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研究成果の刊行物・別刷

Molecular Vision 2008; 14:1037-1040 <http://www.molvis.org/molvis/v14/a124>
Received 14 February 2008 | Accepted 30 May 2008 | Published 5 June 2008

LOXL1 genetic polymorphisms are associated with exfoliation glaucoma in the Japanese population

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Purpose: We performed genetic association studies using a native Japanese population to examine the reproducibility of results of *lysyl oxidase-like 1* (*LOXL1*) genetic association studies for exfoliation glaucoma (XFG) beyond the differences of ethnicity. We also quantified *LOXL1* mRNA expression in the human lens capsule to examine the possible correlation between *LOXL1* expression and XFG pathogenesis.

Methods: We performed a case-control study using 95 Japanese XFG patients and 190 controls. Real-time polymerase chain reaction (PCR) analysis was performed using lens capsules obtained during surgery.

Results: The TT genotype in the single nucleotide polymorphism (SNP) rs1048661 and the GG genotype in the SNP rs3825942 in exon 1 of *LOXL1* were significantly associated with an increased risk of XFG under recessive models (χ^2 test, $p=5.34\times10^{-34}$ and $p=2.1\times10^{-8}$, respectively). Quantification of *LOXL1* mRNA expression demonstrated no significant difference between XFG and senile cataract samples.

Conclusions: Although the functional effects of the *LOXL1* SNP appear to be qualitative rather than quantitative, the amino acid substitution (R141L) caused by SNP rs1048661 is not a simple decisive factor for XFG due to the inverted allele frequency between Japanese XFG and Caucasian XFG patients. Further genetic and functional studies are essential for clarifying XFG pathogenesis.

Exfoliation glaucoma (XFG) is an age-related disorder associated with exfoliation syndrome (XFS), manifested by abnormal fibrillar deposits on the lens and iris epithelium [1]. Recently, a genome wide association study performed for the Caucasian population revealed a strong association between the genotype of single genetic polymorphisms (SNPs) in the *lysyl oxidase-like 1* (*LOXL1*) gene and the occurrence of XFS/XFG [2]. It was reported that the rate of XFG occurrence significantly differed from one ethnic population to another [3], therefore it is logically important to perform a case-control study using another ethnic population such as the Japanese. In this study, we found a strong genetic association between the occurrence of XFG and the *LOXL1* single nucleotide polymorphism (SNP) genotype. One of the nonsynonymous SNP (rs1048661) showed a very strong association with XFG. However, the risk allele was inverse compared to the Caucasian study. To gain further insight into the role of *LOXL1* for XFG, it is important to compare the expression levels of *LOXL1* mRNA in XFG eyes and non-glaucomatous eyes. We obtained anterior lens capsules during combined glaucoma-cataract surgery or during cataract surgery alone from XFG patients and non-glaucomatous senile cataract patients, respectively. We then performed a quantitative analysis of *LOXL1* mRNA expression using these anterior lens capsules.

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METHODS

Subjects: All XFG patients were diagnosed by slit-lamp examination for the existence of exfoliation material on the anterior lens capsule with maximal dilation of the pupils and with glaucomatous optic neuropathy as well as visual field defect. Peripheral blood was obtained from 95 XFG patients 47–93 years of age (mean age: 75.7±8.1 years). The controls were 190 randomly-selected, population-based individuals 54–83 years of age (mean age: 65.0±6.8 years) with no glaucomatous changes or existence of exfoliation materials (Table 1). All of the XFG patients and normal volunteers were recruited at Kyoto Prefectural University Hospital (Kyoto,

Table 1. Clinical characters of the exfoliation glaucoma patients and control.

	XFG	Control
Total number of subjects	95	190
Mean age (range)	75.7 (47–93 years)	65.0 (54–83 years)

Ninety-five exfoliation glaucoma patients 47 to 93 years of age (mean age: 75.7 years), and 190 randomly-selected population-based individuals 54 to 83 years of age (mean age: 65.0 years) with no glaucomatous changes or existence of exfoliation materials were involved in this study.

Table 2. Case-control study of two nonsynonymous single nucleotide polymorphisms in *LOXL1*.

rs1048661 (R141L)* Genotype	Control	XFG	rs3825942 (G153D)** Genotype	Control	XFG
GG	33	0	GG	135	94
GT	114	1	GA	53	1
TT	43	94	AA	2	0

The rs1048661 TT genotype and the rs3825942 GG genotype were significantly associated with an increased risk of XFG under recessive models (χ2 test). The single asterisk indicates TT versus GT+GG, P value=5.34x10–34, OR=321.3, and 95% CI=43.5–2373.2, and the double asterisk indicates GG versus GA+AA, p=2.1x10–8, OR=38.3, and 95% CI=5.2–281.6.

Japan) and examined by glaucoma specialists using slit-lamp microscopy and an automated visual field analyzer. All study subjects were ethnically Japanese. According to the rules of the process committee at Kyoto Prefectural University of Medicine, written informed consent was obtained from all participants before participation in this genetic association study. The study was conducted in accordance with the tenets of the Declaration of Helsinki.

Genotyping: We genotyped two nonsynonymous single nucleotide polymorphisms (SNPs; rs1048661 and rs3825942) in the *LOXL1* gene region according to the previous report [2]. We genotyped the SNPs with both direct sequencing and the TaqMan genotyping assay (Applied Biosystems, Foster City, CA). We used a set of primers (5′-GAT CCA GTG GGA GAA CAA CG-3′ and 5′-GGT ACT CGG GCA GCT CTT C-3′) for direct sequencing. Genotyping was performed using on a 3130xl Sequence Detection System or with 7500 Realtime-time polymerase chain reaction (PCR) system (Applied Biosystems). The TaqMan genotyping assay was performed according to the manufacturer's protocol. All the genotyping procedures were approved by the ethics committee of Kyoto Prefectural University of Medicine.

Statistical analysis: The frequencies of the genotypes were compared between XFG patients and controls in the recessive model. In this model, frequencies of the homozygous genotype for major alleles were compared using a 2x2 contingency table. Here, the association was evaluated using the χ^2 test for the contingency table. A p-value of less than 0.01 was considered to be statistically significant. Odds ratios (OR) and 95% confidence intervals (95% CI) were also calculated.

Real-time polymerase chain reaction analysis: Anterior capsules that were obtained during glaucoma/cataract or cataract surgery with written informed consent were immediately stored with RNAlater reagent (Ambion, Austin, TX) to protect the RNA. All procedures were approved by the ethics committee of Kyoto Prefectural University of Medicine. Total RNA was isolated with the Micro RNA extraction kit (Qiagen Japan, Tokyo, Japan) from the anterior capsules, and then cDNA was prepared as described previously [4]. The anterior capsules were obtained from 10 XFG patients undergoing combined (cataract+glaucoma) surgery (mean age 74.9±8.0 years; male:female=6:4) and 10 non-glaucomatous, senile cataract patients (mean age: 76.5±10.6 years; male:female=4:6). To avoid contamination of blood, anterior lens capsulotomy was performed before glaucoma surgery in cases of combined procedures. We used TaqMan real-time PCR probes and primers specific for human *LOXL1* (Hs00173746_m1), and 18S rRNA from Applied Biosystems (Assay-on-Demand gene expression products). Real-time PCR analysis was performed on a 7500 real-time PCR system. The relative expression of *LOXL1* mRNA in the anterior lens capsule was quantified by the standard curve method using 18S rRNA expression in the same cDNA as the control.

