平成　　年　　月　　日

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氏　　名：＿＿＿＿＿＿＿＿＿＿

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職　　名：＿＿＿＿＿＿＿＿＿＿

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2．看護師	9．臨床工学技士
3．薬剤師	10．管理栄養士
4．臨床検査技師	11．医療ソーシャルワーカー
5．診療放射線技師	12．診療情報管理士
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LOXL1 variants in elderly Japanese patients with exfoliation syndrome/glaucoma, primary open-angle glaucoma, normal tension glaucoma, and cataract

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Purpose: To evaluate the association of lysyl oxidase like 1 (*LOXL1*) gene variants in Japanese patients with open-angle glaucoma.

Methods: We evaluated the association of three *LOXL1* variants (rs1048661, rs3825942, and rs2165241) in 142 Japanese patients with exfoliation syndrome (EX; n=59) and exfoliation glaucoma (EG; n=83) as well as in 251 control patients aged 70 years or older with primary open-angle glaucoma (PG; n=40), normal tension glaucoma (NG; n=54), and cataract (CT; n=157).

Results: In comparison with the CT group, the single nucleotide polymorphisms (SNPs) showed significant association with EX, EG, and EX+EG. The odds ratio (OR)=19.71–28.23 and $p=1.69\times10^{-23}$-3.00×10^{-45} for allele T of rs1048661; OR=28.21–39.78 and $p=1.77\times10^{-8}$-2.42×10^{-22} for allele G of rs3825942; and OR=16.59–23.40 and $p=4.79\times10^{-5}$-1.08×10^{-9} for allele C of rs2165241. In comparison with the controls (CT+PG+NG), the haplotype rs1048661/rs3825942 (T/G) was significantly associated with EX+EG ($p=8.27\times10^{-44}$), and haplotype G/A had a significant protective effect ($p=2.25\times10^{-14}$). None of the three SNPs showed significant differences between the EX and EG groups or between the PG and NG groups.

Conclusions: These SNPs are associated with exfoliation syndrome/glaucoma in the Japanese population. The risk alleles in rs1048661 and rs2165241 are different from other populations. Additional genetic or environmental risk factors other than these *LOXL1* SNPs could be associated with the development of exfoliation syndrome as well as exfoliation glaucoma among exfoliation syndrome patients.

Exfoliation syndrome is the most common identifiable cause of open-angle glaucoma worldwide. It is an age-related, generalized disorder of the extracellular matrix that is characterized by the production and progressive accumulation of a fibrillar extracellular material in many ocular tissues [1]. A recent genome-wide association study demonstrated that one intronic single nucleotide polymorphism (SNP, rs2165241) and two exonic SNPs (rs1048661 [R141L] and rs3825942 [G153D]), which are located in the first exon of the lysyl oxidase like1 (*LOXL1*) gene on chromosome 15q24.1, are highly associated with exfoliation syndrome in Icelandic and Swedish populations while none of these SNPs were associated with primary open-angle glaucoma in either of the two populations [2].

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Until now, several lines of studies have reported significant associations of these *LOXL1* SNPs with exfoliation syndrome [3-16] or the lack of associations of these SNPs with primary open-angle glaucoma [16-18] in various populations. In these studies, subjects in their 40s and 50s are sometimes recruited as controls. The prevalence of clinical exfoliation syndrome increases with age, particularly after the age of 60 years [1]. However, preclinical exfoliation syndromes in younger generations are able to distinguish from normal subjects until patients become older. Accordingly, the level of statistical significance between exfoliation syndrome and controls could be underestimated in studies that include younger patients in the control group. In this study, we evaluated the association of three *LOXL1* SNPs in Japanese patients with exfoliation syndrome/glaucoma, primary open angle glaucoma, normal tension glaucoma, and cataract. To reduce the chance of misclassifying latent syndromes within the control group, only patients aged 70 years or older were recruited as primary open-angle glaucoma, normal tension glaucoma, or cataract subjects in this study. To the best of our knowledge, this is the first study reporting an association of

Table 1. Summary of study populations.

