Table 2. Allelic and genotypic counts and frequencies of rs1048661, rs3825942, and rs2165241 in exfoliation syndrome/glaucoma (EX, EG, and EX+EG), primary glaucoma (PG, NG, PG+NG), and cataract (CT).

	Exfoliation syndrome or glaucoma						Primary glaucoma							
	EX		EG		EX+EG		PG		NG		PG+NG		CT	
	Count	Frequency	Count	Frequency	Count	Frequency	Count	Frequency	Count	Frequency	Count	Frequency	Count	Frequency
rs1048661														
Allele														
T	111	0.941	159	0.958	270	0.951	39	0.488	50	0.463	89	0.473	140	0.446
G	7	0.059	7	0.042	14	0.049	41	0.513	58	0.537	99	0.527	174	0.554
Genotype														
TT	54	0.915	76	0.916	130	0.916	10	0.25	8	0.148	18	0.192	25	0.159
TG	3	0.051	7	0.084	10	0.070	19	0.475	34	0.630	53	0.564	90	0.573
GG	2	0.034	0	0	2	0.014	11	0.275	12	0.222	23	0.245	42	0.268
rs3825942														
Allele														
G	117	0.992	165	0.994	282	0.993	64	0.800	86	0.796	150	0.798	253	0.806
A	1	0.009	1	0.006	2	0.007	16	0.200	22	0.204	38	0.202	61	0.194
Genotype														
GG	58	0.983	82	0.988	140	0.986	25	0.625	33	0.611	58	0.617	100	0.637
AG	1	0.017	1	0.012	2	0.014	14	0.350	20	0.370	34	0.362	53	0.338
AA	0	0	0	0	0	0	1	0.025	1	0.019	2	0.021	4	0.026
rs2165241														
Allele														
C	117	0.992	165	0.994	282	0.993	77	0.963	93	0.861	170	0.904	275	0.876
T	1	0.009	1	0.006	2	0.007	3	0.038	15	0.139	18	0.096	39	0.124
Genotype														
CC	58	0.983	82	0.988	140	0.986	37	0.925	40	0.741	77	0.819	122	0.777
CT	1	0.017	1	0.012	2	0.014	3	0.075	13	0.241	16	0.170	31	0.198
TT	0	0	0	0	0	0	0	0	1	0.019	1	0.011	4	0.026

EX, exfoliation syndrome; EG, exfoliation glaucoma; PG, primary open angle glaucoma aged 70 years or older; NG, normal tension glaucoma aged 70 years or older; and CT, cataract aged 70 years or older.

Table 3. Odds ratios and p values for allelic and genotypic frequencies of rs1048661, rs3825942, and rs2165241 in comparison between cases (EX, EG, and EX+EG) and controls (CT, PG, NG, and PG+NG).

