 (NP_941071) proteins as revealed by BLAST analysis.

(MP4), were first characterized in cosmid genomic libraries derived from 129Sv and Balb/c mice, respectively [9]. Due to its high proline content, this was called MP4. However, there has not yet been a functional study on its hypothetical product, namely the "PRP MP4 precursor" (NP_444481), herein referred to as MP4.

The 300 amino acid long MP4 contains 84 (28.0%) proline residues, 53 (17.7%) glutamine residues, and 52 (17.3%) glycine residues. Similarly, the expressed sequence tag (EST) sequence BF536212 (corresponding to mRNA NM_198669) was predicted based on bioinformatics analysis[10] to encode a 504 amino acid protein proline-rich protein BstNI subfamily 1 (Prb1) (NP_941071) that contains 159 (31.5%) prolines, 108 (21.4%) glutamines, and 102 (20.2%) glycines. High homology exists between these two proteins (Figure 1). The first 292 amino acids of MP4 share 83.7% identity (or 90.8% similarity) to the first 294 residues of Prb1, and the 7 C-terminal residues of both proteins are identical. Like with MP4, no functional studies have been recorded for Prb1. The high-grade upregulation of these two genes' mRNA in fungal-challenged corneal tissues in previous results suggested that they might participate in the innate immunity of corneas against fungal infections, or more broadly, of other tissues against microbes. This study was carried out to verify this possibility.

MATERIALS AND METHODS

Ethic Statement Use of animals was approved by the Ethic Committee of Shandong Eye Institute and observed the Guidelines on the Humane Treatment of Laboratory Animals (Chinese Ministry of Science and Technology, 2006).

Keratitis Model for *Mus Musculus* Predicted Gene 4736 Expression Measurement *Pseudomonas aeruginosa* keratitis (PaK) was established using a well-defined intrastromal injection protocol, as described previously, for fungal infection of the cornea [11]. In brief, specific-pathogen-free Balb/c mice (Beijing Pharmacology Institute, Beijing, China) were utilized, and all animal experiments were carried out in accordance with the Guidelines on the Humane Treatment of Laboratory Animals (Chinese Ministry of Science and Technology, 2006) and the Statement for the Use of Animals in Ophthalmic and Vision Research. Only the left corneas were used for manipulation, with the right eyes used as untreated controls. *P. aeruginosa* ATCC 27853 (American Tissue Culture Collection, Manassas, VA, USA) was cultured and handled following the Shandong Eye Institute Biosafety Code. Inoculates at the exponential phase were harvested, washed, and suspended in a saline buffer at a concentration of 1×10^9/mL. The corneas were pierced near the center with a 30 gauge needle to the depth of the stroma, and a 33 gauge needle with a 30 degree bevel (Hamilton, Reno, NV, USA) was used to inject 1 μL of bacterial suspension (1×10^6) into the center of the cornea. In the sham-infection group, 1 μL of saline buffer was substituted for the microbe suspension. The corneas were monitored using a slit lamp 24h later to confirm successful infection. The mice were euthanized, and the eyeballs were harvested to detect MP4 levels using immunohistochemistry (IHC), Western blot, or reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR).

***Mus Musculus* Predicted Gene 4736 Proteins Assay** For the lack of commercial anti-MP4 antibodies, we first had polyclonal rabbit anti-MP4 antibodies prepared by the Abmart company (Shanghai, China) to enable us to perform IHC and Western blot assay of this protein in tissues. The company utilized a patented protocol to express a recombinant artificial "protein" containing seven 10 amino acid long immune epitopes that were predicted based on the MP4 sequence. After four immunizations of the rabbits with the recombinant proteins, the antibody in antiserum was purified with a protein A column, and the titer of the final solution was measured to be 12 500 to 25 000. The antibody

was used at 1:200 for IHC and 1:300 for Western blot, as recommended by the manufacturer.