	Exfoliation syndrome or glaucoma			Primary glaucoma				
Parameter	EX	EG	EX+EG	PG	NG	PG+NG	CT	p value
No. of subjects	59	83	142	40	54	94	157	
Male:Female								
N	12:47	42:41	54:88	14:26	18:36	32:62	45:112	0.2264*
%	20.3:79.7	50.6:49.4	50.6:49.4	35.0:65.0	33.3:66.7	34.0:66.0	28.7:71.3	
Age (years)								
mean±SD	78.2±8.0	78.8±8.5	78.5±8.2	75.6±5.3	78.3±4.8	77.2±5.1	77.2±5.0	0.1271**
Range	55–95	57–95	55–95	70–87	70–91	70–91	70–90	

The asterisk and double asterisk indicate that the p values were obtained by Pearson's χ^2 test and one-way ANOVA, respectively, among three (EX+EG, PG+NG, and CT) groups. 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; CT, cataract aged 70 years or older.

LOXL1 SNPs with exfoliation syndrome/glaucoma using age-controlled subjects. This is also the first study reporting the association of *LOXL1* SNPs with normal tension glaucoma.

METHODS

Subjects: Three hundred and ninety-three unrelated Japanese subjects presenting exfoliation syndrome without glaucoma (EX), exfoliation syndrome with glaucoma (EG), primary open-angle glaucoma (PG), normal tension glaucoma (NG), and cataract (CT) were recruited at the Shimane University Hospital and Iinan Hospital in Shimane, Japan. This study adhered to the tenets of the Declaration of Helsinki. The research was reviewed and approved by the Institutional Review Boards of both hospitals. Written informed consent was obtained from all of the subjects. All of the subjects underwent a dilated pupil examination of the anterior segments, ocular media, and fundus using a slit-lamp and a funduscope. The subjects with EX and EG exhibited the typical pattern of exfoliation material on the anterior lens surface and/or pupillary margin during slit-lamp examination. The subjects with EG and PG had a history of intraocular pressure (IOP) greater than or equal to 21 mmHg, and the subjects with EX, NG, and CT had a history of IOP never exceeding 20 mmHg. The subjects with PG, NG, and EG presented a typical glaucomatous optic disc cupping or rim thinning and visual field loss. The patients who had a history of IOP greater than or equal to 21 mmHg but no glaucomatous optic disc changes nor visual field loss were assigned as EG in this study. To avoid possible misclassification of latent exfoliation syndromes as PG, NG, or CT, patients younger than 70 years old were not recruited. The number of subjects, each gender, and the mean and range of ages in each group are summarized in Table 1.

DNA genotyping: Genomic DNA was extracted from the peripheral white blood cells of each subject. Polymerase chain reaction (PCR) was performed using primers designed to amplify the genomic region containing both rs1048661 and rs3825942 (forward primer: 5'-AGG TGT ACA GCT TGC TCA ACT C-3' and reverse primer: 5'-TAG TAC ACG AAA CCC TGG TCG T-3') or just rs2165241 (forward primer: 5'-AGA ATG CAA GAC CTC AGC ATG AG-3' and reverse primer: 5'-TAG TGG CCA GAG GTC TGC TAA G-3'). The sequence was determined based on the dideoxy terminator method using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. We used SeqScape Software version 2.5 (Applied Biosystems) to analyze the sequence alignment.

Statistical analysis: The deviation of the genotype distributions from the Hardy–Weinberg equilibrium was assessed in the case and control samples using HAPLOVIEW version 4.0 [19]. Statistical analysis was performed using R version 2.6.2. Fisher's exact test was used to compare the allele or genotype frequencies of each case group with the controls. The odds ratio (OR) and 95% confidence intervals (CIs) were calculated by the logistic regression method. Individual haplotypes and their estimated population frequencies were inferred using HAPLOVIEW version 4.0 [19] with all of the parameters set at the default values.

RESULTS

The allelic and genotypic counts and frequencies of SNPs rs1048661, rs3825942, and rs2165241 within *LOXL1* are shown in Table 2. The ORs and p values for the allelic and genotypic frequencies of the three SNPs in comparison between the cases (EX, EG, and EX+EG) and controls (CT, PG, NG, and PG+NG) are shown in Table 3. In comparison with the CT group, the SNPs showed a significant association with EX, EG, and EX+EG for the T allele of rs1048661 at OR=19.71–28.23 and $p=1.69\times10^{-23}$-3.00×10^{-45}, the G allele of rs3825942 at OR=28.21–39.78 and $p=1.77\times10^{-8}$-2.42×10^{-22}, and the C allele of rs2165241 at OR=16.59–23.40 and $p=4.79\times10^{-5}$-1.08×10^{-9} (Table 3). The genotypes, TT of rs1048661 ($p=4.11\times10^{-25}$-3.78×10^{-43}), GG of rs3825942 ($p=3.53\times10^{-11}$-2.10×10^{-33}), and CC of rs2165241 ($p=1.95\times10^{-4}$-1.07×10^{-8}), also showed significant