	EX				EG				EX+EG			
Parameter	**Versus**	**Versus**	**Versus**	**Versus**	**Versus**	**Versus**	**Versus**	**Versus**	**Versus**	**Versus**	**Versus**	**Versus**
rs1048661	**CT**	**PG**	**NG**	**PG+NG**	**CT**	**PG**	**NG**	**PG+NG**	**CT**	**PG**	**NG**	**PG+NG**
Allele												
p value	1.69×10^{-23}	3.23×10^{-13}	2.55×10^{-16}	4.08×10^{-19}	5.65×10^{-33}	1.92×10^{-17}	1.32×10^{-21}	4.71×10^{-26}	3.00×10^{-45}	2.61×10^{-20}	4.40×10^{-26}	1.18×10^{-33}
OR	19.71	16.67	18.39	17.64	28.23	23.88	26.35	25.27	23.97	20.27	22.37	21.45
95% CI	8.90–43.67	6.91–40.22	7.84–43.14	7.80–39.88	12.83–62.14	9.96–57.27	11.31–61.41	11.25–56.76	13.40–42.87	10.14–40.56	11.60–43.16	11.67–39.43
Genotype												
p value	4.11×10^{-25}	1.16×10^{-11}	1.91×10^{-17}	2.31×10^{-19}	4.80×10^{-32}	2.12×10^{-14}	6.50×10^{-21}	3.42×10^{-24}	3.78×10^{-43}	1.04×10^{-16}	2.74×10^{-25}	3.57×10^{-31}
rs3825942												
Allele												
p value	1.77×10^{-8}	2.10×10^{-33}	2.10×10^{-33}	2.10×10^{-33}	2.42×10^{-22}	6.48×10^{-8}	4.72×10^{-9}	1.23×10^{-16}	3.98×10^{-16}	8.68×10^{-10}	1.47×10^{-11}	1.84×10^{-14}
OR	28.21	29.25	29.93	29.64	39.78	41.25	42.21	41.8	34	35.25	36.07	35.72
95% CI	3.86–205.97	3.79–225.66	3.96–226.36	4.01–219.07	5.46–289–78	5.36–317.49	5.59–318.47	5.67–308.20	8.23–140.45	7.91–157.16	8.31–156.49	8.50–150.11
Genotype												
p value	2.10×10^{-33}	2.10×10^{-33}	2.10×10^{-33}	2.10×10^{-33}	3.53×10^{-11}	7.19×10^{-8}	2.88×10^{-9}	1.11×10^{-10}	6.37×10^{-16}	1.20×10^{-9}	1.00×10^{-11}	1.17×10^{-14}
rs2165241												
Allele												
p value	4.79×10^{-5}	0.3051	1.03×10^{-33}	1.20×10^{-3}	7.87×10^{-7}	0.1022	4.87×10^{-6}	8.28×10^{-5}	1.08×10^{-9}	0.0729	1.52×10^{-7}	3.02×10^{-6}
OR	16.59	4.56	18.87	12.39	23.4	6.43	26.61	17.47	20	5.49	22.74	14.93
95% CI	2.25–122.20	0.47–44.63	2.45–145.49	1.63–94.08	3.18–171.92	0.66–62.81	3.46–204.70	2.31–132.36	4.78–83.62	0.90–33.46	5.11–101.30	3.42–65.14
Genotype												
p value	1.95×10^{-4}	0.3	1.59×10^{-4}	2.82×10^{-3}	5.14×10^{-6}	0.1004	7.16×10^{-6}	1.23×10^{-4}	1.07×10^{-8}	0.0715	2.79×10^{-7}	4.71×10^{-6}

OR, odds ratio; CI, confidence interval; NA, not applicable; EX, exfoliation syndrome; EG, exfoliation glaucoma; PG, primary open-angle glaucoma aged 70 years or older; NG, normal tension glaucoma aged 70 years or older; and CT, cataract aged 70 years or older. p values were obtained by Fisher's exact probability test.

Table 4. Odds ratios and p values for three SNPs in comparison between EX and EG.

	EX versus EG		
	rs1048661	rs3825942	rs2165241
	Allele		
p value	0.5829	1	1
OR	1.43	1.41	1.41
95% CI	0.49–4.20	0.09–22.78	0.09–22.78
	Genotype		
p value	0.2005	1	1

No significant association was found between EX and EG. OR, odds ratio; CI, confidence interval; EX, exfoliation syndrome; and EG, exfoliation glaucoma. p values were obtained by Fisher's exact probability test.

associations with EX, EG, and EX+EG when they were compared to the CT group (Table 2 and Table 3). Significant associations with EX, EG, and EX+EG were detected in comparisons with the primary glaucoma groups (PG, NG, and PG+NG) for the alleles, T of rs1048661, G of rs3825942, and C of rs2165241, as well as the genotypes, TT of rs1048661, GG of rs3825942, and CC of rs2165241, with the exception of the comparisons between EX, EG, or EX+EG and the PG groups for allelic (OR=4.56–6.43 and p=0.3051–0.0729) and genotypic (p=0.3000–0.0715) frequencies in rs2165241 (Table 3).

None of the three SNPs showed a significant difference between the EX and EG groups in these allelic or genotypic frequencies (Table 4). In addition to this, none of the three SNPs showed significant differences in their allelic or genotypic frequencies in comparisons between the primary glaucoma (PG, NG, or PG+NG) and CT groups or between the PG and NG groups (data not shown), excepted for the allele C of rs2165241 (p=0.0233) and the genotype CC of rs2165241 (p=0.0294) in comparison between the PG and NG groups.