At 24h after PaK induction, the eyeballs were harvested, fixed in 4% formaldehyde (pH 6.9), embedded in a paraffin block, and subjected to routine hematoxylin-eosin (HE) staining, gram staining or IHC. In the latter case, polyclonal rabbit anti-MP4 in combination with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (MAXIM BIO, Fuzhou, China) was used. After development with 3, 3'-diaminobenzidine, the sections were counterstained with hematoxylin. To validate the specificity of the primary antibody, normal rabbit serum was used as substitute for the anti-MP4 antibody, and the results are shown as "negative" staining control. To test potential expression of MP4 in tears, lacrimal glands were harvested from normal Balb/c mice and used for immunostaining as well. For Western blotting assay, three corneas from PaK models were pooled as one sample for protein extraction using a lysis buffer (50 mm Tris pH 7.4, 150 mm NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, sodium orthovanadate, and sodium fluoride). Ten microliters of each protein sample were resolved on 15% SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore, Billerica, USA). The blots were blocked in 5% nonfat dry milk dissolved in TBST buffer for 1h, incubated with rabbit anti-MP4 (or anti- GAPDH) antibody in TBST for 1h, followed by incubation with HRP-conjugated goat IgG antibody (MAXIM BIO) for 1h. All incubations were carried out at room temperature, and three washes with 10 mL TBST buffer were applied between each step. The membranes were then developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL, USA) and exposed to X-ray film (Kodak, Rochester, NY, USA).

Real-time Polymerase Chain Reaction Assay of *Mus Musculus* Predicted Gene 4736 Messengers At 24h after PaK induction, total RNA was extracted from pooled corneas using isopropanol precipitation and purified using NucleoSpin ® RNA clean-up columns (MACHEREY-NAGEL, Düren, Germany). RNA from 3 corneas was pooled to yield one RNA sample, and three samples were included per group. Total RNA was reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara, Shiga, Japan). RT-qPCR was performed using the SYBR method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference gene. The primer sets for MP4 were gatgatgaagatggcagtgag (forward) and atggtggtttcctgagtgag (reverse), and for GAPDH being actcacggcaaattcaac (forward) and tagactccacgacatactc (reverse). Cycling conditions were as follows: 10s at 95℃ and 45 cycles of amplification for 15s at 95℃ and 1min at 60℃. After analysis with the endorsed software, the fractional cycle number for threshold fluorescence (threshold cycle, Ct) for each reaction was obtained. The average of three duplicates was used to calculate the relative Ct against GAPDH ($\Delta Ct=Ct_{gene}-Ct_{GAPDH}$) for each sample. The average ΔCt for the three samples in the PaK and control groups was used to calculate the $\Delta\Delta Ct$ of the PaK samples ($\Delta\Delta Ct=\Delta Ct_{PaK}-\Delta Ct_{control}$). The relative expression folds of the PaK samples over controls were calculated as $1/2^{\Delta\Delta Ct}$.

Analysis of *Mus Musculus* Predicted Gene 4736 and Proline-rich Protein BstNI Subfamily 1 Expression by BioGPS BioGPS is a free online software for "extensible and customizable gene annotation" and "a complete resource for learning about gene and protein function" [12] and is used to retrieve potential expression of MP4 in various tissues/organs of mice. Detailed calculation rationales beyond the scope of this paper but available online (www. biogps.org). The accession numbers of the two genes were filled and the output data concerning the relative expression levels were directly subjected for graphing in Excel without any other manipulation.

RESULTS

***Mus Musculus* Predicted Gene 4736 Expression Increased in Infected Corneas** Like with our previous report with fungi-infected corneas[8], RT-qPCR assay showed that MP4 gene mRNA was upregulated in corneas with PaK (Figure 2A). Using the customized polyclonal antibodies and Western blot or IHC, we further showed that the MP4 proteins were also increased in corneas upon infection with bacteria (Figure 2B, 2C). It is noticeable that MP4 was mainly detected in the epithelium in normal corneas, but after bacterial infection, the cells in the stromal layer, supposed stromal fibroblasts and infiltrating leukocytes, also produced MP4 (Figure 2C).