RESULTS

Case-control association study in the LOXL1 gene region: We genotyped 95 XFG cases and 190 control subjects by direct sequencing methods. The rs1048661 TT genotype and the rs3825942 GG genotype in the first exon of *LOXL1* were significantly associated with an increased risk of XFG under recessive models (χ^2 test, raw p value=$5.34x10^{-34}$, OR=321.3, 95% CI=43.5–2373.2 and p=$2.1x10^{-8}$, OR=38.3, 95% CI=5.2–281.6, respectively; Table 2). We further tested reproducibility of the genotyping by TaqMan genotyping assay and confirmed the genotyping results because the results of the rs1048661 genotyping was not concordant with the Hardy–Weinberg equilibrium (HWE). The results obtained by the two methods were identical.

Haplotype analysis of LOXL1 single nucleotide polymorphisms: First, we checked the state of linkage disequilibrium (LD) between rs1048661 and rs3825942 and found that those two SNPs are in a state of strong LD (Table 3). Next, we examined the distribution of two-locus haplotypes in the XFG and control samples (Table 4) using Haploview [5] and PENHAPLO [6] software. Among the two-locus haplotypes of SNPs in exon 1 (rs1048661 G/T and rs3825942 G/A), the TG haplotype showed an increased risk for XFG (TG/TG versus others; p=$6.87x10^{-7}$, OR=312.162) in recessive models. We checked HWE among control subjects using this two-locus haplotype and found that the

two-locus haplotype was concordant with HWE (p=0.0125, χ^2 test).

LOXL1 mRNA quantification using anterior lens capsules: cDNA was synthesized from total RNA isolated from the anterior lens capsules of patients undergoing cataract surgery. We analyzed both XFG (n=10) and senile cataract (n=10) specimens. Figure 1 is a representative result of two independent results of experiments run in duplicate. There was no statistically significant difference between the expression levels of *LOXL1* mRNA in the lens epithelium obtained from XFG patients and senile cataract patients. (Figure 1, p=0.529 by the Mann–Whitney U-test)

DISCUSSION

Similar to previous studies among Caucasian [2,7,8], we found a strong genetic association between the *LOXL1* SNP and XFG patients. The lysyl oxidase protein family has multiple functions including specific oxidative deamination of lysine residues and the cross-linking of elastin [9]. Phenotypic analysis of *LOXL1* knockout mice [10] showed that the LOXL1 protein has an essential role for the homeostasis of elastic fibers, which also contribute to the trabecular meshwork structures [11]. Therefore, it is functionally reasonable to assume that *LOXL1* is one of the causative genes for XFG.

For the rs3825942 genotype, our results are consistent with those of Thorleifsson et al. [2]. The allele frequency of rs3825942 G is consistently higher among Caucasian and Japanese XFG patients so it is reasonable to conclude that rs3825942 A is a protective allele against the occurrence of XFG.

On the other hand, the risk allele for XFG occurrence is rs1048661-"T" among the Japanese population and rs1048661-"G" among the Caucasian population. We double-checked these genotype results (rs3825942 and rs1048661) by both direct sequencing and the TaqMan genotyping assay because the genotype results of rs1048661 within control subjects were not concordant with HWE. The results obtained from two different methods were in complete agreement. Therefore non-concordance with HWE is not due to genotyping errors. We further checked the two-locus haplotype and found that the haplotype was concordant with HWE. Since the two-locus, rs1048661 and rs3825942, was in the state of linkage disequilibrium (D'=1.0), it was appropriate to check the HWE by haplotype.

Inverted genotypes of XFG patients between the Japanese and Caucasian population suggests the following possibilities: (1) The 141st amino acid substitution (R141L) does not have a dominant role for the pathophysiology of XFG, (2) the heterozygote for the rs1048661 G/T genotype may have a protective role against XFG occurrence, or (3) there might be another causative polymorphism in a state of linkage disequilibrium with rs1048661 G/T. If the last possibility is the case, there might be a historical recombination between the SNP rs1048661 and the causative polymorphism.

As a next step, we quantified relative *LOXL1* mRNA expression in anterior lens capsules and found that there was no significant difference between XFG and senile cataract controls. These results are different from the previous study by Thorleifsson et al. [2], which showed a significantly higher *LOXL1* mRNA expression with the TT genotype (the protective genotype against XFG in the Caucasian population) than those with the TG or GG genotype using adipose tissues.

Table 3. Linkage disequilibrium state of rs1048661 and rs3825942.

SNP	D'	r2
Control+XFG	1	0.243
Control only	1	0.196
XFG only	1	1

We calculated the state of linkage disequilibrium between rs1048661 and rs3825942 among the control population, XFG population, and total population. Linkage disequilibrium coefficients were expressed as D' or r2.

Table 4. Structures and frequency of two locus haplotype.

	Frequency	
Haplotype	Case	Control
TG	0.9947	0.5263
GG	0	0.3237
GA	0.0053	0.15

We examined the frequency of two-locus haplotypes in the XFG and control samples using Haploview and PENHAPLO software. The TG haplotype means rs1048661-T and rs3825942-G, the GG haplotype means rs1048661-G and rs3825942-G, and the GA haplotype means rs1048661-G and rs3825942-A.

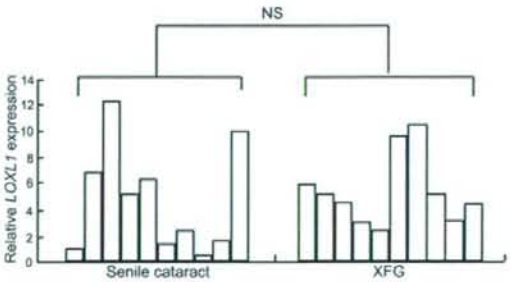

Figure 1. Real-time polymerase chain reaction analysis of *LOXL1* mRNA expression in anterior lens capsules. Total RNA was extracted from the anterior lens capsule of XFG/senile cataracts. Real-time PCR analysis was performed with expression assay probes. The amount of relative expression was normalized to that of 18Sr RNA. (N.S.: Not statistically significant, p=0.529; Mann–Whitney's U-test).

We hypothesize that the difference might be due to the mixed *LOXL1* SNP genotype of our senile cataract surgery samples, which are not able to clarify genotype due to ethical procedure reasons. However, our *LOXL1* mRNA expression analysis reflects a direct pathological site within the ocular tissue and better represents *LOXL1* expression status in the affected eyes than that of adipose tissue [2]. Therefore, we deduced that it is likely that the quantitative difference of *LOXL1* mRNA is not a direct pathogenetic cause of XFG.