The two SNPs, rs1048661 and rs3825942, were in linkage disequilibrium ($D'=1$). In our study populations, only three of the four possible haplotypes in rs1048661/rs3825942 were detected (Table 5 and Table 6). In the comparisons between cases (EX, EG, or EX+EG) and controls (CT+PG+NG), the T and G were significantly associated with EX, EG, and EX+EG, and the G and A had a significant protective effect (Table 5 and Table 6).

DISCUSSION

Based on this study among 393 elderly Japanese patients with exfoliation syndrome, exfoliation glaucoma, primary open-angle glaucoma, and cataract, we confirmed the findings of Thorleifsson and colleagues [2] that three SNPs within *LOXL1* are strongly associated with exfoliation syndrome and glaucoma. In addition to the original study in Icelandic and Swedish populations, allele G of rs3825942 has been consistently suggested as a risk–associated allele of exfoliation syndrome/glaucoma in five studies from the United States [3-7], two studies from Europe [8,9], one study from India [10], one study from Australia [11], and six studies including this one from Japan [12-16]. In this study, we found that allele T of rs1048661 is associated with exfoliation syndrome/glaucoma, which is consistent with other studies in the Japanese population [12-16], while allele G is reported to be risk-associated in studies from other countries [2-4,6-11]. We also found that allele C of rs2165241 is associated with exfoliation syndrome/glaucoma, which is consistent with one study from Japan [13], while allele T is risk-associated in other populations [2,4,5,7,8]. Allelic frequencies of the three SNPs reported in previous studies and the current study are summarized in Figure 1. These results suggest a possibility that the missense changes in *LOXL1* are not actually causative but mark a haplotype that carries variants that may indeed be causative.

This study revealed extremely high ORs and significant p values for the three SNPs in the comparisons between the exfoliation syndrome/glaucoma groups and cataract groups. Only patients aged 70 years or older were recruited as control subjects in this study, enabling us to reduce the chance of misclassifying latent or preclinical exfoliation syndromes into the control group. Accordingly, the inclusion criteria of the control group might contribute to the extremely significant association of the *LOXL1* SNPs with exfoliation syndrome in this study. In contrast, the significance of the association of the SNPs with exfoliation syndrome/glaucoma was relatively low when the PG group was used as a control comparison compared to when the NG or CT groups were used as control comparisons. Although the level of significance was relatively low, we found differences in frequencies between PG and NG for allele C of rs2165241 (p=0.0233) and the genotype CC of rs2165241 (p=0.0294). These may suggest the possible inclusion of late onset exfoliation syndrome in the PG group. Previously, a lack of association between *LOXL1* polymorphisms and primary open-angle glaucoma or primary angle-closure glaucoma were reported in Caucasian, African American, Ghanaian, and Indian populations [17,18]. We did

Table 5. Counts and frequencies of haplotype rs1048661/rs3825942 in cases (EX, EG, and EX+EG) and controls (CT, NG, PG+NG, CT+NG, and CT+PG+NG)

Cases	EX		EG		EX+EG	
	Count	Frequency	Count	Frequency	Count	Frequency
TG	111	0.941	159	0.958	270	0.951
GG	6	0.051	6	0.036	12	0.042
GA	1	0.008	1	0.006	2	0.007

Controls	CT		NG		PG+NG		CT+NG		CT+PG+NG	
	Count	Frequency	Count	Frequency	Count	Frequency	Count	Frequency	Count	Frequency
TG	140	0.443	50	0.463	89	0.473	190	0.448	229	0.454
GG	115	0.364	36	0.333	61	0.324	151	0.356	176	0.349
GA	61	0.193	22	0.204	38	0.202	83	0.196	99	0.196

EX, exfoliation syndrome; EG, exfoliation glaucoma; PG, primary open angle glaucoma aged 70 years or older; NG, normal tension glaucoma aged 70 years or older; and CT, cataract aged 70 years or older.

