·Basic Research·

Phosphorylation of alphaB-crystallin in epiretinal membrane of human proliferative diabetic retinopathy

Yoko Dong, Zhenyu Dong, Satoru Kase, Ryo Ando, Junichi Fukuhara, Satoshi Kinoshita, Saori Inafuku, Yoshiaki Tagawa, Erdal Tan Ishizuka, Wataru Saito, Miyuki Murata, Atsuhiro Kanda, Kousuke Noda, Susumu Ishida

Laboratory of Ocular Cell Biology and Visual Science, Department of Ophthalmology, Hokkaido University Graduate School of Medicine, Nishi 7, Kita 15, Kita-ku, Sapporo 060-8638, Hokkaido, Japan

Correspondence to: Satoru Kase. Laboratory of Ocular Cell Biology and Visual Science, Department of Ophthalmology, Hokkaido University Graduate School of Medicine, Nishi 7, Kita 15, Kita-ku, Sapporo 060-8638, Hokkaido, Japan. kaseron@med.hokudai.ac.jp

Received: 2015-09-10 Accepted: 2015-12-24

Abstract

• AIM: To examine phosphorylation of alphaB-crystallin (p-αBC), a vascular endothelial growth factor (VEGF) chaperone, and immunohistochemically investigate relationship between p-αBC, VEGF and phosphorylated p38-mitogen-activated protein kinase (p-p38 MAPK) in the epiretinal membrane of human proliferative diabetic retinopathy (PDR).

• METHODS: Eleven epiretinal membranes of PDR surgically excised were included in this study. Two normal retinas were also collected from enucleation tissues due to choroidal melanoma. Paraformaldehyde-fixed, paraffin-embedded tissue sections were processed for immunohistochemistry with anti-p-αBC, VEGF, CD31, and p-p38 MAPK antibodies.

• RESULTS: Immunoreactivity for p-αBC was observed in all of the epiretinal membranes examined, where phosphorylation on serine (Ser) 59 showed strongest immunoreactivity in over 70% of the membranes. The immunolocalization of p-αBC was detected in the CD31-positive endothelial cells, and co-localized with VEGF and p-p38 MAPK in PDR membranes. Immunoreactivity for p-αBC, however, was undetectable in endothelial cells of the normal retinas, where p-p38 MAPK immunoreactivity was less marked than PDR membranes.

• CONCLUSION: Phosphorylation of αBC, in particular, phosphorylation on Ser59 by p-p38 MAPK may play a potential role as a molecular chaperon for VEGF in the pathogenesis of epiretinal membranes in PDR.

• KEYWORDS: phosphorylated alphaB-crystallin; vascular endothelial growth factor; neovascularization; proliferative diabetic retinopathy

DOI:10.18240/ijo.2016.08.03

Dong Y, Dong Z, Kase S, Ando R, Fukuhara J, Kinoshita S, Inafuku S, Tagawa Y, Ishizuka ET, Saito W, Murata M, Kanda A, Noda K, Ishida S. Phosphorylation of alphaB-crystallin in epiretinal membrane of human proliferative diabetic retinopathy. *Int J Ophthalmol* 2016;9(8):1100-1105

INTRODUCTION

AlphaB-crystallin (αBC), a predominant protein of the ocular lens [1-2], belongs to the small heat shock protein family. In addition to being a structural protein, recent studies reported that αBC was also expressed in various non-lenticular tissues, which contributes to the protection of cells from stress-induced damage by acting as a molecular chaperone and anti-apoptotic regulator [3-7]. Furthermore, in response to various stresses, αBC is known to be phosphorylated at three serine (Ser) residues, Ser19 by kinase which has not yet been identified, Ser45 by extracellular signal-regulated kinase (ERK), and Ser59 by p38-mitogen-activated protein kinase (p38 MAPK) [8-9]. Among these phosphorylation sites, phosphorylation on Ser59 conferred maximal cytoprotection in various systemic circulation disorders[10-12].