To further clarify the pathophysiological role of *LOXL1* for XFG, we are now performing extensive SNP discovery around *LOXL1* to find out other possible causative polymorphisms. In addition, functional analysis of LOXL1-fibulin5 protein interaction [12] using two types of recombinant LOXL1 precursor protein (141R and 141L) is ongoing. Since LOXL1-fibulin5 interaction is essential for mature cross-linked elastin formation [10,12], we are focused on determining the role of the two variants of *LOXL1* and its mixture to gain further insight into our genetic association results showing that the rs1048661 G/T heterozygote is protective against XFG. It is also important to investigate the protective role of LOXL1 protein variants that possess the 153D amino acid, which is indicated by multiple genetic association studies including our own [2,7,8] as well as to analyze the possible role of the pairs of 141/153 amino acid variants in consideration of our haplotype analysis result.

ACKNOWLEDGMENTS

This study was supported by grants-in-aid for Scientific Research (programs 18890164, 14657449, and 18659516) from the Japanese Ministry of Education, Culture, Sports, Science and Technology. We thank Dr. Shigeo Kamitsuji (Stagen. Co. Ltd., Tokyo, Japan) for statistical analysis and Mr. John Bush for English language editing. During the submission process of this manuscript, Hayashi et al. published a LOXL1 genetic association study using a Japanese XFS population (Am J Ophthalmol 2008;145:582–585).

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The print version of this article was created on 2 June 2008. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.

JOURNAL OF OCULAR PHARMACOLOGY AND THERAPEUTICS
Volume 24, Number 2, 2008

DOI: 10.1089/jop.2007.0016

Effects of Switching from Topical β-Blockers to Latanoprost on Intraocular Pressure in Patients with Normal-Tension Glaucoma

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ABSTRACT

Aims: **The effects of switching from topical β-blockers (β) to latanoprost (LA) on intraocular pressure (IOP) and IOP-reduction rate (IOP-RR) in patients with normal-tension glaucoma (NTG) were investigated.**

Subjects and Methods: **Sixty (60) NTG patients (60 eyes) were divided into three equal groups receiving carteolol hydrochloride (group A), nipradilol (group B), and betaxolol hydrochloride (group C) twice-daily for 3 months. The drugs were changed to topical LA administered once-daily for the next 3 months.**

Results: **Baseline IOP was 14.4 ± 0.9, 14.6 ± 0.6, and 14.6 ± 0.9 mmHg in groups A, B, and C, respectively. At 3 months, IOP was 12.4 ± 0.6, 13.4 ± 0.6, and 12.9 ± 0.8 mmHg and 10.5 ± 0.5, 11.1 ± 0.8, and 11.7 ± 0.8 mmHg at 6 months in groups A, B, and C, respectively. At 3 months, IOP-RR was 10.4 ± 5.5, 9.5 ± 2.6, and 10.8 ± 4.7% and 24.1 ± 4.3, 22.9 ± 5.9, and 19.4 ± 3.8% at 6 months in groups A, B, and C, respectively. The groups did not significantly differ in the first 3 months regarding IOP and IOP-RR. Switching to LA significantly decreased IOP and increased IOP-RR in all groups.**

Conclusion: **In NTG patients, LA reduced IOP more effectively than the β tested.**

INTRODUCTION

GLAUCOMA AFFECTS MORE THAN 66 million people in the world and is the leading cause of blindness.[1] The Tajimi study,[2,3] which was one of the largest glaucoma epidemiology studies in Japan, showed that the glaucoma prevalence rate in Japanese older than 40 years of age is 5.0%, and the rate of open-angle glaucoma is 3.9%. That study also reported that almost 90% of the open-angle glaucoma cases consisted of normal-tension glaucoma (NTG). Thus, NTG is the most prevalent glaucoma type in Japan, and is one where the difficulty of managing treatment needs to be addressed.

In the treatment of NTG, reducing intraocular pressure (IOP) is the only evidence-based therapy,[4–8] though in some NTG patients, trabeculectomy is one of the effective treatments to prevent the progression of visual-field defects.[5,7,9] However, there are some patients whose glaucomatous damages progress while their IOP is kept in the low teens. In such patients, topical beta (β)-blockers with neuroprotective effects[10–14]

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TABLE 1. GROUP BACKGROUNDS

Group	n (M/F)	Age (years)	Baseline intraocular pressure (mm Hg)
A	16 (7/9)	59.5 ± 3.2	14.4 ± 0.9
B	14 (4/10)	60.8 ± 3.7	14.6 ± 0.6
C	13 (2/11)	59.2 ± 4.1	14.6 ± 0.9

Note. In these 3 groups, there was no significant difference among age and baseline intraocular pressure.

are frequently chosen. In this prospective study, we compared the IOP-reduction effects of three types of topical β-blockers (carteolol hydrochloride, nipradilol, and betaxolol hydrochloride), which were supposed to have neuroprotective effects and latanoprost (LA) in the same NTG subjects.

As for nipradilol, it is a nonselective alpha-1 beta-adrenergic antagonist that reduces IOP, and it is as potent as timolol for primary open-angle glaucoma (POAG)[15] because it increases uveoscleral outflow by the alpha-1 blocking effects and decreases aqueous production through the β-blocking effect.[16]

METHODS

At the outpatient clinic of Kyoto Prefectural University of Medicine and the Baptist Eye Clinic (Kyoto, Japan), 60 patients with NTG (60 eyes: 21 males and 39 females; mean age, 61.7 ± 1.9 years), who were newly diagnosed or who used only one antiglaucoma eye drop, were enrolled. This study was carried out between June 2001 and April 2003. They had enough informed consent by a glaucoma specialist and did not suffer from asthma, arrhythmia, or heart disease. In patients who had been treated with other topical medications, 4 weeks were allowed for wash-out before starting topical β-blockers. The diagnostic criteria for NTG were (1) normal iridocorneal open angle, (2) no evidence of IOP higher than 21 mmHg, (3) glaucomatous changes in the visual field with optic-nerve cupping, and (4) absence of other optic neuropathies. The 60 patients were divided into three equally sized groups, and each group received one type of β-blocker (group A, carteolol hydrochloride; group B, nipradilol; group C, betaxolol hydrochloride) twice a day for 3 months. During the subsequent 3 months, each group was treated once a day with topical LA only. Their IOP was measured once a month during this 6-month period between the hours of 9:30 AM and 12:00 PM by glaucoma specialists with a Goldmann applanation tonometer (Haag-Streit, Bern, Switzerland). The IOP-reduction rate (IOP-RR) was then calculated and the effect of switching from the β-blocker to LA was statistically analyzed by using the formula (baseline IOP-current IOP)/baseline IOP × 100. Statistical analysis was performed with the Tukey-Kramer test, the paired- and unpaired *t* test, and the chi-square test.

We defined nonresponders as patients whose IOP-RR was 10%[17,18] or less in each 3-month period and calculated the rate of nonresponse to β-blockers and LA. To analyze IOP, right-eye data was used when data from both eyes was available.