Table 6. P values for haplotype rs1048661/rs3825942 in comparisons between cases (EX, EG, and EX+EG) and controls (CT, NG, PG+NG, CT+NG, and CT+PG+NG).

EX					
	Versus CT	**Versus NG**	**Versus PG+NG**	**Versus CT+NG**	**Versus CT+PG+NG**
TG	9.56×10^{-21}	NA	9.56×10^{-21}	6.19×10^{-17}	1.27×10^{-21}
GG	9.69×10^{-11}	NA	9.69×10^{-11}	1.76×10^{-10}	1.43×10^{-10}
GA	1.01×10^{-6}	NA	1.01×10^{-6}	5.63×10^{-7}	5.63×10^{-7}
EG					
	Versus CT	**Versus NG**	**Versus PG+NG**	**Versus CT+NG**	**Versus CT+PG+NG**
TG	1.83×10^{-28}	4.95×10^{-21}	3.06×10^{-23}	9.77×10^{-30}	4.40×10^{-20}
GG	3.12×10^{-15}	2.51×10^{-11}	4.82×10^{-12}	2.60×10^{-15}	3.70×10^{-15}
GA	5.63×10^{-9}	8.10×10^{-9}	4.08×10^{-9}	3.02×10^{-9}	2.36×10^{-9}
EX+EG					
	Versus CT	**Versus NG**	**Versus PG+NG**	**Versus CT+NG**	**Versus CT+PG+NG**
TG	1.23×10^{-40}	7.84×10^{-29}	1.24×10^{-32}	5.89×10^{-43}	8.27×10^{-44}
GG	5.93×10^{-22}	4.01×10^{-15}	1.03×10^{-16}	2.39×10^{-22}	2.83×10^{-22}
GA	1.17×10^{-13}	3.98×10^{-13}	9.33×10^{-14}	3.68×10^{-14}	2.25×10^{-14}

Significant association was found between cases and controls. EX, exfoliation syndrome; EG, exfoliation glaucoma; PG, primary open angle glaucoma aged 70 years or older; NG, normal tension glaucoma aged 70 years or older; and CT, cataract aged 70 years or older. NA, not available due to a small number of samples.

not find any significant associations between any of the three SNPs with primary open-angle glaucoma, reconfirming the previous observations in our Japanese population. Most recently, one study from Japan reported a lack of association between *LOXL1* polymorphisms and primary open-angle glaucoma in two of three SNPs (e.g., rs1048661 and rs3825942) [16]. In addition to this, the current study suggests a lack of association between rs2165241 and primary open-angle glaucoma as well as a lack of association between any of the three SNPs with normal tension glaucoma.

In this study, none of the SNPs exhibited significant differences in their allelic and genotypic frequencies between exfoliation syndrome and glaucoma, suggesting that these SNPs are associated with exfoliation glaucoma through their association with exfoliation syndrome as reported previously [2]. Accordingly, our data suggest that additional genetic or environmental risk factors are associated with the development of exfoliation glaucoma among exfoliation syndrome patients. Further study is required to clarify these risks.

In summary, we have demonstrated significant associations of *LOXL1* variants with Japanese patients who have exfoliation syndrome/glaucoma. Compared to other populations, the risk alleles in rs1048661 and rs2165241 are unique in this population. The *LOXL1* association of exfoliation glaucoma is through the association of exfoliation syndrome. *LOXL1* lacked any association with primary open-angle glaucoma or normal tension glaucoma in this population. Additional genetic or environmental risk factors

