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Association of Keratin 8 Level in Aqueous Humor With Outcomes of Intravitreal Ranibizumab Treatment for Neovascular Age-Related Macular Degeneration

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Purpose: To investigate keratin 8 (KRT8) level in the aqueous humor (AH) of patients with neovascular age-related macular degeneration (nAMD) and elucidate its association with intravitreal ranibizumab (IVR) treatment outcomes.

Methods: This prospective study involved 58 eyes of treatment-naïve nAMD patients treated with three IVR doses monthly and whose AH samples were collected at baseline and two months after the initial treatment. KRT8 level was determined using the enzyme-linked immunosorbent assay and compared with that of the control group, which comprised patients who underwent cataract surgery during the same period. The nAMD-affected eyes were classified into responder (dry) and poor responder (persistent fluid) groups, according to optical coherence tomography (OCT) findings at month three. Additionally, associations between the KRT8 level and IVR treatment outcomes were analyzed.

Results: The baseline KRT8 level was significantly higher in the AMD group than in the control group. In the AMD group, responders demonstrated significant differences between the KRT8 level at the baseline and month two, whereas poor responders exhibited no significant change. Regression analysis revealed that a higher KRT8 level at month two was significantly associated with persistent fluid upon OCT at months three and six.

Conclusions: Monitoring aqueous KRT8 level may facilitate early determination of the therapeutic effects of IVR in nAMD patients and reflect the conditions of retinal pigment epithelium during the disease course.

Translational Relevance: Monitoring aqueous KRT8 may aid early determination of therapeutic effects of IVR in neovascular AMD patients and reflect the health conditions of retinal pigment epithelium.

Introduction

Neovascular age-related macular degeneration (nAMD) is the leading cause of blindness among elderly individuals in developed countries.[1,2] Although the use of anti-vascular endothelial growth factor (VEGF) agents has resulted in considerable improvements in nAMD treatment, the response to these drugs varies among individuals, and some patients still show a poor or no response to treatment.[3] The reasons for the poor response to anti-VEGF agents are complex and varied.[4] However, as retinal pigment epithelial (RPE) cells are strongly implicated in the pathogenesis of AMD,[5] the functional status of RPE in individual AMD patients may be one of the major contributors in AMD development and progression as well as to treatment outcomes. Various studies have attempted to correlate morphologic changes in the retina, RPE, and choroid determined by optical coherence tomography (OCT) with the clinical aspects and prognosis of AMD.[6,7] However, even with OCT, the morphological study of RPE is difficult, and molecular and proteomic studies of RPE in vivo have been limited.

Proteomic research involves the analyses of the nature of peptides or proteins in various biological samples of multifactorial diseases. It may help access the biology of cells and tissues involved in diseases and thus find new biomarkers and target-based therapies. Recent investigations have demonstrated specific proteomic signatures in nAMD patients. These studies collected and profiled the aqueous humor (AH) of nAMD patients and controls, identified several differentially expressed proteins in nAMD AH, and selected potential biomarker candidates besides VEGF.[8–12] Previously, we identified RPE-secreted proteins in the AH of nAMD patients and showed that, among them, the expression of epithelial marker protein keratin 8 (KRT8) increased by approximately twofold in nAMD patients compared with that in the control subjects, and it varied after anti-VEGF treatments.[8]

KRT8, which is predominantly expressed in epithelium, is known to support the mechanical integrity of cells, modulate response to stress stimuli, and contribute to the resistance of cells to apoptosis.[13–15] In the retina, KRT8 is a well-known epithelial marker of RPE[16,17]; it has been reported to be a major cytokeratin in RPE cells isolated from the human eyeball, and its expression increases in proliferating RPE cells with good maintenance of cuboidal morphology.[17] Additionally, we previously demonstrated increased KRT8 expression in oxidatively stressed RPE cells, along with autophagy, to protect RPE cells from cell death.[18] However, the clinical implications of upregulated KRT8 level in nAMD patients and their changes during treatments were not elucidated in the previous study.[8] Thus the aim of this prospective study was to investigate the associations of visual and anatomical treatment outcomes with changes in the KRT8 level in the AH of treatment-naïve nAMD patients treated with intravitreal ranibizumab (IVR).

Methods

The present prospective study was performed at Severance Hospital and Gangnam Severance Hospital of Yonsei University and Isan Paik Hospital of Inje University, between April 2016 and April 2018 (ClinicalTrial.gov trial number NCT02707575). This study was performed in accordance with the tenets of the Declaration of Helsinki and approved by the ethics committee of each institution (Severance Hospital: 2018-0061-002, Gangnam Severance Hospital: 2015-0611-015, and Ilsan Paik Hospital: 2016-04-010). All study participants provided written informed consent.