RESULTS

In the course of this study, 17 patients dropped out; 6 (35.3%) owing to LA side effects (a reddish conjunctive state, or irritated conjunctiva), 3 (17.6%) due to β-blocker side effects, and 8 (47.1%) owing to other reasons, such as failure to continue visits. All drop-out patients were subsequently excluded from our statistical analysis. Subjects analyzed after exclusion of the dropouts are summarized in Table 1. Baseline IOP was 14.4 ± 0.9, 14.6 ± 0.6, and 14.6 ± 0.9 mmHg in groups A, B, and C, respectively. There was no

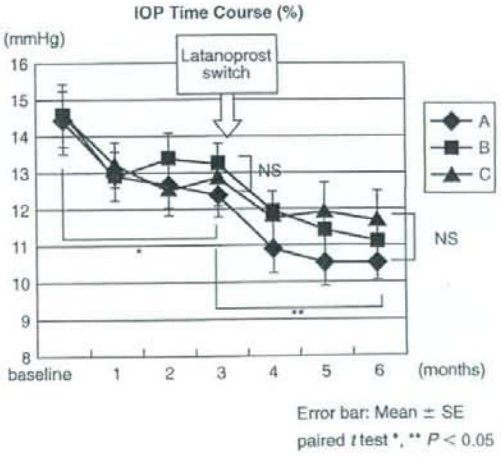

FIG. 1. By switching from β-blocker to latanoprost, intraocular pressure was significantly decreased in all groups.

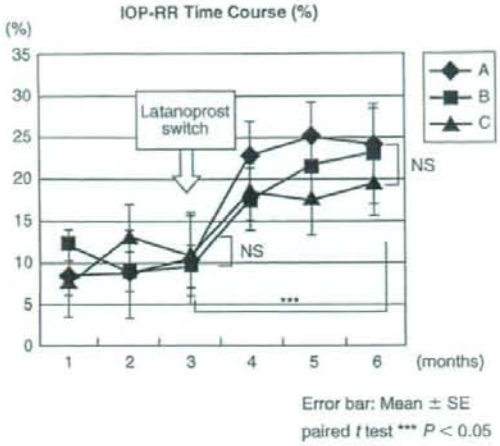

FIG. 2. There were no significant intergroup differences throughout 6 months (Tukey-Kramer test). In all three groups, the intraocular pressure reduction rate was significantly higher at 6 than at 3 months after the inception of the study (paired t test).

TABLE 2. THE CHANGE OF INTRAOCULAR PRESSURE REDUCTION RATE (IOP-RR) AND NONRESPONDERS AT EACH 3-MONTH PERIOD

	β-blockers		*Latanoprost*	
IOP-RR	n	%	n	%
30%	4	9.3	14	32.6
20%	9	20.9	12	27.9
10%<	7	16.3	8	18.6
Nonresponders	23	53.5*	9	20.9*

Asterisk indicates a significant difference in nonresponders between β-blocker and latanoprost (chi-square test, Yates $P = 0.0257$).

significant difference with respect to age or baseline IOP among the three groups. The IOP time courses are shown in Figure 1. At 3 months, IOP was 12.4 ± 0.6, 13.4 ± 0.6, and 12.9 ± 0.8 mmHg, and at 6 months, IOP was 10.5 ± 0.5, 11.1 ± 0.8, and 11.7 ± 0.8 mmHg in groups A, B, and C, respectively. Irrespective of the type of β-blocker administered during the first 3 months, IOP became significantly lower than the baseline; there

TABLE 3. PREVIOUS RESULTS OF MONOTHERAPY SWITCHING β-BLOCKER TO LATANOPROST

Authors	*Glaucoma type*	*Race*	*Before switching eye drop*	n	*pre latanoprost IOP*	*post latanoprost IOP*	*RR (%) from β-blocker to latanoprost*	*Evaluation period*
Zimmerman et al.	POAG	Caucasian	Betaxolol	236	19.9 ± 4.0	16.3 ± 3.3	18.1	6 months
	OH	Black	Carteolol	80	20.1 ± 3.4	15.8 ± 3.3	21.4	
	PE	Hispanic	Levobunolol	177	19.9 ± 3.5	16.9 ± 3.3	15.1	
	PD	Asian	Timolol hemihydrate	173	19.9 ± 3.9	17.6 ± 3.7	11.6	
			Timolol	397	20.6 ± 3.6	17.7 ± 3.3	14.1	
			Timolol gel	816	20.3 ± 3.8	17.4 ± 3.7	14.3	
Bayer et al.	POAG	n/a	Timolol	462	21.1 ± 4.1	17.4 ± 3.1	17.5	24 months
	OH		Levobunolol	79	20.9 ± 3.5	17.4 ± 2.1	16.7	
	PE		Metipranolol	60	20.5 ± 3.5	17.2 ± 2.7	16.1	
	CACG		Betaxolol	40	21.6 ± 4.0	17.1 ± 3.6	20.8	
			Carteolol	33	22.3 ± 2.8	18.0 ± 2.9	19.3	
Haverkamp et al.	POAG OH PE CACG	n/a	Beta-blocker	451	21.3 ± 4.0	17.3 ± 2.7	18.8	3 months
Bron et al.	POAG OH PE	n/a	Timolol	17	26.3 ± 1.2	19.6 ± 1.1	25.5	6 weeks
Ikeda	NTG	Japanese	Carteolol	16	12.4 ± 0.6	10.5 ± 0.5	15.3	3 months
			Betaxolol	14	13.4 ± 0.6	11.1 ± 0.8	17.2	
			Nipradilol	13	12.9 ± 0.8	11.7 ± 0.8	9.3	

POAG, primary open-angle glaucoma; OH, ocular hypertension; PE, pseudoexofoliation; PD, pigment dispersion; CACG, chronic angle-closure glaucoma; NTG, normal-tension glaucoma; carteolol, carteolol hydrochloride; betaxolol, betaxolol hydrochloride.

was no significant intergroup difference. During the 3 months of LA administration, a significant decrease in IOP was again revealed in all groups, irrespective of the type of β-blocker administered before switching. Again, there was no intergroup difference. At 3 months, IOP-RR was 10.4 ± 5.5, 9.5 ± 2.6, and 10.8 ± 4.7%, and at 6 months, IOP-RR was 24.1 ± 4.3, 22.9 ± 5.9, and 19.4 ± 3.8% in groups A, B, and C, respectively. In all three groups, the IOP-RR was significantly higher at 6 than at 3 months after the inception of the study (Fig. 2). All data are presented as the mean ± standard error.

Table 2 shows the change of IOP-RR and nonresponders rate from each β-blocker to LA. These results confirm that the IOP-reduction effect of LA is significantly greater than that of the β-blockers tested. For the rate of nonresponders, LA is significantly lower than β-blockers.