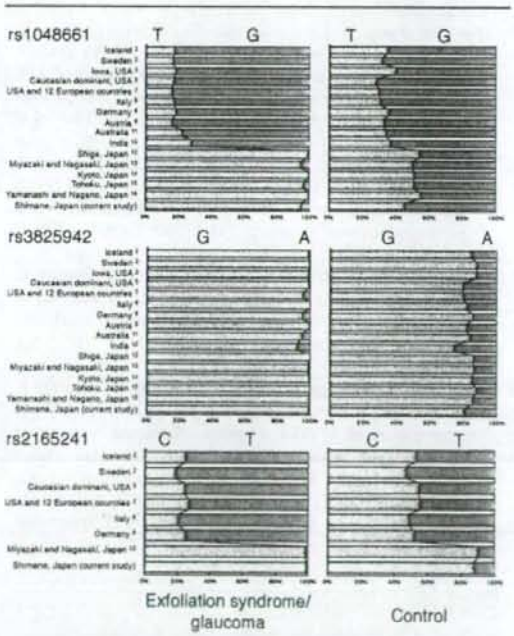

Figure 1. Allelic frequencies of the three SNPs reported in previous studies and the current study. Allelic frequencies of three SNPs in cases (exfoliation syndrome and exfoliation glaucoma) and controls (cataract or normal population) that have been reported in literatures are summarized.

other than *LOXL1* are likely to be associated with an increase in exfoliation glaucoma among exfoliation syndrome patients.

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Comparative Proteomic Analyses of Macular versus Peripheral Retina in Cynomolgus Monkeys (*Macaca fascicularis*)

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Keywords

Macula, Retina, Age-related macular degeneration, Tropomyosin, Synuclein,

Ribonucleoprotein, Mn-superoxide dismutase, Photoreceptor

Abstract

Macula is the specialized region of the retina responsible for high resolution visual acuity in primates. To investigate regional differences between macula and peripheral retina, proteomic analyses of tissues from cynomolgus monkeys were compared. By two dimensional gel electrophoresis and mass spectrometry, twenty six proteins were identified from spots detected only in the macular tissues. These proteins may play a role in the pathogenesis and progression of macular disease.

Introduction

The central region of the retina is called the macula and is approximately 1.5 to 2.0 mm in diameter in humans (1) and 0.6 to 0.9 mm in cynomolgus monkeys (2, 3). The fovea is located at the center of the macula approximately 0.35 mm in diameter where no blood vessels are formed. The density of cone photoreceptors in retina is highest in the fovea (4), and they are connected with large number of retinal ganglion cells (RGCs) in this small region (1). Any damage to the macula can have devastating effect on the central visual acuity e.g., in patients with macular dystrophies and age-related macular degeneration (AMD).

AMD is a multifactorial disease in which multiple genes and environmental factors are involved to progress mainly to two types of diseases (5). The wet-form of AMD is characterized by the loss of central vision caused by choroidal neovascularization (CNV) in subretinal space, while the dry-form of AMD is characterized as geographic atrophy of the retinal pigment epithelium (RPE) cells (1). The deposit called the "drusen" accumulates between the RPE and Bruch's membrane and is considered as hallmark of dry-form of AMD. Although previous reports have shown drusen as risk factor for AMD, explanation of its preferential accumulation in the macula still remains undetermined.

The uniqueness of the macular area is also demonstrated by the preferential development of CNV in the wet-form of AMD. These findings indicate that the macular region of the retina is different from the peripheral retina not only by morphology but also by protein distribution. It can be interpreted as difference of macula versus peripheral retina. Based on this hypothesis, comprehensive gene expression studies of the macula in comparison with peripheral retina using DNA microarray or serial analysis of gene expression (SAGE) have been performed (6-10). Sharon et al. have reported several genes preferentially expressed in the human macula and RPE by SAGE. Most of these genes were associated with the function of the RGC, and presumably detected because of the high density of RGC in the macular area (6). Rickman et al. also performed SAGE

on human retinas and isolated RPE cells and identified genes that were abundantly expressed in cones, RGC, and RPE cells (7). Ishibashi et al. performed 4 K DNA microarray analysis on RPE cells in the macula reporting five differentially expressed genes which was confirmed by real time PCR (8). Recently, Radeke et al. (9) and van Soest et al. (10) used 22 K DNA microarray and identified number of genes that were differentially expressed in the macula and peripheral retina. Few of these genes were highly expressed in the RPE cells in the macula. van Soest et al. showed that WAP four-disulfide core domain 1 was one of the highly expressed proteins in the RPE cells in the macular area by immunohistochemistry (10). However in many cases, the expression level of mRNA did not correlate with the expression level of the protein demonstrating the limitation of mRNA analyses for translational and posttranslational evaluations.