Study Participants

Each enrolled nAMD patient was required to be least 50 years of age with newly diagnosed (treatment-naïve) nAMD, with a recent onset of disease confirmed by history and clinical findings. Excluded eyes exhibited one of the following features: myopia with a refractive error of $> \pm 3.0$ diopters or evidence of pathologic myopia (preoperative refractive data were used to assess pseudophakic eyes); any history of vitrectomy, anti-VEGF therapy, laser treatment, or photodynamic therapy; a history of cataract surgery within three months before presentation; evidence of end-stage AMD such as subfoveal fibrosis or atrophy; eyes with large submacular hemorrhage (SMH) >1 disc diameter; evidence of other retinal diseases, including central serous chorioretinopathy, diabetic retinopathy, hypertensive retinopathy, and other neovascular maculopathies; glaucoma; poor imaging data caused by media opacity; or unstable fixation. Patients with uncontrolled systemic diseases, using immunosuppressive drugs, or with malignant tumors in any location, were also excluded. The control group comprised patients who underwent cataract surgery during the same period. Through preoperative evaluation, the eyes with ophthalmic diseases other than cataracts or those that met the exclusion criteria were excluded from the control group.

Baseline Evaluation, Treatment, and AH Sampling

At baseline, each patient in the nAMD group underwent a comprehensive ophthalmological examination for the assessment best-corrected visual acuity (BCVA) and intraocular pressure (IOP), autorefractometry/keratometry (ARK), slit lamp biomicroscopy, indirect ophthalmoscopy, color fundus photography (FP), fluorescein angiography (FA), indocyanine green angiography (ICGA) (Optos P200Tx; Optos PLC, Dunfermline, UK), and OCT (Swept Source OCT DRI OCT Triton; Topcon, Tokyo, Japan). After the baseline evaluation, three consecutive monthly injections of 0.5 mg IVR (Lucentis; Novartis, Basel, Switzerland) were administered to nAMD patients. At each visit for injection, and one month after the third injection (month three), ophthalmic examination, including BCVA, IOP, slit lamp biomicroscopy, FP, and OCT, were performed to monitor treatment outcome.

AH samples were collected at baseline and two months after the initial treatments (month two). Before surgery, each eye was anaesthetized topically with 0.5% proparacaine hydrochloride. Patients received standard disinfection with povidone-iodine scrub of the eyelids and surrounding skin and povidone-iodine eye drops to the conjunctival sac. After a sterile lid speculum was inserted, a 30-gauge needle was inserted bevel up through the peripheral cornea and 0.1 mL of AH was collected. Consecutively, 0.5 mg IVR was administered through the pars plana. Antibiotic eyedrops (0.5% moxifloxacin hydrochloride) were administered after surgery for three days.

The control subjects also underwent a comprehensive ophthalmological examination, including BCVA, IOP, ARK, slit lamp biomicroscopy, indirect ophthalmoscopy, FP, and OCT before surgery. AH samples of the control group were obtained immediately before cataract surgery.

Measurement of KRT8 Level in AH

Immediately after collection, AH samples were transferred into sterile plastic tubes (safe-lock microcentrifuge tubes, 1.5 mL) and immediately frozen and stored at −80°C until analysis.

The level of KRT8 in the AH was quantitatively assessed using a sandwich enzyme-linked immunosorbent assay kit (Cloud-Clone Corporation, Houston, TX, USA). The assays were performed according to the manufacturers' protocols. Samples were added into the wells of 96-well microplates, and the plates were then incubated for 2.5 hours at room temperature (RT), followed by gentle shaking for two hours at 37°C. Biotinylated antibodies were incubated for one hour at RT with gentle shaking at 37°C. Horseradish peroxidase-streptavidin solution was incubated for 45 minutes at RT, followed by gentle shaking for 30 minutes at 37°C. Tetramethylbenzidine dihydrochloride substrates were added to each well for 30 minutes in the dark. The enzyme-substrate reaction was terminated by adding sulphur acid solution, and the color change was measured at a wavelength of 450 nm. The level of KRT8 in the samples was then determined by comparing the optical density of the samples to values on the standard curve.

Imaging and Data Analysis

Neovascular AMD was diagnosed based on the results of FP, FA, ICGA, and OCT, with evidence of hyperfluorescence and late leakage associated with pigmented epithelium detachment, serous retinal detachment, subretinal exudation, and SMH. Choroidal neovascularization (CNV) types were subdivided into four categories as follows: (a) Polypoidal choroidal vasculopathy was diagnosed based on the finding of ICGA with the presence of a branched vascular network and on evidence of terminal polypoidal lesions in the subpigment epithelial layer with orange-red protrusions corresponding to the polypoidal lesions revealed by ICGA, or both. (b) Type 1 CNV was characterized by new vessels located beneath the RPE. (c) Type 2 CNV was defined as new vessels penetrating the RPE layer and localized in the subretinal space as observed upon OCT. (d) Type 3 CNV, retinal angiomatous proliferation, was defined as the intraretinal proliferation of new vessels, which may originate from both retinal and choroidal circulation. The presence of retinal-choroidal anastomosis was identified by ICGA or intraretinal hemorrhage in FP or intraretinal fluid (IRF) on OCT. The size of the CNV area was calculated by defining the boundaries of the CNV in FA. The central macular thickness (CMT) was automatically calculated in OCT as the average retinal thickness within a circle of diameter 1000 µm centered on the fovea (the central circle of the Early Treatment Diabetic Retinopathy Study grid). All images were reviewed before measurement, and re-segmentation or re-centration of the fovea was performed if there were significant errors. The choroidal thickness was measured under the foveal center vertically from the outer border of the hyperreflective line of the RPE to the inner border of the sclera. Morphological features in FP or OCT were also evaluated. These parameters included the presence of IRF, subretinal fluid (SRF), SMH, drusen, or hard exudate. We defined the presence of drusen as one or

more large (>125 µm) druse or extensive (20 soft or 65 hard without any soft) intermediate-sized drusen (63–124 µm), assessed within two disc diameters of the center of the macula. All measurements and diagnosis were conducted by two retinal specialists (J.Y.S. and J.L), and averaged values were used for evaluation.