DISCUSSION

As for POAG and ocular hypertension, there have previously been similar studies regarding switching from β-blocker to LA.[19–22] However, and to the best of our knowledge, our study is the first to compare LA and the three types of β-blockers in the switching condition, while previous reports compared between the two discrete NTG groups.[23–25] The IOP-RR from β-blocker to LA in the NTG patients shown in our data was smaller (9.3%–17.2%) than that in the POAG patients (11.6%–25.5%) presented in previous studies (Table 3).

When discussing drug effectiveness, it is very important to compare that effectiveness in the same patients, not in different patients, because each patient has a possibility to react differently to each drug. However, a switching study is very difficult to perform because of the high dropout rate owing to the long study period or drug side effects. Our study also suffered the relatively high dropout rate, mainly because the patients could not come regularly owing to the long study period. Other reasons why the patients dropped out were the side effects of the medications; LA is twice as much as β-blockers.

We chose three topical β-blockers, carteolol hydrochloride, nipradilol, and betaxolol hydrochloride, because they were frequently used to treat NTG patients in Japan, considering the neuroprotecitve effects and the increase of ocular blood flow.[26,27] However, from our clinical impression, these drugs seem to be less effective for IOP reduction, when compared to LA, even in the NTG patients. Therefore, in this current study, we just focused on the IOP reduction effects of the three β-blockers and LA administered, and found that latanoprost is more effective than any of the three β-blockers, which showed the same IOP reduction during the administration period, and that the types of β-blockers administered prior to switching had no effect on patients' response to LA.

Recently, attention has been paid to LA nonresponders who show little or no IOP reduction effects by latanoprost. However, few reports mentioned the β-blocker nonresponders. In our study, the nonresponder rate of β-blockers among NTG patients was higher than that of LA, and more than half of the β-blocker nonresponders (53.5%) responded to LA after switching, while only 1 patient did not respond to LA but responded to β-blockers. There were 7 double nonresponders for both β-blockers and LA, each of whose baseline IOP was significantly lower (12.0 ± 0.8 mmHg) than that of the responders (15.7 ± 0.4 mmHg) ($P < 0.001$; unpaired t test). This result leads to the facts that the NTG patients whose baseline IOP is in the low teens, or lower, are more difficult to reduce IOP than those of mid-teens or higher.

In cases where a NTG patient was found to be a double nonresponder, the available treatment options for managing and controlling that patient's IOP are quite limited. One possible option is to use other types of drugs, such as carbonic anhydrase inhibitors; however, that treatment might prove to be inadequate in many cases. Another option is a combination therapy, although the IOP-reduction power of each individual portion of the therapy might be weak.

CONCLUSION

This study suggests that LA provides a much greater IOP-RR and lower nonresponder rate than that of β-blockers in Japanese NTG patients.

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Received: February 21, 2007
Accepted: November 19, 2007

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Jpn J Ophthalmol 2008;52:231–238

Anterior Segment Optical Coherence Tomography Findings of Acute Angle-Closure Glaucoma in Vogt-Koyanagi-Harada Disease

Ultrasound biomicroscopy (UBM) is reported to be useful for the evaluation of the anterior chamber, chamber angle, and ciliary body. However, a UBM examination is difficult and invasive for the patient. The new Visante (Carl Zeiss Meditec, Oberkochen, Germany) imaging system using anterior segment optical coherence tomography (AS-OCT) makes it possible to visualize the anterior segment noninvasively to obtain quantitative information. We report a patient with acute angle-closure glaucoma associated with Vogt-Koyanagi-Harada (VKH) disease in whom imaging with AS-OCT was carried out to evaluate the anterior segment.

Case Report

A 50-year-old woman presented with a 2-day history of headache and blurred vision in both eyes. Her visual acuity in the right eye was 1.0 when corrected by –4.25 diopters, and 0.9 corrected by –3.50 diopters in the left eye. Intraocular pressure was 44 mmHg in the right eye and 42 mmHg in the left eye. Slit-lamp examination disclosed a shallow anterior chamber and narrow chamber angle in both eyes. Signs of iridocyclitis were absent. Fundus examination disclosed serous retinal detachment, edema around the optic disc, and circumferential detachment of the ciliary body in both eyes. (Fig.1A). She was diagnosed as having acute angle-closure glaucoma in VKH disease. Fluorescein angiography (Fig. 1B) and the human leukocyte antigen pattern of positive DR-4 (DRB1*04) also supported the diagnosis. AS-OCT images showed a shallow anterior chamber, narrow chamber angle, and supraciliary fluid in both eyes. The iris showed anterior bowing consistent with a pupillary block in both eyes (Fig. 2A–C).

These findings improved after two separate pulsed treatments with methylprednisolone at the initial daily dosage of 1000 mg followed by oral prednisolone. After the initial pulsed treatment, intraocular pressure was 13 mmHg in the right eye and 15 mmHg in the left. Three weeks after corticosteroid treatment, the patient's visual acuity was 0.9 in both eyes, and the refraction state became –2.00 diopters in the right eye and –1.00 diopters in the left.

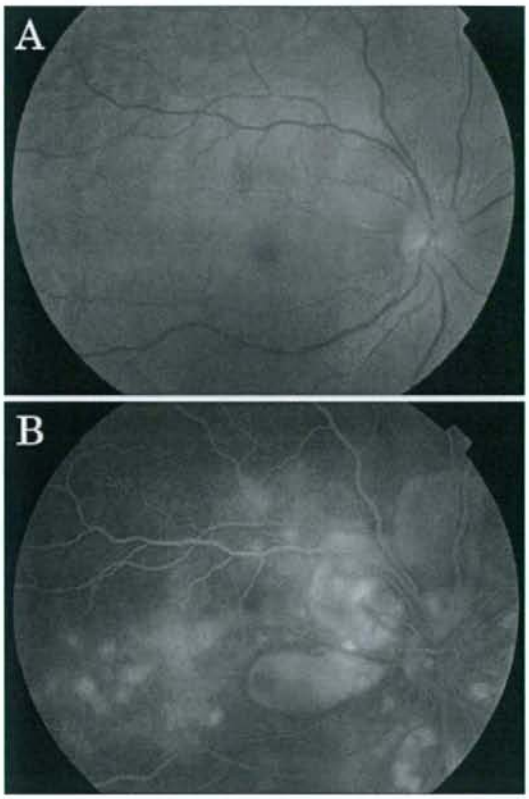

Figure 1. **A** Fundus photograph showing serous retinal detachment in the right eye of a Vogt-Koyanagi-Harada patient. **B** Fluorescein angiogram showing hyperfluorescent spots consistent with dye leakage and dye pooling in the subretinal space.

Comments

VKH disease is a bilateral panuveitis with serous retinal detachment and symptoms of meningeal irritation, dysacusia, and poliosis.[1] The disease is accompanied by various degrees of inflammation of the iris, ciliary body, and choroid, but changes in the anterior segment of the eyeball have been examined only by means of slit-lamp microscopy. UBM examination is a useful method for evaluating these changes of the anterior segment, and the diagnostic utility of UBM has been reported in various studies of many kinds of disease.