Preferential Expression of *Mus Musculus* Predicted Gene 4736 in Ocular and Periocular Tissues We then checked the potential expression of MP4 in all tissues by screening public data using the public online BioGPS software. Surprisingly, in the datasets that profiled gene expression in most mouse tissues [13], MP4 was most abundantly expressed in lacrimal glands, followed by the lens, salivary glands, and eyecups (Figure 3). This implicated that, though cornea was the tissue that first caught our attention on MP4 in previous study, this gene might be more closely related with lacrimal glands and, to lesser significance, with salivary gland. Encouragingly, lacrimal glands from normal mice were positive for MP4 staining, and most of the positive staining was limited to acinar cells (Figure 4). Prb1 data were not available in the BioGPS system.

DISCUSSION

The MP4 and Prb1 cDNA sequences were discovered in 1991 and 2002, respectively, but their functions have not yet been elucidated. The current study represents the first

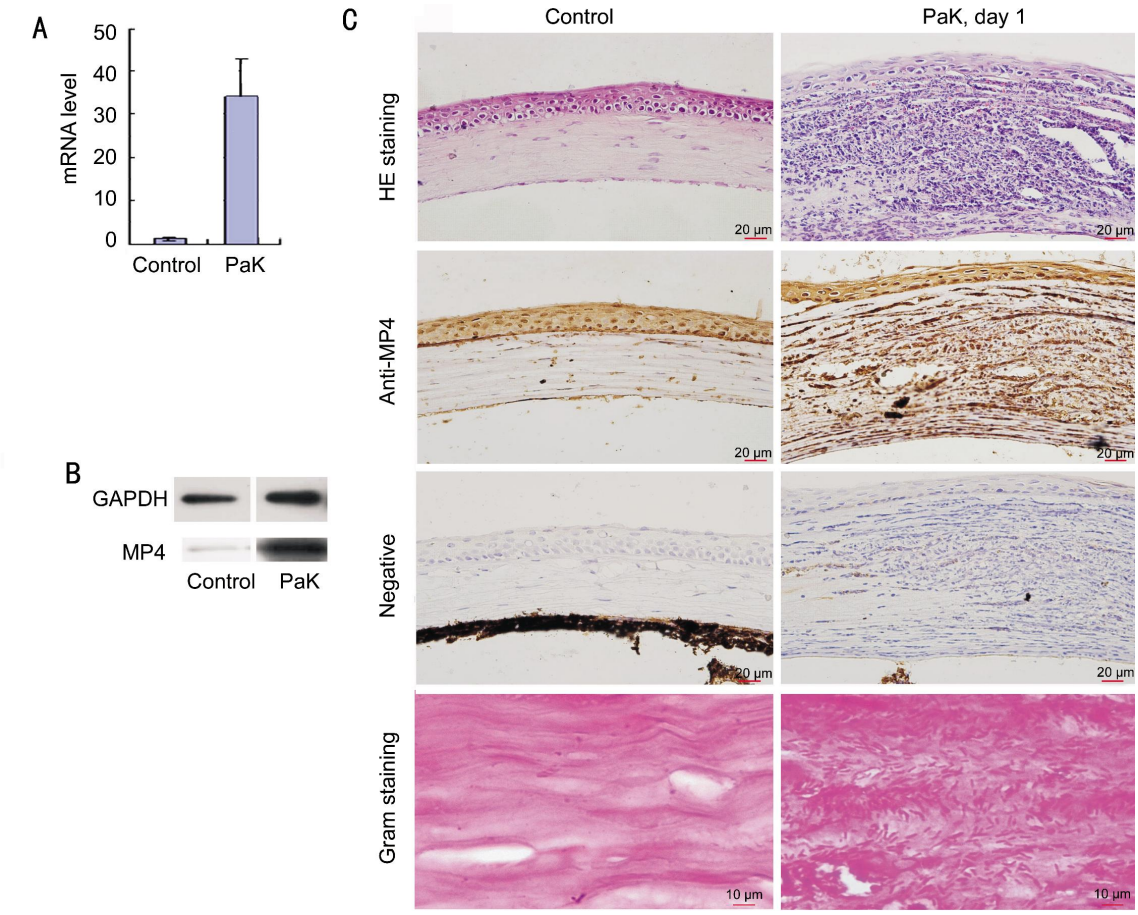

Figure 2 Expression of MP4 in murine corneas PaK was established in mice. One day later, corneas were harvested for mRNA (A) or protein extraction (B), or subjected to HE or gram staining or IHC (C). In the latter case, normal rabbit serum was used as substitute for the anti-MP4 antibody to validate the specificity of the primary antibody, and the results are shown as "negative" staining control. In A and B, three corneas from PaK models or normal control were pooled as one sample respectively. All experiments were conducted at least twice.

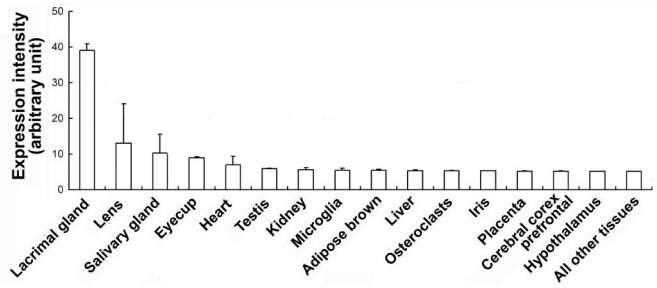

Figure 3 Preferential expression of MP4 in lacrimal glands as revealed by BioGPS Expression intensity was given by BioGPS. "All other tissues" represents the tissues or cells that were included in BioGPS but not detailed here, including white adipose, adrenal gland, amygdala, bladder, bone, bone