Proliferative diabetic retinopathy (PDR) is the advanced stage of diabetic retinopathy, characterized by pathological retinal angiogenesis and neovascular epiretinal membrane formation. As a result, PDR membrane formation can lead to subsequent retinal detachment and irreversible visual loss[13-14]. Among various molecules, a large amount of evidence has shown that vascular endothelial growth factor (VEGF)-A plays a critical role in the pathogenesis of retinal neovascularization and epiretinal membrane associated with PDR[15-17].

Recently, we showed that αBC played a critical role in the promotion of angiogenesis as a molecular chaperone of VEGF, and regulated pathological angiogenesis together with VEGF, using a murine oxygen-induced retinopathy (OIR) model and epiretinal membranes of human PDR [11,18]. Moreover, we examined the three phosphorylation sites of

Table 1 Clinical characteristic of patients with PDP

No.	Age (a)	Sex	TRD	VH	DME	Type of DM	HbA1c (%)	Immunoreactivity of pSer59-αBC	Immunoreactivity of pSer19-αBC	Immunoreactivity of pSer45-αBC	Intravitreal injections of bevacizumab
1	65	F	-	+	-	II	12.0	++	++	++	-
2	20	F	+	-	-	II	5.4	+	+	+	+
3	35	M	-	+	-	II	9.2	+	+	+	+
4	41	M	+	+	-	II	5.7	++	+	+	+
5	59	M	+	-	-	II	5.9	++	++	++	+
6	52	M	+	+	-	II	6.8	+	+	++	-
7	69	F	-	+	+	II	7.2	++	++	++	-
8	60	M	+	+	-	II	5.8	++	++	++	+
9	56	M	+	-	+	II	6.1	++	+	++	-
10	49	F	+	-	+	II	6.4	++	+	++	–
11	34	M	-	+	-	II	8.2	++	+	++	+

PDR: Proliferative diabetic retinopathy; TRD: Tractional retinal detachment; VH: Vitreous hemorrhage; HbA1c: Hemoglobin A1c; DM: Diabetes mellitus. ＋＋: Strong; ＋: Weak.

αBC in human conjunctival squamous cell carcinomas, and suggested that phosphorylation on Ser59 played a crucial role in the tumor angiogenesis of the ocular tumor [19]. However, phosphorylated αBC (p-αBC) in PDR membrane, particularly in terms of its association with expression of VEGF and phosphorylated p38 (p-p38) MAPK, has not yet been clarified.

In this study, we immunohistochemically examined p-αBC in the epiretinal membrane of PDR. Moreover, the co-localization of p-αBC and VEGF, as well as p-p38 MAPK was also analyzed by double staining immunohistochemistry, and compared with findings in normal retinas.

SUBJECTS AND METHODS

Human Surgical Samples Eleven epiretinal membranes were surgically removed from patients with PDR between 2009 and 2013 at the Department of Ophthalmology, Hokkaido University Hospital, Sapporo, Japan. The clinical characteristics of all patients are summarized in Table 1. Two normal retinas obtained by enucleation in two patients, a man aged 58 and aged 69, due to choroidal melanoma without medical history of diabetic mellitus, were also examined as controls. Excised membranes and retinas were fixed in 4% paraformaldehyde for immunohistochemistry. This study was conducted in accordance with the tenets of the Declaration of Helsinki. After receiving approval from the institutional review board of Hokkaido University Hospital (IRB #014-0294), written informed consent was obtained from all patients.

Immunofluorescence Microscopy The slides were dewaxed in xylene, dehydrated in ethanol of various concentrations, and rinsed in phosphate-buffered saline for 10min. As pretreatment, microwave-based antigen retrieval was performed in 10 mmol/L citrate buffer (pH 6.0). These slides were incubated with 0.1% bovine serum albumin for 30min, and then incubated with the following primary antibodies: rabbit anti-pSer19-αBC (1:100 dilution; Novus Biologicals, Littleton, CO, USA), rabbit anti-pSer45-αBC (1:100 dilution; Stressgen, Ann Arbor, MI, USA), rabbit anti-pSer59-αBC (1:100 dilution; Abcam, Tokyo, Japan), mouse anti-VEGF (1:50 dilution; Abcam), and mouse anti-p-p38 MAPK (1:50; Abcam) antibodies. Secondary antibodies for fluorescent detection were AlexaFluor 488 and 546 (Life Technologies). Sections were visualized under a BIOREVO microscope (Keyence, Osaka, Japan). Immunoreactivity was compared for each of three pSer-αBCs among patients, and was evaluated as strong (represented as ++), weak (represented as +), or negative (back ground staining only, represented as -) by two masked investigators.

