

Article

GCAP1, Rab6, and HSP27: Novel Autoantibody Targets in Cancer-Associated Retinopathy and Autoimmune Retinopathy

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Purpose: Autoantibodies (AABs) with different retinal specificities were reported in cancer-associated retinopathy (CAR) and autoimmune retinopathy (AR). The goal was to identify the small retinal proteins of apparent molecular mass of 23-kDa often recognized by patients' AABs.

Methods: Sera specific for a 23-kDa retinal protein of 173 patients were investigated retrospectively by Western blotting and double immunofluorescence confocal microscopy. A proteomic analysis revealed new 23-kDa protein candidates, including guanylyl cyclase-activating proteins (GCAPs), heat shock protein 27 (HSP27), and Rab6A GTPase (Rab6A).

Results: Among the cohort of 173 patients, only 68 had anti-recoverin AABs and the remaining 105 reacted with 4 unique proteins, which were identified as a Rab6A, HSP27, GCAP1, and GCAP2. Confocal images from a double labeling study confirmed the reactivity of AABs with different types of cells in human retina, consistent with the target protein's respective cellular functions. Patients (62/173) had been diagnosed with various kinds of cancer, including 20% of patients who had anti-recoverin, 11% anti-Rab6A, and 5% anti-HSP27 AABs. Only 50% of recoverin-seropositive patients had cancer and the individuals with anti-recoverin AABs had a significantly higher likelihood to be diagnosed with cancer than patients with other anti-23-kDa AABs.

Conclusions: The newly discovered retinal autoantigens may be involved in pathogenicity of CAR and AR. The recognition of AABs against various retinal proteins associated with autoimmune retinal degeneration broadens the group of proteins related with these entities.

Translational Relevance: Patients with anti-recoverin, anti-GCAP1, anti-Rab6A, and anti-HSP27 AABs represented diverse clinical phenotypes, so the presence of disease-associated AABs provides important information for molecular diagnosis.

Introduction

Cancer-associated retinopathy (CAR) and autoimmune retinopathy (AR) are rare conditions that are immunologically heterogeneous, produce a number of different symptoms, and are associated with a diverse anti-retinal autoantibody repertoire.^{1–6} Autoimmune retinopathy resembles CAR but patients with AR do not have diagnosed cancer at the initial ocular exam, or the cancer may be in too early a stage of development to be detected by conventional methods.⁷ However, tumors may be detected in subsequent years with the retinopathy and autoantibodies (AABs) preceding

diagnosis of cancer by years.⁸ The specificities of antiretinal AABs range from photoreceptor-specific proteins, such as recoverin or arrestin, to metabolic proteins, such as enolase or carbonic anhydrase II. Surprisingly, the antigenic proteins mostly represent intracellular proteins involved in important cellular functions ranging from phototransduction to glycolysis.⁸ Different antiretinal AABs frequently coexist in a single patient, creating antibody arrays related to the syndrome.³ However, not all AAB target antigens have been identified.

Recoverin was one of the first autoantigens identified in CAR, a paraneoplastic syndrome in which immunologic events lead to the degeneration of

Table 1. Demographics of Patients Based on Seropositivity with Recoverin, Rab6A, HSP27, and GCAP1 and the Presence of Cancer

Patients	Anti-REC		Anti-Rab6A		Anti-HSP27		Anti-GCAP1/2	
	Cancer (+)	Cancer (-)	Cancer (+)	Cancer (-)	Cancer (+)	Cancer (-)	Cancer (+)	Cancer (-)
Number of patients	34	34	19	34	8	37	0	7
Average age (range in y)	66 (35–89)	55 (14–86)	65 (47–82)	56 (20–84)	62 (25–86)	55 (15–82)	ND	57 (29–78)
Age difference, y	11		9		7		ND	
Female/male	23/12	17/16	11/6	20/16	5/3	27/9	ND	4/4

(+), Patients diagnosed with cancer prior to antibody testing; (–), Patients without cancer at the time of antibody testing.

photoreceptors in some individuals with cancer.^{9,10} Recoverin is a calcium-binding protein that regulates rhodopsin phosphorylation in a calcium-dependent manner.¹¹ Potential CAR antigens continue to be discovered and include: tubby-like protein 1 (TULP1, 78-kDa), interphotoreceptor retinoid-binding protein (IRBP, 145-kDa), heat shock cognate proteins, such as hsp60 and hsc70, photoreceptor cell-specific nuclear receptor (PNR, 41-kDa), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 36-kDa), aldolase (40-kDa), transducin- α (40-kDa), and others. These AAbs in symptomatic patients may be an indication of autoimmune disease of the retina, although their significance in pathogenicity of retinopathy is unknown.^{12,13}