marrow, cerebellum, cerebral cortex, ciliary bodies, cornea, dorsal root ganglia, dorsal striatum, epidermis, hippocampus, lung, lymph nodes, lactating mammary gland, non-lactating mammary gland, nucleus accumbens, olfactory bulb, ovary, Pancreas, pituitary, prostate, retina, skeletal muscle, spinal cord, spleen, stomach, umbilical cord, small intestine, uterus, and large intestine.

attempt to define the functions of these two genes. Although still preliminary, the presented data strongly suggest that these genes are involved in the function of the ocular surface. Public data about the comparative expression of MP4 in tissues also implies that it might be more closely related with lacrimal gland function. Thus, this study is the first one to provide a functional linkage between PRP expression and ocular surface diseases. This observation supplements several previous studies that proposed a role of PRPs in tear film function, or further, in dry eye syndrome. For example, mRNA encoding a 134-residue protein (NP_009175) with 45.5% homology with human salivary acidic PRP1 was designated as human lacrimal-specific proline-rich protein (LPRP)[14], and later this protein was detected in tear film[15]. Similar to MP4, functional or disease relevance studies are lacking except for one recent report that LPRR was "down regulated significantly in all types of dry eye cases, correlating with the disease severity as measured by clinical investigations" independent of the causative factor in all patients [16]. Zhou *et al* [17] described four other PRPs in tear fluid. But the tear fluid used in Zhou study was collected from it as who had undergone pterygium surgery 1-3d prior, and consequently, the high abundance of PRPs detected in these samples may not reflect the levels characteristic of physiological conditions. Later, Tsai *et al*[18] detected the PRP1 precursor and PRP4 in Meibomian gland secretions collected from healthy subjects. Grus *et al*[19]