Evaluation of Microvessel Density in Proliferative Diabetic Retinopathy Membranes The number of CD31-positive microvessels in PDR membranes was directly counted at high magnification (objective lens: 40×) in all fields of specimen, and the area-adjusted number of microvessels per 1 mm^2 area was calculated as the microvessel density (MVD). We compared the MVD among the patients and examined the correlation with pSer59-αBC immunoreactivity. Results are presented as mean±standard deviation (SD). Statistical analysis was performed using the two-tailed unpaired Student's t-test, and the level of significance was $P<0.05$.

RESULTS

First, we confirmed immunoreactivity of all p-αBCs in CD31-positive endothelial cells in the epiretinal membranes of human PDR (Figure 1), whereas their immunoreactivity varied among patients. Eight patients showed strongly positive immunoreactivity of pSer59-αBC (Table 1). Double staining immunohistochemistry revealed that pSer59-αBC immunoreactivity was co-localized in VEGF-positive endothelial cells in PDR membranes (Figure 2A-2D).

Furthermore, based on the previous report demonstrating that p-p38 MAPK was responsible for the phosphorylation of Ser59-αBC [9], we also performed double-staining of pSer59-αBC and p-p38 MAPK, and confirmed their

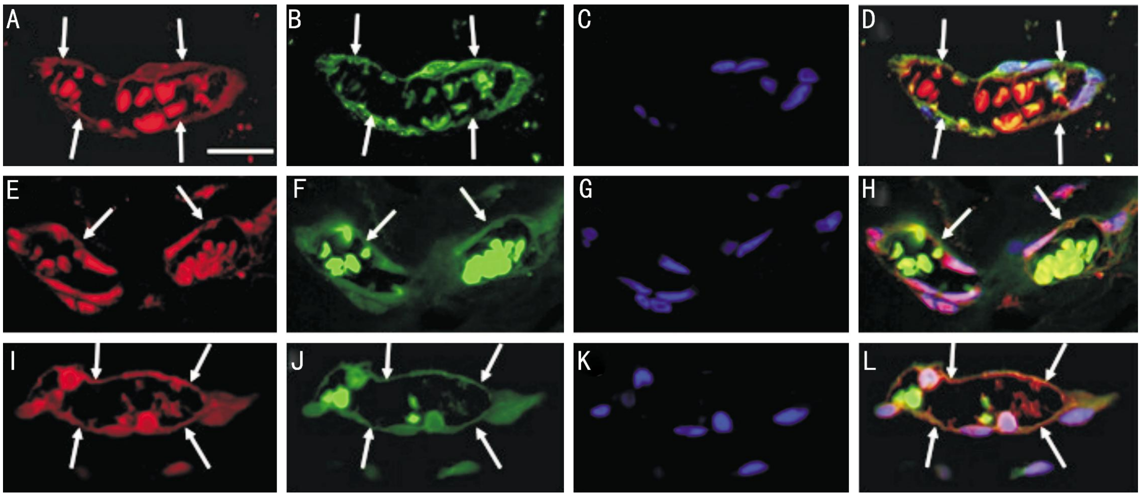

Figure 1 Immunohistochemistry for p-αBCs and CD31 in the epiretinal membrane of PDR A, E, I: pSer59-αBC, pSer19-αBC, pSer45-αBC, arrows indicate the expression of p-αBCs (red); B, F, J: Endothelial cells are clearly detected with anti-CD31 antibody (arrows, green); C, G, K: Spindle-shaped nuclei of endothelial cells are detected with 4', 6-diamidino-2-phenylindole (DAPI) nuclear staining (blue); D, H, L: Merging of A, B, C, and E, F, G, and I, J, K, respectively. Note that p-αBCs expression is detected in endothelial cells (arrows). Scale bar indicates 25 μm.