Statistical Analysis

To compare the baseline characteristics and KRT8 level between the nAMD and control groups, an independent *t* test was used for continuous variables and χ^2 test for categorical variables. To evaluate the relationship between baseline KRT8 level and baseline characteristics, Pearson correlation was used for continuous variables, whereas an independent *t* test was used for comparisons between the baseline KRT8 level and categorical variables. To investigate the changes in the KRT8 level according to the treatment response, the nAMD group was classified into responders and poor responders according to OCT findings at month three. Poor responders were defined by the presence of any persistent fluid (IRF or SRF) in OCT (raster scan covering the lesion more than 20 × 10°) at month three. To compare the KRT8 level before and after IVR, the paired *t* test was used, and the independent *t* test was used for comparisons of the KRT8 level between responders and poor responders.

To analyze the association between the KRT8 level and treatment outcomes, visual and anatomical outcomes were evaluated three and six months after initial injection based on the measurement of BCVA and OCT. The BCVA using the Snellen visual acuity chart was converted to logarithm of the minimum angle of resolution (logMAR) units, whereas anatomical outcomes were classified into good and poor anatomical outcome. Poor anatomical outcome was defined as the presence of any persistent fluid (IRF or SRF) in OCT (raster scan covering the lesion more than 20 × 10°) at months three and six. The treatment outcome after six months was evaluated by reviewing BCVA and OCT retrospectively after the completion of the prospective study. To investigate the association between the KRT8 level and visual outcome, linear regression analysis was performed, and logistic regression analysis was used for associations between the KRT8 level and anatomical outcome. In multivariate regression analysis, treatment outcome was the dependent variable, and clinically significant parameters significantly associated with treatment outcome in the univariate analysis were used as independent variables. Statistical analyses were performed using SPSS for Windows (version 21.0; IBM Corp., Armonk, NY, USA). Results with a P value = 0.05 were considered statistically significant.

Results

Demographics and Baseline Characteristics

In this prospective case-control study, we evaluated 58 eyes of 58 patients with treatment-naïve nAMD and 46 eyes of the control patients who underwent cataract surgery during the same period. The characteristics of nAMD patients and those in the control group are summarized in Table 1. There was no significant difference in age ($P = 0.19$), sex ($P = 0.23$), or presence of systemic hypertension (HTN) ($P = 0.80$) or diabetes mellitus (DM) ($P = 0.30$) between the nAMD and control groups. Baseline visual acuity was better in the control group than in the nAMD group ($P < 0.001$).

In the nAMD group, baseline visual acuity was 0.65 ± 0.41 logMAR, and the size of the CNV area was 8.75 ± 9.46 mm^2. Eighteen eyes (31%) were phakic and 40 eyes (69%) were pseudophakic. In terms of CNV type, 29 eyes (50%) had polypoidal choroidal vasculopathy, nine (15.5%) had type 1 CNV, nine had type 2 CNV, and 11 (19.0%) had type 3 CNV. In terms of morphologic features at baseline, 46 eyes (79.3%) had SRF, 24 (41.4%) had IRF, 17 (29.3%) had SMH, 17 (29.3%) had drusen, and nine (15.5%) had hard exudate.

Baseline KRT8 Level in the nAMD and Control Groups

At baseline, the mean KRT8 level in AH was 8.48 ± 1.21 ng/mL in the nAMD group, which was significantly higher than that in the control group (4.99 ± 0.82 ng/mL, $P < 0.001$) (Table 1). In the control group, the baseline KRT8 level did not correlate with age ($P = 0.83$), and there was no difference in the KRT8 level in terms of sex ($P = 0.22$), and the presence of HTN ($P = 0.27$) or DM ($P = 0.54$). In the nAMD group, the baseline KRT8 level did not correlate with age ($P = 0.10$), and there was no difference in the KRT8 level based on sex ($P = 0.74$), the presence of HTN ($P = 0.14$) or DM ($P = 0.54$), or lens status ($P = 0.59$). In addition, the baseline KRT8 level showed no correlation with CMT ($P = 0.89$), choroidal thickness ($P = 0.73$), or CNV size ($P = 0.92$). There was no significant difference in the baseline KRT8 level in terms of the type of CNV ($P = 0.25$) or other morphologic characteristics in OCT, including SRF ($P = 0.33$), IRF

Table 1. Baseline Characteristics and Baseline KRT8 Level in the nAMD and Control Groups

	nAMD	Control	*P* Value
n	58	46	NA
Age (years)	75.7 ± 9.5	73.5 ± 7.6	0.19
Sex (male; n, %)	39 (73.6)	25 (54.3)	0.23
HTN (n, %)	11 (18.9)	7 (15.2)	0.80
DM (n, %)	6 (10.3)	2 (4.3)	0.30
Visual acuity (logMAR)	0.65 ± 0.41	0.11 ± 0.16	<0.001*
(Snellen equivalents)	20/89	20/26	
Baseline KRT8 (ng/mL)	8.48 ± 1.21	4.99 ± 0.82	<0.001*

NA, not applicable.
*$P < 0.05$.