Recent technical advances in proteomics have allowed direct determination of the protein profile of body fluids and tissue homogenates. Proteomic analyses of the retina were first performed by Nishizawa et al. (11), and soon several groups catalogued the retinal proteins using single or two-dimensional (2D) gel electrophoresis followed by mass spectrometry analysis (12-14). Ethen et al. examined cadaver eyes with AMD by proteomic analyses and reported that the expression of proteins change with the progression of AMD, and the changes in the macula was different from that in the

peripheral retina (15).

Proteomic studies of the macula are difficult because of the lack of fresh human eyes, and the small area of the macula. To overcome these problems, we selected non-human primate eyes of the Cynomolgus monkey (*Macaca fascicularis*), which have a well defined macula. Monkeys with characteristics of AMD have been reported by many investigators (16-21) including our laboratory on early-onset and late-onset Cynomolgus monkey AMD models (22-26). Our proteomic study has shown that the protein profile of monkey eyes with drusen were similar to that in human drusen for many of the key molecules including complement components C5 and C9. In addition, the terminal C5b-9 complement complex was confirmed to present in drusen by immunostaining (27).

To identify the proteins present at high levels in the macular area and to understand the biology of the macula, we performed proteomic analyses to determine the protein profile of the macula and peripheral retina and posttranslational modification specific to the macular area.

Experimental section

Preparation of Cynomolgus monkey eyes

All primate experiments were approved by the Animal Ethics Committee of the Tsukuba Primate Research Center and were conducted in accordance with the The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Eight eyes from eight normal female Cynomolgus monkeys (*Macaca fascicularis*) whose ages ranged between 13- to 19 years were studied. Eyes were removed approximately one hour after death and treated with RNAlater (Applied Biosystems, Tokyo, Japan). Then a 3 mm diameter macular and peripheral retina containing the RPE and choroidal layer were punched out and frozen until use. The proteins extracted from the tissues of eight eyes were pooled for analyses.

Protein extraction and 2D-gel electrophoresis

The proteins from the macula and peripheral retina were extracted after homogenization and sonication in sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 40 mM Tris, 0.2% Bio-Lyte 3/10 (Bio-Rad Hercules, CA)). After centrifugation for 15 min x 3 at 14,000 rpm (20,800 x g), the lysate was precipitated using Ready Prep 2D cleanup kit (Bio-Rad) and redissolved in sample buffer. The supernatant was collected. The protein concentrations were determined with the RC-DC protein assay kit (Bio-Rad) according to the manufacturer's instruction. Protein samples (300 μg) were separated by IEF using 17

cm IPG strips. After 12 to 16 hours of rehydration at 20° C, IEF was used for the first dimension with an initial voltage of 250 V for 15 min and then increased to 10,000 V for 3 hours and held until 60,000 volt-hours was reached. Immediately after the IEF, the IPG strips were stored at -20º C until the equilibration step was carried out. The IPG strips were equilibrated for 20 min in buffer containing 6 M urea, 2% SDS, 0.375 M Tris (pH 8.8), and 20% glycerol under reduced conditions with 2% DTT, followed by another incubation for 10 min in the same buffer under alkylating conditions with 2.5% iodoacetamide. Equilibrated IPG strips were electrophoresed for the second-dimension using 12% acrylamide gel. 2D-gel electrophoresis was performed at four different pH ranges, viz., pH 3-10, pH 4-7, pH 5-8 and pH7-10. After 2D-gel electrophoresis, the proteins were stained with SYPRO Ruby (Bio-Rad). The images of the macular and peripheral retina were compared with ImageMaster 2D Platinum ver.5.0 (GE Healthcare Bio-Sciences, Piscataway NJ) followed by a visual inspection. Then, the gel spots, as shown in Figure 1-4 (arrows) were excised. Butt et al. have described the difficulties of IEF separation by RNAlater because of contaminations (28). Our samples were desalted prior to the IEF separation by Ready Prep 2D cleanup kit (Bio-Rad) using TCA acetone precipitation which resulted in successful separations.