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 CT**	**Versus PG**	**Versus NG**	**Versus PG+NG**	**Versus CT**	**Versus PG**	**Versus NG**	**Versus PG+NG**	**Versus CT**	**Versus PG**	**Versus NG**	**Versus PG+NG**
rs1048661												
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^{-10}	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^{-20}	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

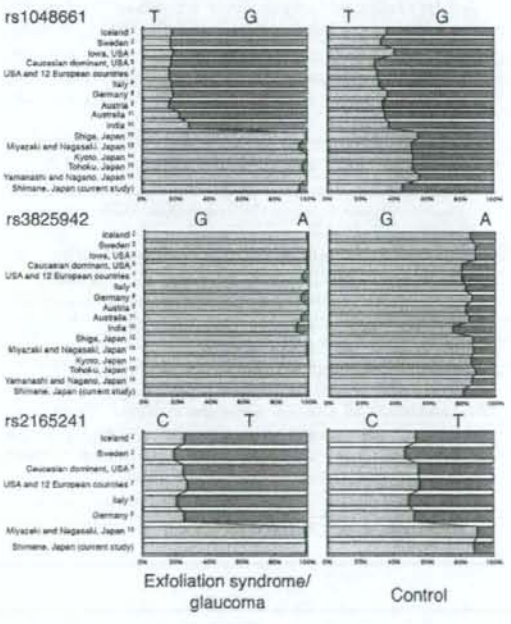

Figure 1. Allelic frequencies of the three SNPs reported in previous studies and the current study. Allelic frequencies of three SNPs in cases (exfoliation syndrome and exfoliation glaucoma) and controls (cataract or normal population) that have been reported in literatures are summarized.

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

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Letter to the Editor

Immunohistochemical analysis of aldehyde-modified proteins in drusen in cynomolgus monkeys (*Macaca fascicularis*)☆

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Abstract

Protein modifications resulting from reactive aldehydes are thought to be involved in the pathogenesis of various degenerative diseases. Aged cynomolgus monkey (*Macaca fascicularis*) spontaneously develop drusen in the macula, consistent with the phenotype observed in early-stage age-related macular degeneration (AMD), indicating that this animal is an optimum model for AMD. In retinal sections from three monkeys with macular degeneration, regardless of their size, drusen were consistently positive with immunohistochemical labeling against protein modifications by 4-hydroxynonenal and 4-hydroxyhexenal, end products of non-enzymatic oxidation of n-6 and n-3 polyunsaturated fatty acids, respectively. Positive labeling for both modifications was observed in the ganglion cell layer, the inner nuclear layer, the outer nuclear layer, and the retinal pigment epithelium. However, no consistent differences in location or intensity of the labeling were observed between monkeys with normal macula and macular degeneration. The results suggest a possible association between drusen formation and protein modifications by aldehydes in the pathogenesis of AMD.

Keywords: aldehydes; 4-hydroxynonenal; 4-hydroxyhexenal; cynomolgus monkey; drusen; age-related macular degeneration; protein modification

Age-related macular degeneration (AMD) is the leading cause of legal blindness in elderly individuals in industrialized countries (Fine et al., 2000). Accumulation of extracellular deposits between the retinal pigment epithelium (RPE) and Bruch's membrane, referred to as drusen, is regarded as a hallmark risk factor for development of AMD (de Jong, 2006). Various lipids, polysaccharides, and glycosaminoglycans have been identified as constituents of drusen (Hageman et al., 2001). Recent studies have revealed that drusen contains various protein molecules that are related to inflammation, immune responses, and oxidative stresses (Mullins et al., 2000; Crabb et al., 2002); yet the mechanism of formation is not fully understood.

Aged monkeys spontaneously develop macular degenerative changes such as pigment mottling, hyperpigmentation or hypopigmentation, and drusen in the macula, consistent with the phenotype observed in early-stage AMD (Stafford et al., 1984; Ishibashi et al., 1986). Previous proteomic analysis indicated that a number of protein components are common in drusen from monkeys and humans (Crabb et al., 2002; Umeda et al., 2005). Thus, these animals are thought to be an optimum animal model for AMD.