($P = 0.80$), SMH ($P = 0.20$), drusen ($P = 0.50$), and exudate ($P = 0.49$) (Supplementary Table S1).

Changes in the KRT8 Level During Monthly Intravitreal Ranibizumab Injections in the nAMD Group

A significant decrease in the KRT8 level was observed between baseline and month two ($P = 0.017$) (Fig. 1). A comparison of the KRT8 level between responders and poor responders revealed that the difference in the baseline KRT8 level failed to reach statistical significance between the groups ($P = 0.053$). However, poor responders showed significantly higher KRT8 level than responders at month two ($P < 0.001$). In addition, responders showed a significant decrease in the KRT8 level between baseline and month two ($P = 0.002$), whereas poor responders showed no significant change in the KRT8 level ($P = 0.73$) (Figs. 2 and 3). The changes in the KRT8 level between baseline and month two showed a significant difference between responders and poor responders (−0.56 and −0.10, respectively; $P = 0.048$).

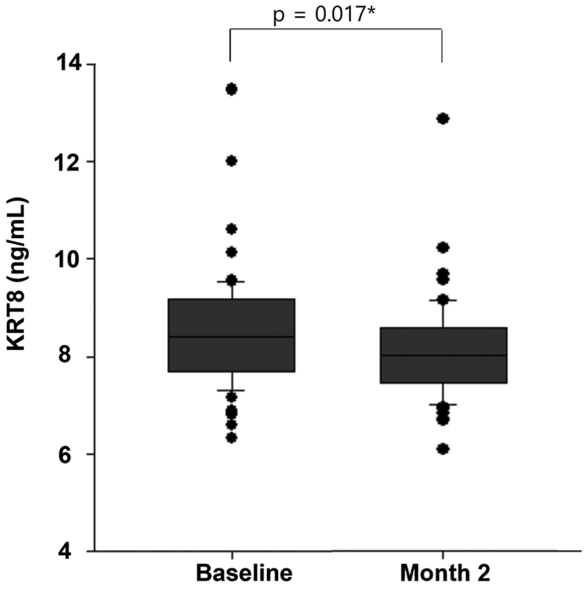

Figure 1. Changes in the KRT8 level in the eyes treated with intravitreal ranibizumab for neovascular age-related macular degeneration. The KRT8 level decreased significantly between baseline and month two. *Box* indicates median and inter-quartile range.

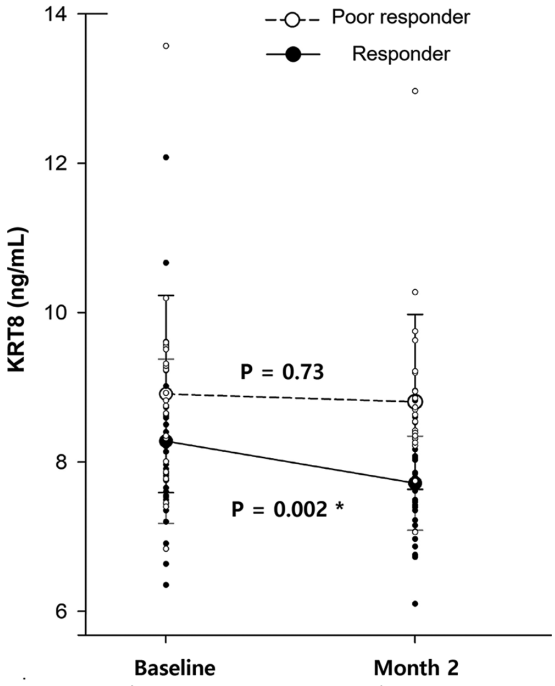

Figure 2. Changes in the KRT8 level in responders and poor responders to IVR treatment for neovascular age-related macular degeneration. Responders exhibited a significant decrease in the KRT8 level between baseline and month two, whereas poor responders exhibited no significant changes in the KRT8 level.