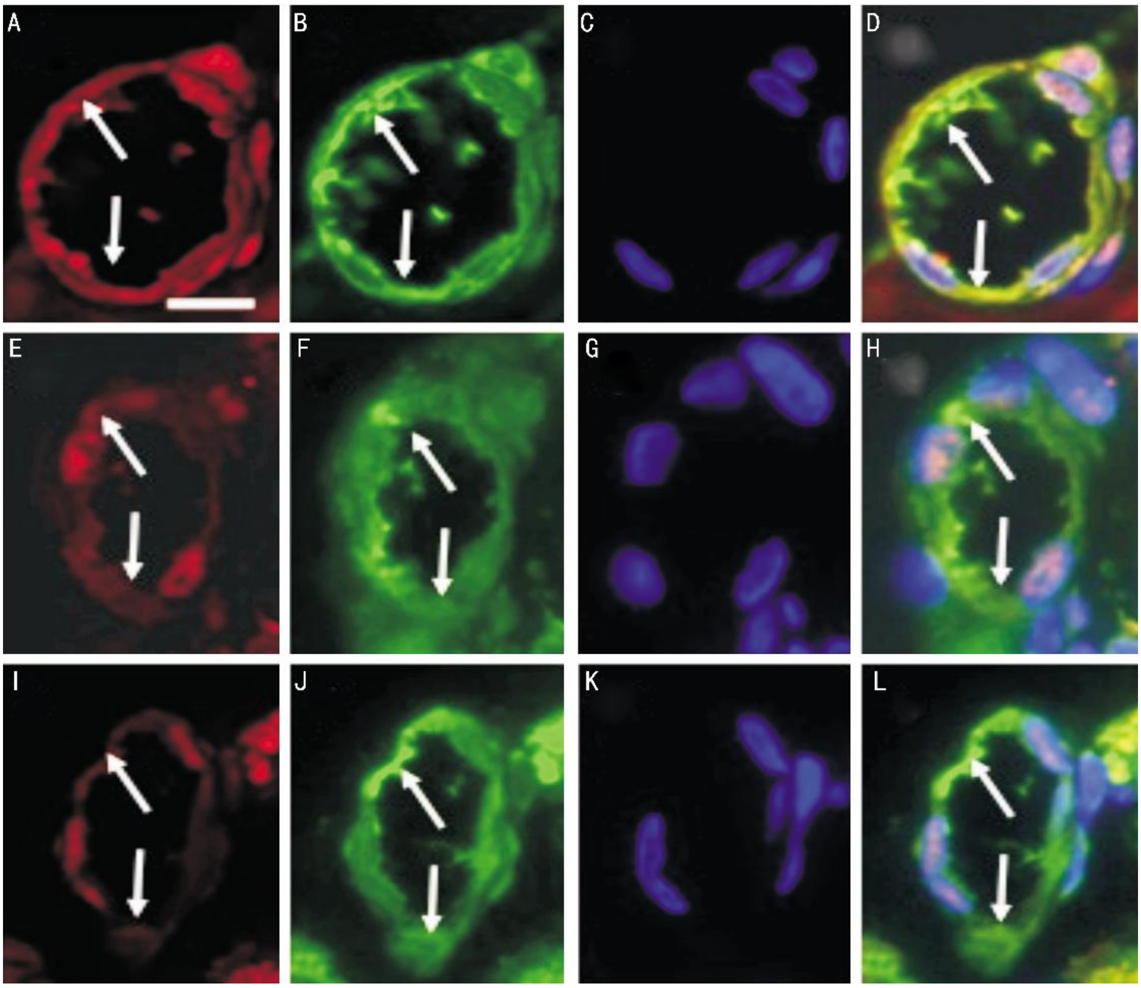

Figure 2 Immunohistochemistry for p-αBCs and VEGF-A in the epiretinal membrane of PDR A, E, I: pSer59-αBC, pSer19-αBC, pSer45-αBC, arrows indicate the expression of p-αBC (red); B, F, J: VEGF expression is clearly detected in the epiretinal membrane (arrows, green); C, G, K: Spindle-shaped nuclei of endothelial cells are detected with DAPI nuclear staining (blue); D, H, L: Merging of A, B, C, and E, F, G, as well as I, J, K, respectively. Note that both p-αBCs and VGEF expressions are detected in neovascular endothelial cells. Scale bar indicates 10 μm.