4-Hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE) are α,β-unsaturated aldehydes that are end products of non-enzymatic oxidation of n-6 and n-3 polyunsaturated fatty acids, respectively (Esterbauer, 1993). These highly reactive aldehydes can react readily with histidine, cysteine, or lysine

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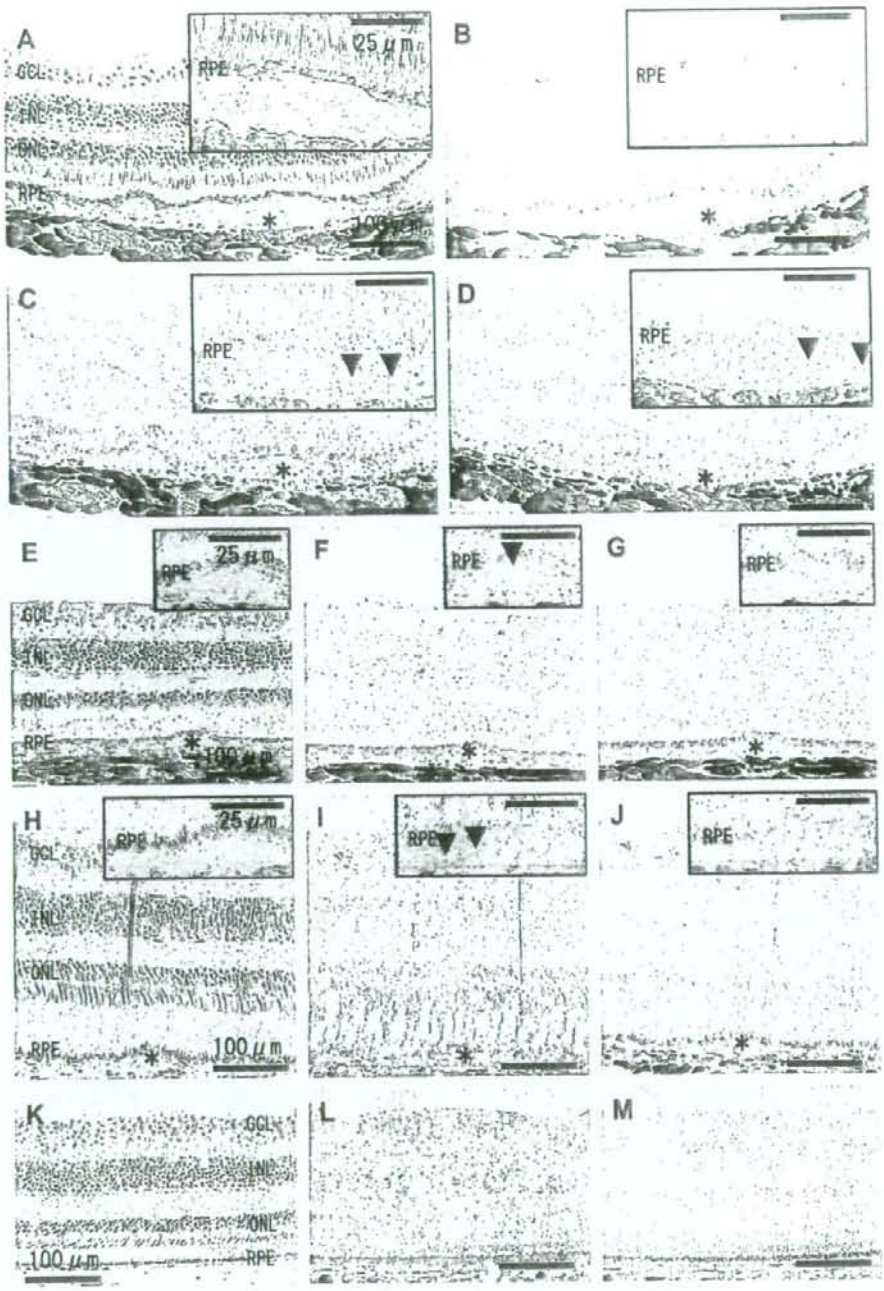

Fig. 1. Retinal sections from monkeys with macular degeneration and normal macula. Representative retinal sections from three monkeys with macular degeneration (panels A–D, E–G, and H–J, respectively) and those from a monkey with a normal macula (panels K–M) are shown. Representative sections stained with hematoxylin and eosin (panels A, E, H, and K) labeled with normal mouse IgG (panel B), 4-HNE-modified proteins (panels C, F, I, and L) and 4-HHE-modified proteins (panels D, G, J, and M). Asterisks and arrowheads indicate drusen and granular labeling, respectively. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; and RPE, retinal pigment epithelium.