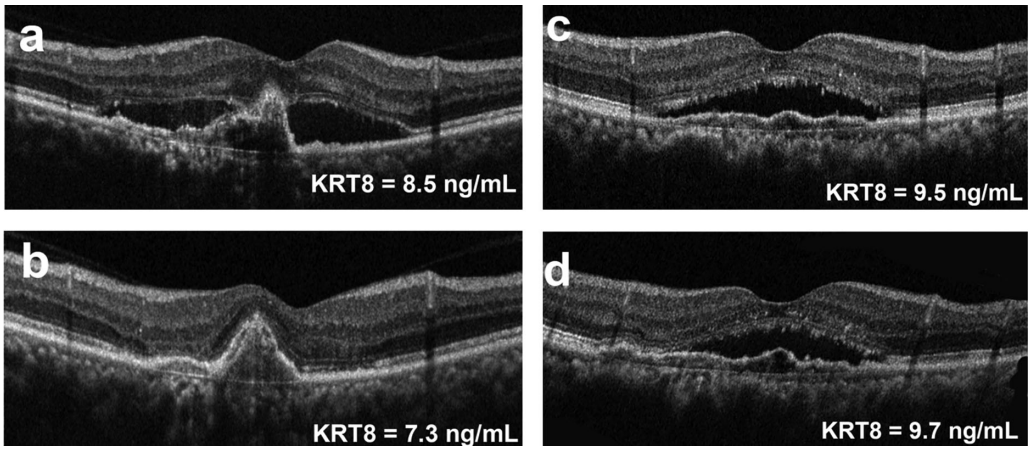

Figure 3. Treatment outcomes and KRT8 level after IVR injection for neovascular age-related macular degeneration. In a responder, OCT showed SRF before IVR (a), whereas SRF was resolved after IVR (b). KRT8 level decreased from 8.5 ng/mL to 7.3 ng/mL after treatment. In a poor responder, baseline OCT showed SRF (c), and the KRT8 level was 9.5 ng/mL. Persistent fluid was observed in OCT after treatment (d), and the KRT8 level was 9.7 ng/mL, which was slightly higher than the baseline measurement.

KRT8 Level and Treatment Outcomes After Intravitreal Ranibizumab Injection

After three monthly injections of IVR (month three), visual acuity improved from 0.65 ± 0.41 logMAR to 0.47 ± 0.38 ($P = 0.002$). In OCT, 33 eyes (56.9%) were dry, whereas 25 (43.1%) showed persistent fluid at month three. CMT was significantly improved from 455.9 ± 252.3 µm to 263.9 ± 110.1 µm ($P < 0.001$).

The relationship between treatment outcome at month three and KRT8 level is shown in Table 2. Visual outcome was not associated with the KRT8 level at baseline ($P = 0.63$) and changes in the KRT8 level between baseline and month two ($P = 0.11$), but worse visual outcome was associated with higher KRT level at month two ($P = 0.045$). The association of anatomical outcome with the KRT8 level failed to reach statistical significance at baseline ($P = 0.07$) and changes in the KRT8 level between baseline and month two ($P = 0.057$), but a significant association was observed between poor anatomical outcome (persistent fluid on OCT) and higher KRT level at month two ($P = 0.001$).

Association Between the KRT8 Level at Month Two and Treatment Outcomes After Intravitreal Ranibizumab Injection

In the univariate analysis, a worse visual outcome was associated with a higher KRT8 level at month two ($P = 0.045$) and the presence of IRF ($P = 0.002$). However, these associations were not significant in the multivariate analysis (Supplementary Table S2).

Poor anatomic outcome was associated with a higher KRT8 level at month two ($P = 0.001$) and larger CNV size ($P = 0.04$) in the univariate analysis. In the multivariate logistic regression analysis, poor anatomical outcome was still found to be associated with a higher KRT8 level at month two (odds ratio [OR] 8.32, 95% confidence interval [CI] 2.02–34.2, $P = 0.003$) (Table 3).

Table 2. Association Between the KRT8 Level and Treatment Outcome After Intravitreal Ranibizumab Injection

	Visual Outcome			Anatomical Outcomes		
	B ± SE	95% CI	*P*	OR	95% CI	*P*
KRT8 at baseline	0.02 ± 0.04	−0.07–0.11	0.63	1.61	0.96–2.69	0.07
KRT8 at Month 2	0.10 ± 0.05	0.003–0.20	0.045*	7.97	2.48–25.66	0.001*
Changes in KRT8	0.09 ± 0.06	−0.02–0.21	0.11	2.30	0.97–5.42	0.057

Anatomical outcome, persistent fluid on OCT at month 3; B, unstandardized beta coefficient; SE, standard error.
*$P < 0.05$.

Table 3. Association Between the KRT8 Level at Month Two and Anatomic Outcome at Month Three

	Univariate			Multivariate		
	OR	95% CI	*P*	OR	95% CI	*P*
KRT8 (ng/mL)	7.97	2.48–25.7	0.001*	8.32	2.02–34.2	0.003*
Baseline characteristics for adjustments						
Age (years)	0.99	0.94–1.05	0.74	0.96	0.87–1.05	0.32
Sex (male)	0.42	0.13–1.38	0.15	0.62	0.07–5.33	0.66
HTN	0.51	0.12–2.15	0.36			
DM	3.47	0.58–20.8	0.17			
ChT (μm)	1.00	0.99–1.01	0.26			
CNV size (mm^2)	1.14	1.01–1.30	0.04*	1.16	0.95–1.41	0.15
CNV type						
PCV		Ref			Ref	
Type 1	0.49	0.11–2.22	0.35	0.35	0.05–2.73	0.32
Type 2	4.89	0.54–44.6	0.16	1.74	0.09–32.58	0.71
Type 3	0.51	0.13–2.07	0.67	0.99	0.06–17.44	0.99
Morphologic characteristics of baseline OCT						
SRF	2.31	0.55–9.65	0.25			
IRF	2.84	0.95–8.44	0.06	2.11	0.34–13.20	0.42
SMH	0.53	0.16–1.79	0.31			
Drusen	0.77	0.24–2.48	0.66			
Exudate	1.26	0.30–5.30	0.75			

ChT, subfoveal choroidal thickness; PCV, polypoidal choroidal vasculopathy; Ref, reference.
*$P < 0.05$.