co-localization in endothelial cells situated in human PDR membranes. By contrast, immunoreactivity of pSer59-αBC was not detected in normal human retinal endothelial cells (Figure 3). In addition, the immunoreactivity of p-p38

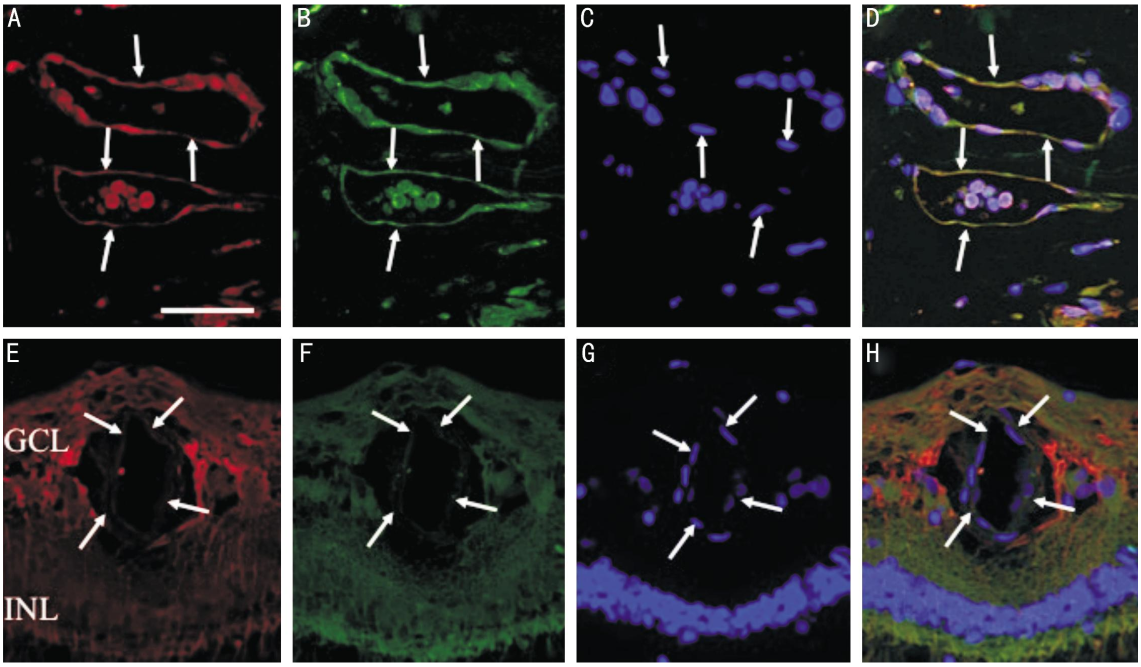

Figure 3 Immunohistochemistry for pSer59-αBC and p-p38 MAPK in the epiretinal membrane of PDR (A, B, C, D) and normal retina (E, F, G, H). A, E: Arrows indicate cytoplasmic immunoreactivity for pSer59-αBC (red); B, F: p-p38 MAPK expression is clearly detected in the epiretinal membrane (arrows, green); C, G: Arrows indicate spindle-shaped nuclei of vascular endothelial cells using DAPI nuclear staining (blue); D, H: Merging of A and B, and C and D, respectively. Note that both pSer59-αBCs and p-p38 MAPK expressions are detected in neovascular endothelial cells (D), while almost no pSer59-αBCs or relatively weak p-p38 MAPK expressions are detected in normal retina vessels (H). GCL: Ganglion cell layer; INL: Inner nuclear layer. Scale bar indicates 50 μm.