In our laboratory, we detected AAbs in patients with symptoms of CAR and AR that bound to a 23-kDa protein on the blot, but subsequently did not react with a purified recoverin. We hypothesized that there are other retinal proteins of a similar molecular weight that can be targeted by autoimmunity in retinopathy. Indeed, a proteomic analysis of the gel with separated human retinal proteins revealed a few potential 23-kDa protein candidates, including guanylyl cyclase-activating protein 1 (GCAP 1), heat shock protein 27 (HSP27), a series of Ras-related proteins Rab GTPases, glutathione S-transferase P, ATP synthase O subunit, mitochondrial precursor, and others. Our studies focused on patients who had AAbs against four 23-kDa-autoantigens that included HSP27, Rab6A, GCAP1, and GCAP2 as possible targets in CAR/AR.

(OHSU) Serum Repository for the presence of AAbs against 23-kDa proteins. We evaluated 640 CAR patients and 2315 AR patients between 2005 and 2014, and identified 173/2955 (6%) individuals (107 women and 66 men, ratio F/M = 2.6; average age, 59 years) whose serum AAbs reacted with a retinal protein of approximately 23-kDa molecular mass on the Western blot with proteins extracted from human retina. However, 105 serum AAbs did not react with purified recoverin in subsequent assays. Demographics of patients with anti-23-kDa AAbs are presented in Table 1. The study was performed according to the guidelines of the Declaration of Helsinki. The OHSU Institutional Review Board approved the project. On average, the patients already diagnosed with cancer were 9 years older than patients without cancer identified at the time of testing.

Western Blotting

Sera from patients were examined for reactivity with retinal proteins by Western blot analysis using proteins extracted from human retinas as described previously.⁷ After initial screening, a confirmation of antiretinal specificity was performed with purified recombinant human proteins as follows: recoverin (purified in the Adamus Laboratory), HSP27 (Abcam, Cambridge, United Kingdom), Rab6A (Sino Biological, Inc., North Wales, PA, USA), as well as GCAP1 and GCAP2 (expressed from pET11d vector in a BLRDE3pLysS Escherichia coli strain and purified using previously described procedures^{14,15}). A sample of 0.25 μ g protein was loaded per lane of 16% Bio-Rad Criterion XT Bis-Tris gel (Bio-Rad Laboratories, Hercules, CA). After electrophoresis in Tris/glycine buffer, the proteins were transferred to Immobilon membrane (Millipore, Billerica, MA) using a semidry apparatus. Then, individual strips

Methods

Patients

We retrospectively analyzed CAR and AR patients' sera from the Oregon Health and Science University

were incubated with patient serum that was initially identified as having anti-23-kDa AAbs. The following control primary antibodies were used: rabbit anti-recoverin R2 (1:50,000; developed in the Adamus Laboratory), rabbit anti-GCAP1 and GCAP2^{16,17} (1:10,000), rabbit anti-rab6A (1:2000; Abcam), mouse anti-HSP27 (1:2000; Thermo Fisher Scientific, Waltham, MA). The secondary antibody, goat anti-human IgG (H+L chain; Thermo Fisher Scientific), goat anti-rabbit IgG (H+L chain; Invitrogen), and goat anti-mouse IgG (H+L chain; Invitrogen), all conjugated to alkaline phosphatase were diluted 1:2000.