Association Between the KRT8 Levels at Month Two and Treatment Outcomes at Month Six After Intravitreal Ranibizumab Injection

After completion of the prospective study, treatment outcomes were retrospectively reviewed in 51 patients who were followed up at month six. In the univariate analysis, visual outcomes at month six were not associated with the KRT8 level at month two ($P = 0.87$); however, anatomical outcomes at month six were associated with the KRT8 level at month two ($P = 0.039$). In the multivariate analysis, poor anatomical outcomes at month six were still associated with a higher KRT level at month two (OR 2.63, 95% CI 1.18–5.88, $P = 0.019$) (Table 4).

Discussion

In the present study, 58 treatment-naïve nAMD patients were enrolled, and the AH level of KRT8 before and after IVR treatments was examined. Significantly increased KRT8 level in nAMD eyes compared with that in the controls was observed before treatments. After IVR, responders showed a significant decrease in the KRT8 level, whereas poor responders showed no change. A higher KRT8 level was associated with the presence of persistent fluid in OCT after IVR, suggesting a potential role for KRT8 as a prognostic indicator in patients with nAMD.

KRT8, a well-known epithelial marker protein, has been known to support the mechanical integrity of cells, modulate stress response, and contribute to cell resistance to apoptosis.[13–15,19] KRT8 has been identified as an RPE marker,[16,17] and in our previous study, we found that the KRT8 level is elevated approximately twofold in nAMD patients compared with that in the controls.[8] In this study, the KRT8 level in nAMD patients was 1.7-fold higher than that in the controls, which is in line with the findings of the previous study. Within the retina, only RPE cells are immunoreactive to keratin; thus the disruption of integrity (or rupture) of RPE cells by CNV membrane (CNVM) could release KRT8 into AH. This assumption could explain our results, that is, decreased KRT8 level after the treatments in responders, because the additional RPE damage would be limited by treatments in these patients.

Table 4. Association Between the KRT8 Level at Month Two and Anatomical Outcomes at Month Six

	Univariate			Multivariate		
	OR	95% CI	*P*	OR	95% CI	*P*
KRT8 (ng/mL)	2.13	1.04–4.39	0.039*	2.63	1.18–5.88	0.019*
Baseline characteristics for adjustments						
Age (years)	0.96	0.90–1.02	0.19	0.95	0.87–1.03	0.20
Sex (male)	0.35	0.09–1.28	0.11	1.01	0.16–6.27	0.99
HTN	0.15	0.02–1.32	0.09			
DM	3.63	0.60–3.63	0.16			
ChT (μm)	1.00	1.00–1.02	0.06			
CNV size (mm^2)	1.03	0.97–1.09	0.37	1.07	0.97–1.17	0.18
CNV type						
PCV		Ref			Ref	
Type 1	0.75	0.14–4.10	0.74	0.55	0.06–4.73	0.58
Type 2	8.00	0.86–74.22	0.07	0.79	0.04–13.96	0.87
Type 3	2.67	0.57–12.56	0.22	0	NA	> 0.99
Morphologic characteristics of baseline OCT						
SRF	$>10^3$	NA	>0.99			
IRF	0.99	0.32–3.08	0.99	1.30	0.22–7.65	0.77
SMH	0.61	0.17–2.12	0.43			
Drusen	0.21	0.05–0.88	0.03*			
Exudate	0.92	0.19–4.35	0.91			

Ref, reference.
*$P < 0.05$.

In addition, oxidative or mechanical stress can trigger cytoskeleton activation,[20,21] and several RPE cells are strongly positive for this marker in surgically excised nAMD-related CNVMs.[22] We previously reported that oxidative stress in human RPE cells induces the upregulation of KRT8 and autophagy, resulting in the protection of RPE cells from apoptotic cell death under oxidative stress.[18] CNV in nAMD shares with the process of wound-healing response,[22] and proliferation of RPE is speculated to serve as a reparative process to cover and regenerate damaged tissue and seal off leaking vascular channels.[23] Therefore elevated KRT8 level in the AH is likely to be related to a good reparative mechanism in these treatment-naïve nAMD patients.

After two consecutive IVR treatments, only responders demonstrated a significant decrease in the KRT8 level after IVR, whereas poor responders showed persistent elevated KRT8 level. Furthermore, a higher KRT8 level at month two was associated with the presence of persistent fluid in OCT at month three after adjusting for other variables. Elevated KRT8 level in nAMD eyes early in the disease course is possibly a reparative or protective mechanism; however, prolonged elevation of KRT8 level might be detrimental, as it could be related to epithelial-mesenchymal transition (EMT).[18] In EMT, polarized epithelial cells convert to motile mesenchymal cells, and transdifferentiated RPE cells are the principal nonvascular stromal cells in vascular and fibrotic nAMD-related CNVMs.[22] EMT ultimately results in the loss of RPE characteristics,[17] which is concomitant with a rearrangement of the cytoskeleton.[24] Our previous study showed that under prolonged oxidative stress, a high KRT8 level induces EMT via its phosphorylation, resulting in loss of RPE cell junction integrity and degeneration of the RPE.[18] Similar results have been reported in pancreatic and gastric cancer cells.[25] Although there has been no study on how EMT causes resistance to treatment in nAMD patients, several studies have shown that EMT is associated with resistance to anti-VEGF treatment in various tumors, including pancreatic cancers,[26] genitourinary cancers,[27] and brain tumours.[28] In gastric cancer, KRT8 overexpression leads to EMT and enhances the proliferation and migration of cancer cells, and patients with a high KRT8 level tend to have unfavorable outcomes.[29] Moreover, EMT in RPE contributes to retinal fibrosis in nAMD eyes,[30] and fibrosis often develops in poor responders to anti-VEGF treatment.[31] Based on these