MAPK in normal human retinal blood vessels was less marked than that in PDR membranes (Figure 3). MVD was 304±274 and 126±77 in cases with strong and weak-positive for pSer59-αBC, respectively, while there was no statistically significant difference (*P*=0.12).

In this study, other phosphorylation sites were also examined in PDR membrane tissues. Eight patients showed strong immunoreactivity of pSer45-αBC, while the remaining 3 patients showed relatively weak immunoreactivity. By contrast, only 4 out of 11 patients showed strong, and the remaining 7 patients showed relatively weak immunoreactivity of pSer19-αBC. Furthermore, 4 patients (patient 1, 5, 7 and 8) showed strong immunoreactivity of all three phosphorylation sites of αBC, and 2 patients (patient 2 and 3) showed weak immunoreactivity of all sites. pSer45-αBC and pSer19-αBC showed marginal correlation with VEGF expression compared with pSer59-αBC (Figure 2E-2L).

We further investigated whether anti-VEGF intravitreal injection treatment could affect immunoreactivities of phosphorylated αBCs. Regardless of anti-VEGF intravitreal injection treatment, no apparent difference could be identified among the immunoreactivities of αBCs phosphorylated at Ser59, Ser19 and Ser45 (*P*=0.66, 0.84 and 0.07, respectively).

DISCUSSION

We recently showed that αBC expression was detected in the neovessels of PDR membranes [18], regardless of its phosphorylation. This study demonstrated phosphorylation of αBCs in the neovessels of all human PDR membranes examined. Our results also indicate that phosphorylation of αBC, especially phosphorylation of αBC on Ser59, may be associated with p-p38 MAPK in PDR membrane, which was not observed in the normal retinas.

We previously demonstrated that αBC binding to VEGF protein promoted intraocular neovascularization in a murine OIR model [11], and also reported the co-localization of αBC and VEGF in epiretinal membranes of human PDR [18]. Furthermore, previous reports have revealed that phosphorylation on Ser59 by p-p38 MAPK contributes most to the function of αBC as a molecular chaperon [10-11]. Reddy *et al* [20] demonstrated that pSer59-αBC was significantly up-regulated as compared to normal retinas in a rat model of diabetes. Based on the reports, we conducted this study, focusing on the phosphorylation of αBC, particularly phosphorylation on Ser59, and hypothesizing that phosphorylation on Ser59 by p-p38 MAPK, which is activated by diabetes [21], would also contribute to the pathogenesis of neovascularization in human diabetic retinopathy.

Consistent with our hypothesis, almost no expression of pSer59-αBC could be detected in normal retinal vessels of humans, while expression of pSer59-αBC was markedly detected in neovessels in all PDR membranes examined. Double staining immunohistochemistry clearly demonstrated that pSer59-αBC was co-localized with VEGF in neovessels of PDR membranes. Similarly, immunoreactivity of p-p38 MAPK showed less marked signals in normal retinal vessels than in neovessels of PDR, where αBC was phosphorylated on Ser59. These results indicate p-p38 MAPK might contribute to the phosphorylation of αBC on Ser59 in the formation of PDR.

Recent reports suggest that αBC can lead to angiogenesis in various tissues [11,22]. We further examined MVD in the PDR membranes in order to determine whether αBC phosphorylation was related with the neovascularization. As a result, we found no significant correlation between pSer59 and MVD in human PDR membranes. In contrast, it is known that VEGF immunoreactivity is associated with the MVD [23], indicating that VEGF directly exerts angiogenesis within the PDR membrane as an angiogenic factor. Therefore, phosphorylation of αBC may contribute to angiogenesis by functioning as a VEGF chaperone rather than by itself, although the number of patients examined in this study is limited.