In gel digestion and LC-MS/MS analyses

Each gel piece was cut to approximately one cubic millimeter and washed twice with 50 mM ammonium bicarbonate/50% acetonitrile. After destaining, the gel pieces were rinsed with distilled water, and incubated with acetonitrile for 20 min. The supernatant was discarded and the gel pieces were completely dried before incubation with 10 mM DTT in 100 mM ammonium bicarbonate for 45 min at 56° C. The supernatant was discarded and the pieces were incubated in the dark with 55 mM iodoacetamide in 100 mM ammonium bicarbonate (30 min, at room temperature). The supernatant was discarded, and the gels were washed three times. Finally, the gel pieces were completely dried before tryptic digestion in a solution of sequencing grade modified trypsin (12.5 ng/ul; Promega, Madison, WI) in 50 mM ammonium bicarbonate. The digestion was performed at 37° C overnight, and the extraction step was performed once with 25 mM ammonium bicarbonate, twice with 5% formic acid, and finally with distilled water. The extracted peptides were pooled and dried. After re-suspending in 40 μl of aqueous 0.01% trifluoroacetic acid/2% acetonitrile, the samples were analyzed by LC-MS/MS. LC-MS/MS was performed with a combined system of Paradigm system (Michrom BioResources, Auburn, CA) and an ESI mass spectrometer (LCQ Deca XP plus or Finnigan LTQ; Thermo Fisher Scientific, Yokohama, Japan; assembled by AMR Inc.,

Tokyo, Japan). For the LCQ analysis, sample peptides were separated on nano column (AMR Inc.) with solvent A (2% acetonitrile/0.1% formic acid) and B (90% acetonitrile/0.1 % formic acid) at a flow rate of 0.6 μl/min, gradient of 5 to 95% solvent B over 40 min. For the LTQ analysis, peptides were separated on Magic C18 (Michrom BioResources) with solvent A and B, a flow rate 1.5 μl/min, gradient of 5 to 95% solvent B over 30 min. The identification of the proteins from the MS/MS spectra was performed using protein identification software (Bioworks ver.3.1, Thermo Fisher Scientific) and UniProtKB/Swiss-Prot database (Release 48.8) which was preliminarily extracted by the species "human" (13361 entries). Peak list generation and database search were performed with the following parameters; mass tolerance for precursor ions, 2.5 amu; mass tolerance for fragment ions, 0.00 amu;, enzymatic cleavage position before lysine or arginine; number of missed cleavage sites permitted 2; fixed modification, carbamide-methylation (+57.02Da) for cysteine; variable modification, oxidation (+16Da) for methionine. The peptide sequences were filtered by peptide Cross Correlation (XC) score and delta Cn score. The threshold level of delta Cn score were >0.1 for peptide sequences from both measurement devices. The threshold level of XC score for each charge (+1/+2/+3) were >1.5/2.0/3.7 for LCQ and >1.9/2.2/3.7 for LTQ. Then correlation between observed in gel images and theoretical molecular weight and pI were

considered. When peptides matched multiple members of the protein family, the protein which had the most number of peptides matched to the amino acid sequence was selected. In case of equal numbers, both proteins were listed.

Western blotting and immunohistochemical analysis of macula

Five to fifteen micrograms of macula or peripheral retina homogenates were diluted in double volume of SDS buffer and separated by either 1D or 2D-PAGE followed by PVDF membrane transfer. Membranes were blocked with a Blocking Solution Concentrate (KPL, Gaithersburg, MD) and probed with one of the following primary antibodies (Abs): chicken Ab to human arrestin-C (GenWay Biotech, San Diego, CA), rabbit Ab to human Mn- superoxide dismutase (Mn-SOD) (upstate, Temecula, CA), rabbit Ab to human synuclein gamma (Novus Biologicals, Littleton, CO), rat Ab to human epidermal fatty acid binding protein (E-FABP) (R&D Systems, Inc. Minneapolis, MN), rabbit Ab to tropomyosin Br-1, Br-3 (CHEMICON International, Temecula, CA), mouse Ab to chicken tropomyosin TM311 (Abcam, Cambridge, UK), goat Ab to human heterogeneous nuclear ribonucleoproteins (hnRNPs) A2/B1 (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse Ab to human hnRNPs C1/C2 (abcam). The specific signals were detected with one of the following secondary antibodies: goat Ab to mouse IgG horseradish peroxidase