findings, we speculate that in responders, KRT8 expression is elevated as a reactive RPE change with the development of CNV and then decreases when the wound healing process proceeds and CNV regresses with anti-VEGF treatments. An unsuccessful treatment response could result in progression of tissue injury, inflammation, and prolonged loss of RPE cell-to-cell contact, which are responsible for initiating EMT and fibrosis. These processes might contribute to the persistence of KRT8 upregulation in poor responders. It remains to be determined whether upregulated KRT8 expression reflects the consequences or the causes of poor treatment response to IVR; in other words, prolonged KRT upregulation in poor responders might induce EMT, leading to resistance to anti-VEGF treatments.

Although anti-VEGF agents have shown remarkable results in nAMD treatment, some patients show poor or no response to anti-VEGF agents or experience a loss of efficacy of anti-VEGF after repeated administration. Several proteins or pathways, other than VEGF, could cause variability in behavior of the disease and response to anti-VEGF treatment, and thus, could be therapeutic targets for nAMD patients, particularly those who show a poor response to treatment. For example, our previous study suggested that the upregulation of KRT8 and downregulation of phosphorylated KRT8 may promote cell survival while suppressing EMT[18]; thus KRT8 could be a novel target for the treatment of nAMD, which is supported by our present findings.

The limitations of this study include its small sample size and short follow-up period. With a short follow-up period, it is difficult to elucidate the association between KRT8 and recurrence or long-term treatment responses. Although there were no significant differences in age and sex between the nAMD and control groups, matched case-control studies with larger sample sizes based on sample size calculation are required. Although statistically significant, the relatively low beta coefficients and wide CIs for the associations between the KRT8 level and treatment outcomes suggest that more research is needed on nAMD pathophysiology and confounding factors before using KRT8 level in clinical practice. In addition, as the level of both VEGF and KRT8 in the AH could not be obtained due to technical limitations, whether the change in the KRT8 level in the AH is an independent marker for treatment or whether it is associated with the change in VEGF levels could not be determined. Despite these limitations, our results suggest that KRT8 could be a possible prognostic biomarker in nAMD patients. It would also be necessary to investigate whether there is a relationship between the KRT8 level in AH and that in tear or serum, which can be assessed less invasively than AH collection.

In summary, in this study, monitoring the AH level of KRT8 during IVR treatment showed an association between decreasing KRT8 level and better treatment responses to anti-VEGF. An increase in the KRT8 level before treatment may suggest that RPE cells proliferate to envelop CNV and thus regress; the KRT8 level seems to decrease once they have proliferated to some degree. Although long-term data are needed to show that the level of KRT8 in nAMD patients returns to that in the controls after regression of CNV, monitoring aqueous KRT8 level may be a practical approach to predict the therapeutic effects during early treatment. In addition, it may also help in determining the treatment strategy with anti-VEGFs, including treatment intervals, as an aid to image biomarkers. In addition, identification of poor responders to anti-VEGF treatments will help clinicians decide whether to switch to other agents available in the near future, thereby facilitating optimization of customized treatments for nAMD.

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References

1. Bressler NM. Age-related macular degeneration is the leading cause of blindness. *JAMA*. 2004;291:1900–1901.
2. Pascolini D, Mariotti SP, Pokharel GP, et al. 2002 Global update of available data on visual impairment: a compilation of population-based prevalence studies. *Ophthalmic Epidemiol*. 2004;11:67–115.
3. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med*. 2006;355:1419–1431.
4. Yang S, Zhao J, Sun X. Resistance to anti-VEGF therapy in neovascular age-related macular