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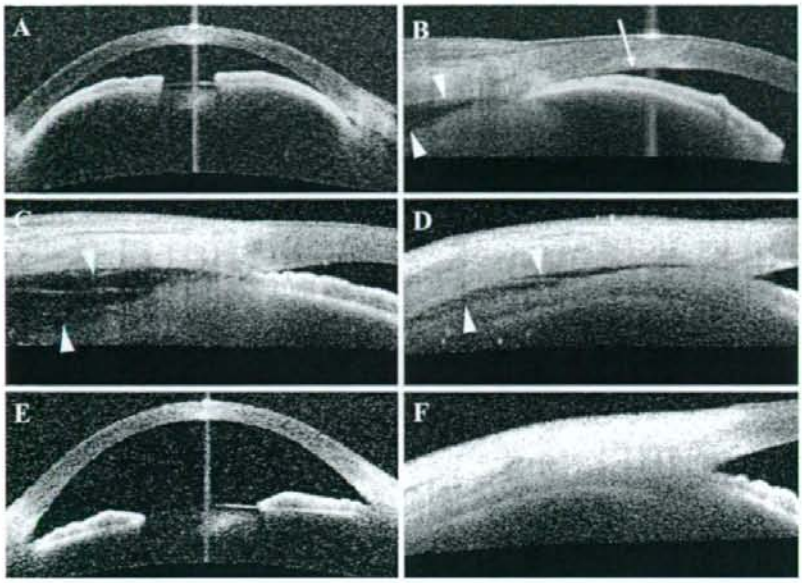

Figure 2A–F. Right eye. **A, B** Anterior segment optical coherence tomography images showing shallow anterior chamber, narrow chamber angle (*arrow*), and supraciliary fluid (*arrowheads*). **C** The supraciliary fluid (*arrowheads*) was evident before steroid treatment. **D** Ten days after steroid treatment, the supraciliary fluid (*arrowheads*) decreased. **E, F** Fourteen days after steroid treatment, the anterior chamber returned to the normal depth and supraciliary fluid had disappeared.

Anterior segment imaging is a rapidly advancing field in ophthalmology.[2] AS-OCT is a new imaging system that provides quantitative information and qualitative imaging of the cornea and anterior chamber with a short measurement time. This system does not require contact with the eye to obtain measurements, so it is a noninvasive examination. AS-OCT can measure corneal thickness, anterior chamber depth, and chamber angle. AS-OCT has also been used for measurements of corneal flap depth following laser in situ keratomileusis (LASIK), of anterior chamber width prior to phakic intraocular lens implantation, and of morphologic changes occurring in eyes after glaucoma surgery.[2]

Many studies have reported UBM findings in VKH disease,[3-5] yet to the best of our knowledge this is the first report about AS-OCT findings of acute angle-closure glaucoma in VKH disease. After systemic corticosteroid treatment, AS-OCT images showed that the anterior chamber returned to the normal depth, the ciliary body reverted to its normal position, and the supraciliary fluid disappeared in both eyes (Fig. 2D–F). Supraciliary fluid secondary to inflammation of the uvea is considered to be related to the development of a shallow anterior chamber.[3-5] This study shows that AS-OCT is a simple and noninvasive imaging technique with high resolution that is useful for observing the response to corticosteroid treatment in a VKH patient with acute angle-closure glaucoma.

Key Words: acute angle-closure glaucoma, anterior segment optical coherence tomography, supraciliary fluid, Vogt-Koyanagi-Harada disease

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DOI 10.1007/s10384-007-0523-5

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Journal of Proteomics & Bioinformatics - Open Access
www.omicsonline.com Research Article JPB/Vol.1/October 2008

Applying Magnetic Bead Separation / MALDI-TOF Mass Spectrometry to Human Tear Fluid Proteome Analysis

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Received August 27, 2008; Accepted October 06, 2008; Published October 10, 2008

Citation: Sekiyama E, Matsuyama Y, Higo D, Nirasawa T, Ikegawa M, etal. (2008) Applying Magnetic Bead Separation / MALDI-TOF Mass Spectrometry to Human Tear Fluid Proteome Analysis. J Proteomics Bioinform 1: 368-373.

Key words: tear fluid; magnetic bead; Proline-rich protein 4; ClinProt; MALDI-TOF-MS

Abstract

The proteins and peptides in tears play an important role in preserving the integrity and stability of the ocular surface. Proteomic analysis of tear films will enable us to detect early biological markers of eye diseases, however, it is often hampered by the small amount of tear volume and the low protein concentration. Here we adopted magnetic bead-based purification (ClinProt system) followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to profile human tear proteins. Basal and reflex tear fluids were collected from normal healthy volunteers using glass microcapillary tubes. Reversed phase (C8) and weak cation exchange (WCX) magnetic beads were applied to obtain multiple components detected as clear signals. Principal component analysis showed a clear differentiation between basal and reflex tears. Among the key alterations, two markedly increased peaks in the reflex tear fluids at m/z 2422.12 and m/z 2721.29 were subsequently analyzed by tandem MS analysis and their source to be proline-rich protein 4 (PRP4). We conclude that magnetic bead-based separation combined with MALDI-TOF-MS (ClinProt MALDI-TOF) appears to be ideally suited for the first-line screening of peptides and proteins in tears.

The search for biomarkers of human diseases has been increasingly successful because of emerging new techniques in the field of proteomics (Hu, 2006; Villanueva, 2004; Zhang, 2004; Ketterlinus, 2005; Koo, 2005; Cheng AJ, 2005; Mirr EN, 2005). Proteins and peptides in tears are reported to play important roles in preserving the integrity and stability of the ocular surface, and changes in tear proteins eins are associated with various pathological eye conditions (Koo, 2005). Among the molecules identified are candidates for biomarkers of dry-eye diseases (Grus, 2005; Tomosugi N, 2005). Earlier investigations of tear film proteins have included extensive analysis using high-performance liquid chromatography (HPLC) or two-dimensional (2-D) gel electrophoresis, combined with mass spectrometry-based protein identification (Koo, 2005; Cheng, 2005; Mirr, 2005; Grus, 2005; Tomosugi, 2005; Kijlstra 1989; Zhou, 2006; Li, 2005; Fung, 2004; de Souza, 2006), but these protocols are sometimes hampered by the small amount of tear fluid and its low protein concentration. For high-throughput analysis, surface-enhanced laser desorption / ionization time-of-flight (SELDI-TOF) MS analysis was developed (Grus, 2005; Tomosugi N, 2005). With this technique, very small sample volumes can be directly applied to chip-based array surfaces; however, its limitations include the difficulty of further protein identification. Here we show that the combination of magnetic bead separation and MALDI-TOF MS spectrometry (ClinProt system) is a reasonably efficacious, simple method for profiling and identifying proteins from eluted tear fluids.