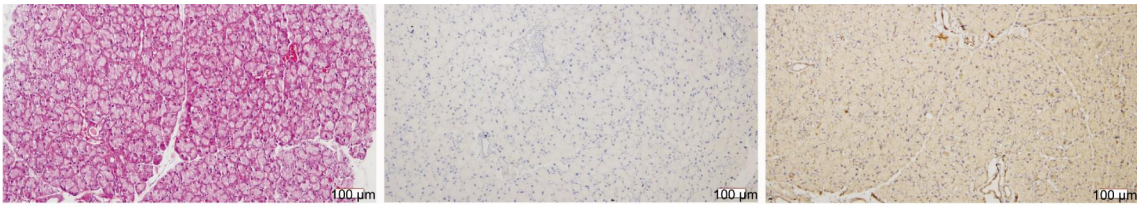

Figure 4 Immunostaining of MP4 in normal mouse lacrimal glands Sections were subjected to HE staining and immunostaining respectively. Parallel handling with normal rabbit serum but without anti-MP4 antibodies was run as "negative" control.

further demonstrated reduced levels of two PRPs, PRP3 and PRP4, in tears from patients with DED. By examining patients who wear contact lenses, Nichols *et al* [20-21] showed that PRP4 was deposited in the contact lens and that its concentration in tears was significantly decreased in patients with contact lens-related dry eye compared with that in controls. However, another group of PRPs, namely small proline-rich proteins (sPRPs), which had been correlated with dry eye [22-24] should not be mixed with the PRPs discussed here. sPRPs are regarded biomarkers of squamous cells thus involved in various skin diseases [24-25] or squamous metaplasia of corneal epithelial cells.

The current study, along with a previous one [8], imply that MP4 and Prb1 might be acute-phase reactants of corneas upon infection. IHC showed that besides epithelial cells, residential corneal stromal cells or infiltrating leukocytes could be the main producers of MP4 in the stromal layer, but no effort was made to identify the actual cellular source of such MP4. However, when we checked other microarray data obtained in this lab from other research projects, we found that MP4 and Prb1 showed changes opposite to that in infection models. For example, in a sterile penetrating corneal trauma model caused by a three-edged needle in Balb/c mice, MP4 and Prb1 levels were significantly downregulated at day 3 post-trauma, a time point when the corneas were similarly infiltrated with leukocytes. The intensity ratio of MP4 in two replicated arrays of injured corneas were 0.17 and 0.20 compared with normal corneas, while the ratios for Prb1 were 0.17 and 0.16, respectively. By day 14, when the injured corneas healed except for a scar, the levels of MP4/Prb1 returned to normal (unpublished data). Since both the infection model and the trauma models were characterized with leukocyte infiltration, the opposite change of MP4/Prb1 in infection and trauma suggests that the MP4/Prb1 expression in the corneas is regulated by mechanisms that do not depend on leukocytes, but rather act differentially in infectious and noninfectious situations.

The current study has significance in advancing our knowledge about the ocular surface. First, in recent years, the significance of dry eye diseases in vision problems has been recognized, and research on the composition and biology of tear films (TF) bears promise when it comes to uncovering the remove of and suggest possible cures for such diseases [26-27]. However, as Green-Church *et al* [28] stated in a recent review, "we are only beginning to understand the specific molecular components of the TF and their relationship with disease and TF stability". We think the potential contributions of various prolin-rich proteins (including PRPs, LPRP, sPRPs, *etc*) deserve more attention. As aforementioned, PRPs are among the main antimicrobial constituents in saliva. Based on our findings in this study, we have good reason to hypothesize that PRPs, including MP4 and Prb1, play similar roles in tear fluid.

In summary, by integrating the data from various projects and resources, we infer that two PRPs, namely MP4 and Prb1, are involved in the physiology or pathological process concerning the ocular surface, such as cornea and lacrimal gland. We propose that these proteins contribute to the homeostatic and antimicrobial environment in the conjunctival sac, but extensive studies into their functions will contribute to an understanding of the biology of the ocular surface, or even lead to the development of novel protocols for managing related disease, such as corneal infection or dry eye.

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