degeneration: a comprehensive review. *Drug Des Devel Ther.* 2016;10:1857–1867.
5. Handa JT, Rickman CB, Dick AD, et al. A systems biology approach towards understanding and treating non-neovascular age-related macular degeneration. *Nat. Commun.* 2019;10(1):3347.
6. Lai TT, Hsieh YT, Yang CM, Ho TC, Yang CH. Biomarkers of optical coherence tomography in evaluating the treatment outcomes of neovascular age-related macular degeneration: a real-world study. *Sci Rep.* 2019;9(1):529.
7. Simader C, Ritter M, Bolz M, et al. Morphologic parameters relevant for visual outcome during anti-angiogenic therapy of neovascular age-related macular degeneration. *Ophthalmology*. 2014;121:1237–1245.
8. Kang GY, Bang JY, Choi AJ, et al. Exosomal proteins in the aqueous humor as novel biomarkers in patients with neovascular age-related macular degeneration. *J. Proteome Res.* 2014;13:581–595.
9. Kim TW, Kang JW, Ahn J, et al. Proteomic analysis of the aqueous humor in age-related macular degeneration (AMD) patients. *J Proteome Res.* 2012;11:4034–4043.
10. Lee H, Choi AJ, Kang GY, et al. Increased 26S proteasome non-ATPase regulatory subunit 1 in the aqueous humor of patients with age-related macular degeneration. *BMB Rep.* 2014;47:292–297.
11. Yao J, Liu X, Yang Q, et al. Proteomic analysis of the aqueous humor in patients with wet age-related macular degeneration. *Proteomics Clin Appl.* 2013;7(7-8):550–560.
12. Kersten E, Paun CC, Schellevis RL, et al. Systemic and ocular fluid compounds as potential biomarkers in age-related macular degeneration. *Surv Ophthalmol.* 2018;63:9–39.
13. Zatloukal K, Stumptner C, Lehner M, et al. Cytokeratin 8 protects from hepatotoxicity, and its ratio to cytokeratin 18 determines the ability of hepatocytes to form Mallory bodies. *Am. J. Pathol.* 2000;156(4):1263–1274.
14. Ku NO, Omary MB. A disease- and phosphorylation-related nonmechanical function for keratin 8. *J Cell Biol.* 2006;174:115–125.
15. Caulin C, Ware CF, Magin TM, Oshima RG. Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis. *J Cell Biol.* 2000;149:17–22.
16. Zhao C, Yasumura D, Li X, et al. mTOR-mediated dedifferentiation of the retinal pigment epithelium initiates photoreceptor degeneration in mice. *J Clin Invest.* 2011;121:369–383.
17. Hunt RC, Davis AA. Altered expression of keratin and vimentin in human retinal pigment epithelial cells in vivo and in vitro. *J Cell Physiol.* 1990;145:187–199.
18. Baek A, Yoon S, Kim J, et al. Autophagy and KRT8/keratin 8 protect degeneration of retinal pigment epithelium under oxidative stress. *Autophagy*. 2017;13(2):248–263.
19. Tao GZ, Looi KS, Toivola DM, Strnad P, Zhou Q, Liao J, et al. Keratins modulate the shape and function of hepatocyte mitochondria: a mechanism for protection from apoptosis. *J Cell Sci.* 2009;122:3851–3855.
20. Girouard MP, Pool M, Alchini R, Rambaldi I, Fournier AE. RhoA proteolysis regulates the actin cytoskeleton in response to oxidative stress. *PloS One*. 2016;11(12):e0168641.
21. Wu S, Lu Q, Wang N, et al. Cyclic stretch induced-retinal pigment epithelial cell apoptosis and cytokine changes. *BMC Ophthalmol.* 2017;17:208.
22. Lopez PF, Sippy BD, Lambert HM, Thach AB, Hinton DR. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. *Invest Ophthalmol Vis Sci.* 1996;37:855–868.
23. Hu DN, Gentile RC, McCormick SA, et al. Role of RPE cells in pathogenesis of proliferative vitreoretinopathy and age-related macular degeneration: cell culture study of surgical excised pre- and subretinal membranes. *J Clin Ophthalmol Eye Disord.* 2017;1(1):1002.
24. Grisanti S, Guidry C. Transdifferentiation of retinal pigment epithelial cells from epithelial to mesenchymal phenotype. *Invest Ophthalmol Vis Sci.* 1995;36:391–405.
25. Busch T, Armacki M, Eiseler T, et al. Keratin 8 phosphorylation regulates keratin reorganization and migration of epithelial tumor cells. *J Cell Sci.* 2012;125:2148–2159.
26. Carbone C, Moccia T, Zhu C, et al. Anti-VEGF treatment resistant pancreatic cancers secrete proinflammatory factors that contribute to malignant progression by inducing an EMT cell phenotype. *Clin. Cancer Res.* 2011;17:5822–5832.
27. Hammers H, Fu C, Gerber S, et al. Epithelial-mesenchymal transition: A mechanism of resistance to VEGF pathway inhibition in genitourinary cancers. *J Clin Oncol.* 2012;30:390.
28. Piao Y, Liang J, Holmes L, Henry V, Sulman E, Groot JF. Acquired resistance to anti-VEGF therapy in glioblastoma is associated with a

mesenchymal transition. *Clin Cancer Res.* 2013;19:4392–4403.
29. Fang J, Wang H, Liu Y, Ding F, Ni Y, Shao S. High KRT8 expression promotes tumor progression and metastasis of gastric cancer. *Cancer Sci.* 2017;108:178–186.
30. Ishikawa K, Kannan R, Hinton DR. Molecular mechanisms of subretinal fibrosis in age-related macular degeneration. *Exp Eye Res.* 2016;142:19–25.
31. Dikmetas O, Kadayıfcılar S, Eldem B. The effect of CFH polymorphisms on the response to the treatment of age-related macular degeneration (AMD) with intravitreal ranibizumab. *Mol Vis.* 2013;19:2571–2578.