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residues of proteins, leading to formation of stable Michael adducts with a hemiacetal structure (Uchida and Stadtman, 1992). Formation of these adducts leads to a variety of cytopathological effects such as inhibition of enzyme activity, inhibition of protein, RNA, and DNA synthesis, cell cycle arrest, and apoptosis (Awasthi et al., 2004). The use of specific antibodies to recognize the hemiacetal structure of Michael adducts enables their detection in tissues (Uchida et al., 1993). Previous studies have suggested that modifications by these aldehydes of proteins common to drusen components in humans are molecular events preceding light-induced retinal degeneration in rats (Tanito et al., 2005, 2006). Recently, proteins modified by 4-HNE were detected in the neural retina of patients with AMD (Ethen et al., 2007). We evaluated immunohistochemically the localization of proteins modified by these aldehydes on retinal sections from aged monkeys.

Cynomolgus monkeys (*Macaca fascicularis*) were reared in large-scale breeding facilities at the Simian Conservation Breeding and Research Center, Inc., Manila, the Philippines. Maintenance of monkeys and preparation of paraffin-embedded retinal sections have been described previously (Umeda et al., 2005). At the breeding facilities, 278 aged female monkeys (age range, 13–25 years; average age, 16.9 years) were funduscopically examined three times from 2001 to 2004. Of the 278 animals, 67.6% had a normal macula with no detectable pigmentary abnormalities, 10.8% were diagnosed with mild macular degeneration (<5 drusen), 11.2% with moderate macular degeneration (5–20 drusen), and 10.4% with severe macular degeneration (>20 drusen) (Umeda et al., 2005).

In the current study, retinal sections from three monkeys with normal macula and three monkeys with severe macular degeneration were examined. The immunohistochemistry methods were described previously (Tanito et al., 2005). Mouse anti-4-HNE- and anti-4-HHE-modified protein antibodies were purchased from NOF Corporation (Tokyo, Japan). Hematoxylin and eosin staining was performed to observe accumulation of drusen of various sizes between the RPE and choriocapillaries in the macular region in retinal sections from the three monkeys with severe disease (Fig. 1A, E, and H), whereas no drusen accumulated in any retinal sections from three monkeys with normal macula (Fig. 1K). By immunohistochemistry, regardless of size, drusen were consistently positive with diffuse labeling against both protein modifications by 4-HNE (Fig. 1C, F, and I) and 4-HHE (Fig. 1D, G, and J) in retinal sections from three monkeys with macular degeneration. Using high magnification, drusen frequently contained granular labeling against both protein modifications by 4-HNE (insets, Fig. 1C, F, and I) and 4-HHE (inset, Fig. 1D). Positive labeling for both antibodies was observed in the ganglion cell layer, the inner nuclear layer, the outer nuclear layer, and the RPE. However, no consistent differences in localization or intensity of labeling were observed between monkeys with normal macula and those with macular degeneration. Retinal sections incubated with normal mouse IgG as a negative control showed no positive staining over all retinal layers. The results clearly showed that the drusen contained proteins modified by the reactive aldehydes, 4-HNE and 4-HHE.

AMD progression can be slowed with antioxidant and zinc (The AREDS Research Group, 2001), thus oxidative stress has been suspected of contributing to the pathogenesis of AMD (Beatty et al., 2000). The relationship between abnormal protein oxidation/modification and macular degeneration has been reported, including detection of cross-linked species of tissue metalloproteinase inhibitor 3 and vitronectin, docosahexaenoic acid-derived carboxyethylpyrrole protein adducts in drusen from patients with AMD (Crabb et al., 2002), and protein modifications from oxidized carbohydrate such as carboxymethyl-lysine and pentosidine in ocular tissues from aged donors and those with AMD (Ishibashi et al., 1998; Handa et al., 1999). In addition to the previous evidence, we identified possible involvement of protein modifications by 4-HNE and 4-HHE in the formation of drusen in AMD. Protein modification by biologically active molecules including reactive aldehydes may be a critical process in drusen formation and the development of AMD.

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Mullins, R.F., Russell, S.R., Anderson, D.H., Hageman, G.S., 2000. Drusen associated with aging and age-related macular degeneration contain proteins

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