Open-eye basal tear fluids were collected from twenty normal healthy volunteers who did not wear contact lenses and had no evidence of ocular disease. The subjects ranged in age from 20 to 29 years, old enough to collect properly physiological tears as described below. Informed consent was obtained from all volunteers participating in the study, and the protocols were approved by the institutional ethics committee and conformed to the provisions of the Declaration of Helsinki. The ophthalmic examination included subjective symptoms, Schirmer's test, biomicroscopy with careful examination of the lid margin and meibomian glands, and tear break-up time. Each volunteer was questioned about subjective symptoms such as burning, itching, foreign body sensation, dryness, and photophobia. Tear fluid was collected in the afternoon using 1-µL glass micro-capillary tubes (Corning, New York, NY, USA) without touching the lid margins or eye-lashes. After basal tear fluids were collected, reflex tear fluids were elicited by nasopharyngeal scrub and collected. The collected samples were stored at -80 °C until analysis.

For analysis, the tear fluid samples were thawed and purified with a reagent set that included two kinds of chemically coated magnetic beads: reversed phase (C8) and weak cation exchange (WCX) (ClinProt™ Bruker Daltonics). We used œ-cyano-4-hydroxycinnamic acid as the matrix solution. All these procedures were performed at room temperature with moderate humidity. The eluted samples were then dropped onto a MALDI sample plate (600 µm Anchorchip™: Bruker Daltonics), and spectra were obtained by an Autoflex II MALDI-TOF mass ss spectrometer (Bruker Daltonics) operated in positive-ion linear mode. All spectra were obtained randomly over the surface of the matrix spot. The criteria for peak detection were: signal-to-noise ratio >5 and 2-Da peak-width filter. Approximately 10-20 peaks were produced after the treatment with the WCX or C8 beads (Fig. 1A). Multiple components were detected as clear signals in the mass range of 0-20 kDa, which includes proteins such as lysozyme and lipocalin. Inducible secreted tear proteins are believed to consist primarily of three entities that account for 85% of the total protein content: lysozyme, lactoferrin, and the tear-specific lipocalins (Kijlstra, 1989). Lysozyme and lipocalin were previously identified as protein fragments at *m/z* 14,687.8 and *m/z* 17,438, respectively (Fung, 15 2004). In the present study, we detected signals with the same *m/z* ratios in the basal tear fluid samples (Fig.1A). However, we could not detect lactoferrin by MALDI-TOF MS analysis with WCX or C8 beads, or by electrospray ionization (ESI)-MS analysis, for unknown reasons (Kijlstra, 1989).

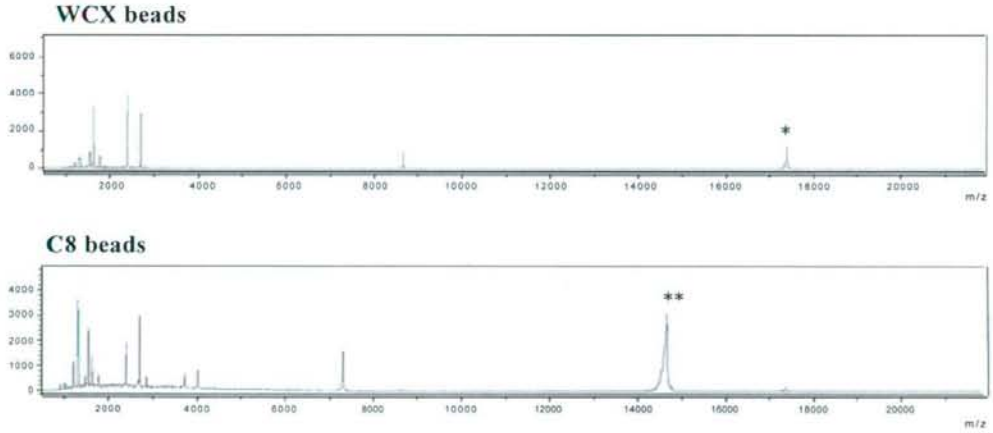

Figure 1: Protein/Peptide profiling of tear fluid samples from twenty healthy volunteers using ClinProt Mass Spectrometry.

(A) Typical ClinProt profiles of basal tear fluids eluted from WCX and C8 beads in the mass range 0-20 kDa m/z and subjected to flexAnalysis™. Multiple components were detected as clear signals, including lipocalin (*: m/z 17438) and lysozyme (**: m/z 14687.8).

The obtained data were graphed as columns representing normalized peak intensities (Fig.1B; pseudo-gel view) and further analyzed by a multivariate statistical analysis including principal component analysis (PCA) by the *ClinProTools*TM software (Bruker Daltonik) (Zhang, 2004; Ketterlinus, 2005). The results showed a differential distribution of samples from basal tears ars from reflex tears (Fig.1C).

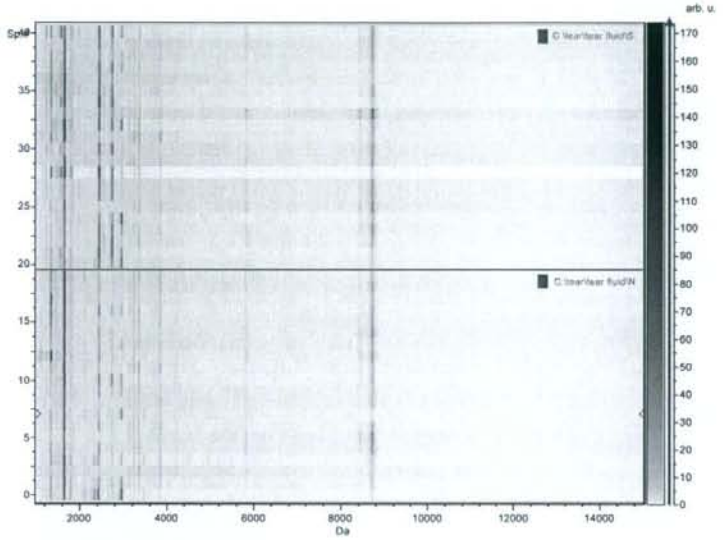

Figure 1: (B) Psuedo-gel views of the mass spectrum of basal tear fluids (lower column) and the reflex tear fluids (upper column) were shown with the calculated molecular weight (m/z values) along the x-axis and relative intensity along the y-axis using ClinProtools™.

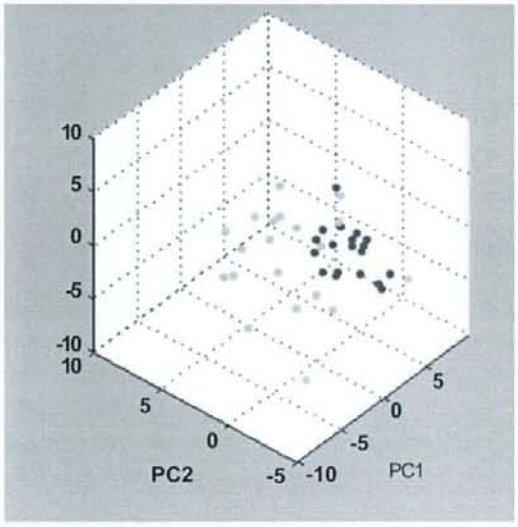

Figure 1: (C) 3-D view of PCA scores plot analyzed by *ClinProtools*™. Green spots represent reflex tears and the red spots represent basal tears.