Interestingly, strong pSer45-αBC immunoreactivity was detected in as many PDR membranes as pSer59-αBC immunoreactivity. In contrast, immunoreactivity of pSer19-αBC was less marked than that of pSer59-αBC or pSer45-αBC. Ser45-αBC phosphorylation is regulated by ERK, while mechanisms underlying the phosphorylation on Ser19 remain unknown [8]. Although pSer45-αBC and VEGF immunolocalization seemed to be marginal correlation rather than pSer59-αBC, further studies are needed to clarify a role of pSer45 in retinal neovascularization *in vivo* and *in vitro*.

In this study, we could not find any statistically significant effects of anti-VEGF intravitreal injection treatment on phosphorylation immunoreactivities of αBCs. However, immunoreactivities of αBCs phosphorylated at Ser45 tend to be higher in patients given the treatment (P=0.07). Due to the limitation of this study, a precise effect of anti-VEGF intravitreal injection on the expression of phosphorylated αBC is required to be clarified in a larger study.

αBC might be an interesting therapeutic target, given that it prevents VEGF from being degraded as a molecular chaperon [11]. Indeed, down-regulation of αBC by siRNA transfection led to suppression of tumor growth [24-25], as well as significantly low expression of VEGF *in vitro* [11]. Thus, reducing αBC expression or regulating its phosphorylation may be beneficial for controlling the development of PDR. In this view, further investigations will be required to clarify the precise mechanism of αBC phosphorylation and its involvement with VEGF in the pathogenesis of retinal neovascularization.

In conclusion, this study suggests that phosphorylation of αBC is associated with VEGF in the development of diabetic retinopathy, where phosphorylation of Ser59-αBC by p-p38 MAPK might be involved.

ACKNOWLEDGEMENTS

The authors thank Ikuyo Hirose and Shiho Yoshida for their technical support for this study.

Foundations: Supported by the Research foundation of the Japan Society for the Promotion of Science (JSPS) (No. 15K10856); Scientific Research from The Ministry of Education, Culture, Sports, Science, and Technology (MEXT).

Conflicts of Interest: Dong Y, None; **Dong Z,** None; **Kase S,** None; **Ando R,** None; **Fukuhara J,** None; **Kinoshita S,** None; **Inafuku S,** None; **Tagawa Y,** None; **Ishizuka ET,** None; **Saito W,** None; **Murata M,** None; **Kanda A,** None; **Noda K,** None; **Ishida S,** None.

REFERENCES

1 Wistow GJ, Piatigorsky J. Lens crystallins: the evolution and expression of proteins for a highly specialized tissue. *Annu Rev Biochem* 1988;57: 479-504.

2 Clark JI. Functional sequences in human alphaB crystallin. *Biochim Biophys Acta* 2016;1860(1 Pt B):240-245.

3 Kase S, Parikh JG, Rao NA. Expression of heat shock protein 27 and alpha-crystallins in human retinoblastoma after chemoreduction. *Br J Ophthalmol* 2009;93(4):541-544.

4 Ousman SS, Tomooka BH, van Noort JM, Wawrousek EF, O'Connor KC, Hafler DA, Sobel RA, Robinson WH, Steinman L. Protective and therapeutic role for alphaB-crystallin in autoimmune demyelination. *Nature* 2007;448(7152):474-479.

5 Andley UP. Crystallins in the eye: Function and pathology. *Prog Retin Eye Res* 2007;26(1):78-98.

6 Watanabe G, Kato S, Nakata H, Ishida T, Ohuchi N, Ishioka C. alphaB-crystallin: a novel p53-target gene required for p53-dependent apoptosis. *Cancer Sci* 2009;100(12):2368-2375.

7 Arrigo AP. Pathology-dependent effects linked to small heat shock proteins expression: an update. *Scientifica (Cairo)* 2012;2012:185641.

8 Ito H, Okamoto K, Nakayama H, Isobe T, Kato K. Phosphorylation of alphaB-crystallin in response to various types of stress. *J Biol Chem* 1997; 272(47):29934-29941.

9 Li R, Reiser G. Phosphorylation of Ser45 and Ser59 of αB-crystallin and p38/extracellular regulated kinase activity determine αB-crystallin-mediated protection of rat brain astrocytes from C2-ceramide-and staurosporine-induced cell death. *J Neurochem* 2011; 118(3):354-364.