(HRP) conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA), goat Ab to rabbit IgG HRP conjugated (Pierce, Rockford, IL), rabbit Ab to chicken/turkey IgG HRP conjugated (Zymed Laboratories, South San Francisco, CA), donkey Ab to goat IgG HRP conjugated (Jackson ImmunoResearch Laboratories), goat Ab to rat IgG HRP conjugated (Zymed Laboratories). The signals were made visible by chemiluminescence reactions and examined with a chemiluminescence imager (Lumi-Imager F1; Roche Diagnostics, Tokyo, Japan). The detection of Mn-SOD by 2D western blotting of macular or peripheral retina samples was made by 2D gel electrophoresis. Thirty micrograms of protein from each sample were separated using 7cm IPG strip at pH 3 to 10. The second dimensional separation was made on 12.5% polyacrylamide gel and then transferred to PVDF membranes. For quantitative comparisons of the band intensities of the western blots, the blots were scanned and measured by Lumi Analyst ver.3.1 (Roche Diagnostics).

Protein detection followed 1D western blotting

Enucleated eye from normal female cynomolgus monkey (age 13 yrs) were fixed in 10% neutralized and buffered formaldehyde solution at 4° C overnight and then dehydrated. The specimens were embedded in paraffin and serial sectioned at 4 μm thickness. The

specimens were treated for antigen retrieval by autoclaving in Target Retrieval Solution (Dako, Carpinteria, CA) for 20 min at 121° C. The sections were then blocked with Dako protein block (Dako) or skim milk or BSA in PBS. The primary antibodies were the same as used for the western blotting. For signal detection after rinsing off the primary antibodies, the sections were incubated with one of following secondary antibodies: Alexa 488 conjugated goat Ab mouse IgG (invitrogen, Tokyo, Japan), Alexa 568 conjugated goat anti-rabbit IgG (invitrogen), Alexa 488 conjugated donkey anti-goat IgG (invitrogen), Alexa 568 conjugated goat anti-chicken IgG (invitrogen), Alexa 488 conjugated goat anti-rat IgG (invitrogen). After rinsing off the antibodies, the sections were examined by confocal laser scanning microscope (Radiance 2100, Bio-Rad). The cell nuclei were stained with DAPI (4',6-diamino-2-phenylindole). To determine the location of the signals, one of sections was stained with hematoxylin and eosin.

Results

Identification of macula enriched proteins

Approximately 700 spots were detected in the macular and peripheral retinal tissues by 2D-gel (pH range 3-10; Fig. 1). Sixty percent of these spots were found in both

samples. The gel spots indicating high expression of proteins are listed in Table 1. Forty eight known proteins from earlier proteomics studies of the retina were identified (12-14, 29, 30). Fifteen spots were specific to the macula. The neurofilament triplet L protein identified in this study has not been previously reported by proteomic approach but by previous SAGE analysis (6). To obtain a further separation, the pH range of the 2D-gel electrophoresis was narrowed to three ranges; 4-7, 5-8, and 7-10 (Fig. 2-4). Under these conditions, 40 spots specific to macula were further detected and 26 proteins were identified (Table 2). To obtain the relative expression levels of the identified proteins, western blotting analysis was performed.

Quantification of macula enriched proteins

Western blotting was performed on the following seven proteins identified by mass spectrometry; arrestin-C, Mn-SOD, γ-synuclein, E-FABP, tropomyosin1a chain, nhnRNPs A2/B1, and hnRNPs C1/C. Arrestin-C showed a 1.5-fold higher expression in the macula than the peripheral retina (Fig. 5-1). Arrestin-C has been previously identified as a cone photoreceptor specific protein (31), and thus served as a positive control for this study. Mn-SOD, an anti oxidative enzyme located in mitochondria (32), showed equal expression in the macular and peripheral retina (Fig. 5-2). For Mn-SOD, 2D-gel western