We next examined the profiles of proteins smaller than 3.5 kDa obtained from seven representative samples each of basal and reflex tear fluid (Fig.1D). For this purpose, the selected peak must have sufficient intensity to generate a valuable MS/MS fragment spectrum, and a spectrum is acquired in the high-resolution reflectron mode to determine the exact mass of the molecule of interest. Although it is clear from visual inspection (Fig.1D), the two peaks in the spectra obtained from the reflex tears seemed to be the key protein / peptides peaks contributing the most towards the group selection by PCA loading plots as well (data not shown). Subsequently, the TOF/TOF fragment spectrum is acquired from the same sample spot and used for de-novo sequencing or database search. Before the analysis, the tear fluids were concentrated using a ZipTip (Millipore). In the MALDI-TOF/TOF mode, precursor ions were accelerated to 8 kV and selected in a timed ion gate. The fragments were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after the ion reflector passage. S/MS spectra were searched against the human NCBI database using the *MASCOT* search algorithm (http://www.matrixscience.com/home.html), with a mass tolerance of 0.2Da for MS and 0.75 for MS/MS. No enzyme was selected and methionine oxidation and acetylation of the N terminus were used as variable modifications

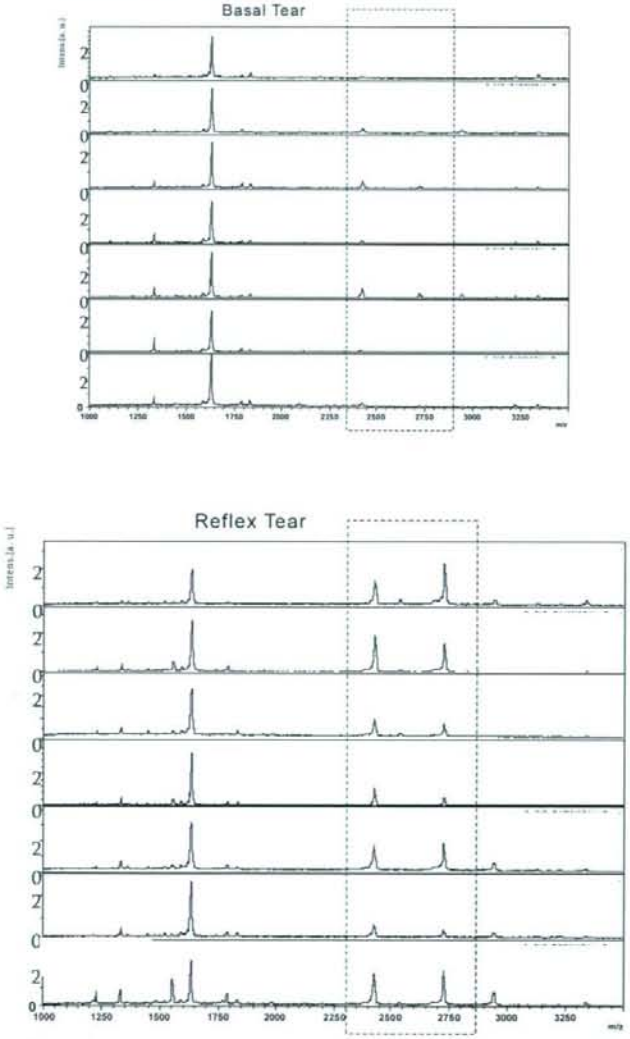

Figure 1: (D) ClinProt profiles of basal and reflex tear fluids eluted from WCX beads in the mass range 1000-3500 Da m/z (n=7). In the reflex tear fluid samples, the height of two peaks increased markedly (m/z 2422 and 2721) (inside the dotted squares).

The Mascot probability score ore for the peak with m/z =2,422 was 66, indicating a reasonably high confidence in identifying the peptide sequence. The sequence was determined to be QEASSFFRRDRPARHPQEQP, which matched the C-terminal fragment of proline-rich protein 4 (aa. 113-132) (locus number AAB26584) (Supplementary data A) (Fung, 2004). A complete MS/MS spectrum of the peak with m/z =2,721 was not obtained; however, the peptide sequence was determined to be RRDRPARH ~W, which partially matched the C-terminal fragment of proline-rich protein 4 (aa.120-134) in the NCBI BLAST protein-protein database (locus number AAB26584) (Supplementary data B).

We also analyzed the protease-digested HPLC fractions of our samples by 15 ESI-LC/MS/MS (esquire HCT; Bruker Daltonics). Not surprisingly, several well-known abundant tear proteins, such as lysozyme, lacritin, lipocalin, and secretoglobin, were detected, and a total of proteins, including PRP4, were identified in the reflex tear fluids. In this study, lactoferrin was not among the abundant proteins detected in tear fluids (Kijlstra, 1989).

Proline-rich proteins (PRPs) are believed to play a significant role in the oral mucosal defense system, in which they affect the aggregation of microorganisms, thereby decreasing the organisms' capacity to colonize tissue surfaces (Fung, 2004; de Souza, 2006). In addition, bacterial proteases are known to clip the *N*-5 -terminus of PRPs, releasing two peptides that have cytokine-like properties, by which they up-regulate the host defense against potential pathogens. PRP4 is expressed in the lacrimal acinar cells and other anterior exocrine glands (Dickinson, 1995). Since the reflex tear fluids were collected soon after the nasopharyngeal scrub, the PRP4 detected in the reflex tear fluids may have been stored in the acinar cells and released quickly after the stimulation. In addition to PRPs, lysozyme is reported to mediate protective functions in the eye (Kijlstra, 1989; Zhou, 2006; Li, 2005; Fung, 2004; de Souza, 2006). Lysozyme serves as a non-specific innate opsonin by binding to the bacterial surface, reducing the negative charge, and facilitating phagocytosis of the bacterium before opsonins from the acquired immune system arrive at the scene. In contrast to PRP4, the peak height of lysozyme showed no remarkable difference between the basal and reflex tear fluids. Thus, it is possible that PRP4 is the first molecule that rapidly confronts foreign antigens at the ocular surface.

In conclusion, the key finding of this study is the up-regulation of a C-terminus of PRP4 in the reflex tear fluids from normal healthy subjects. Accordingly, the magnetic bead separation and MALDI-TOF analysis in combination with bioinformatics software is useful for the high-throughput protein profiling of tear fluids. This is the first study demonstrating the usefulness of the *ClinProt beads system* for r this purpose. This simple and easy approach may be applicable to the discovery of biomarkers in ocular diseases as well.

Acknowledgements

We are grateful to Dr. Seta J. (Bruker Daltonics) and Miss Tanaka M. and Omi N. for technical support. We also thank Dr. Yoneda K. for sample collection. We are indebted to Drs. Nakano, Taniguchi and Yagi for discussion and Mrs. Kamisako and Miss Tsuda for their excellent clerical work.

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