10 Morrison LE, Hoover HE, Thuerauf DJ, Glembotski CC. Mimicking phosphorylation of alphaB-crystallin on serine-59 is necessary and sufficient to provide maximal protection of cardiac myocytes from apoptosis. *Circ Res* 2003;92(2):203-211.

11 Kase S, He S, Sonoda S, Kitamura M, Spee C, Wawrousek E, Ryan SJ, Kannan R, Hinton DR. alphaB-crystallin regulation of angiogenesis by modulation of VEGF. *Blood* 2010;115(16):3398-3406.

12 López-González I, Carmona M, Arregui L, Kovacs GG, Ferrer I. alphaB-crystallin and HSP27 in glial cells in tauopathies. *Neuropathology* 2014;34(6):517-526.

13 Resnikoff S, Pascolini D, Etya'ale D, Kocur I, Pararajasegaram R, Pokharel GP, Mariotti SP. Global data on visual impairment in the year 2002. *Bull World Health Organ* 2004;82(11):844-851.

14 Klein BE. Overview of epidemiologic studies of diabetic retinopathy. *Ophthalmic Epidemiol* 2007;14(4):179-183.

15 Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 1994;331(22):1480-1487.

16 Wirostko B, Wong TY, Simó R. Vascular endothelial growth factor and diabetic complications. *Prog Retin Eye Res* 2008;27(6):608-621.

17 Aiello LP. Angiogenic pathways in diabetic retinopathy. *N Engl J Med* 2005;353(8):839-841.

18 Dong Z, Kase S, Ando R, Fukuhara J, Saito W, Kanda A, Murata M, Noda K, Ishida S. Alphab-crystallin expression in epiretinal membrane of human proliferative diabetic retinopathy. *Retina* 2012;32(6):1190-1196.

19 Dong Z, Kase S, Ando R, Fukuhara J, Kinoshita S, Dong Y, Takashina S, Kanda A, Noda M, Noda K, Ishida S. Expression of αB-crystallin and vascular endothelial growth factor in conjunctival squamous cell carcinoma. *Anticancer Res* 2013;33(9):3745-3751.

20 Reddy VS, Raghu G, Reddy SS, Pasupulati AK, Suryanarayana P, Reddy GB. Response of small heat shock proteins in diabetic rat retina. *Invest Ophthalmol Vis Sci* 2013;54(12):7674-7682.

21 Du Y, Tang J, Li G, Berti-Mattera L, Lee CA, Bartkowski D, Gale D, Monahan J, Niesman MR, Alton G, Kern TS. Effects of p38 MAPK inhibition on early stages of diabetic retinopathy and sensory nerve function. *Invest Ophthalmol Vis Sci* 2010;51(4):2158-2164.

22 van de Schootbrugge C, Bussink J, Span PN, Sweep FC, Grénman R, Stegeman H, Pruijn GJ, Kaanders JH, Boelens WC. αB-crystallin stimulates VEGF secretion and tumor cell migration and correlates with enhanced distant metastasis in head and neck squamous cell carcinoma. *BMC Cancer* 2013;13:128.

23 Abu El-Asrar AM, Missotten L, Geboes K. Expression of hypoxia-inducible factor-1alpha and the protein products of its target genes in diabetic fibrovascular epiretinal membranes. *Br J Ophthalmol* 2007;91(6):822-826.

24 Shin JH, Kim SW, Lim CM, Jeong JY, Piao CS, Lee JK. alphaB-crystallin suppresses oxidative stress-induced astrocyte apoptosis by inhibiting caspase-3 activation. *Neurosci Res* 2009;64(4):355-361.

25 Stegh AH, Kesari S, Mahoney JE, Jenq HT, Forloney KL, Protopopov A, Louis DN, Chin L, DePinho RA. Bcl2L12-mediated inhibition of effector caspase-3 and caspase-7 via distinct mechanisms in glioblastoma. *Proc Natl Acad Sci U S A* 2008;105(31):10703-10708.