Fluorescent Double Immunolabeling

Human retinal cryosections (12 μm) in optimal cutting temperature (OCT) compound were postfixed with 4% paraformaldehyde for 10 minutes followed by blocking with 10% normal goat serum with 1% bovine serum albumin and 0.2% Tween in phosphate buffered saline (PBS) for 60 minutes at room temperature (RT). Then, human sera (diluted 1:50), each specific to one of 23-kDa protein as revealed by Western blotting analysis, were added and incubated overnight at 4°C. The next day, after washing with PBS anti-human IgG conjugated to Alexa Fluor 488 (1:1000; Invitrogen, Carlsbad, CA) was added for 1 hour of incubation. Then, the sections were washed and incubated with various specific primary antibodies for 1 hour at RT as follows: rabbit anti-bovine recoverin R2 that cross-reacted with human recoverin (diluted 1:500), anti-human Rab6A, anti-human HSP27, and anti-bovine CGAP1¹⁶ that cross-reacted with human GCAP1 (diluted 1:200). After washing, the appropriate fluorescent secondary antibodies conjugated to Alexa Fluor 594 (1:2000, Invitrogen) were added for an additional 1 hour of incubation. The sections were washed in PBS and a mounting reagent containing 4',6-diamidino-2-phenylindole (DAPI) was added to seal the sections, inhibit fluorescence quenching, and stain the nuclei. The immunofluorescent images were acquired using an Olympus Fluoview1000 confocal microscope, and pseudocolors were applied for analysis by Olympus FluoView FV10-ASW software (Olympus, Center Valley, PA). A negative control contained secondary antibodies only.

Identification of a Library of 23-kDa Retinal Antigens

The identification of reactive 23-kDa molecular mass protein antigens was performed by Dr. Larry

David in the OHSU Proteomics Shared Resource facility as described previously.¹⁸ Briefly, 30 μg portions of human retinal proteins were separated in 3 lanes of an SDS-PAGE using a Bio-Rad Criterion 10% gel stained with Coomassie brilliant blue and 2-mm wide slices excised from the bottom of the gel. The excised gel slices were destained twice for 30 minutes by shaking in 50 mM ammonium bicarbonate buffer, 50% acetonitrile, dried, and then reduced by addition of 10 mM dithiothreitol, 100 mM ammonium bicarbonate, and incubation at 56°C for 30 minutes. Proteins then were alkylated by addition of 55 mM iodoacetamide, 100 mM ammonium bicarbonate, and incubation at RT for 30 minutes in the dark. Gel slices then were again washed with destain solution, dried, and reswelled on ice for 15 minutes in digestion buffer containing 100 mM ammonium bicarbonate, 5 mM CaCl_2 , and 25 ng/ μL sequencing grade modified trypsin (ProMega, Madison, WI). Following reswelling, excess trypsin solution was removed, gel slices covered with digestion buffer without trypsin, and samples incubated overnight at 37°C with shaking. Peptides then were recovered by addition of 3 μL of formic acid, shaking for 15 minutes, and the digestion buffer removed from the gel slices and placed in autosampler vials. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) of peptides was performed by capillary reverse phase separation using a 95-minute acetonitrile gradient and an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific) to generate data-dependent MS/MS. The resulting LC-MS/MS data then were analyzed to identify peptides and proteins using the Protein Discoverer 1.4 Suite (Thermo Fisher Scientific) with Sequest HT and Percolator programs to identify peptides and calculate peptide false discovery rates, respectively. Searches used a decoy database strategy and a human-only version of the Swiss-Prot database (Swiss Institute of Bioinformatics, Geneva, Switzerland) containing 20,163 entries and downloaded in July 2015. Only peptides with Percolator q scores below 0.01 (approximate false discovery rate <1%) and proteins with 2 or more unique peptide identifications in each gel slice were accepted. Protein Discoverer .msf files then were loaded into Scaffold 4.2.1 (Proteome Software, Portland, OR, USA) and numbers of MS/MS spectra assigned to each protein (spectral counts) in each gel slice exported. Under these criteria, a total of 1033 human retinal proteins were identified with 19 matches to decoy database entries, resulting in a protein false discovery rate of approximately 2%. The

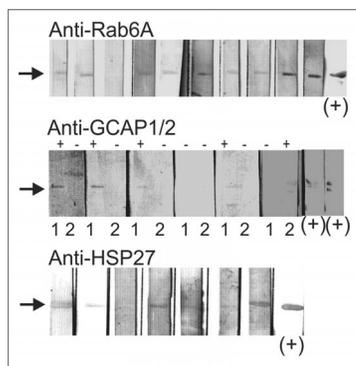


Figure 1. Representative blots showing immune reactivity with purified Rab6A, GCAP1/2, and HSP27 proteins. Individual blots containing purified proteins were incubated with patient serum diluted 1:30 or specific antibodies, marked here as “(+)” producing positive reaction. Arrows point at positive bands. In the case of GCAP proteins, patient’s serum was incubated with a blot containing GCAP1 and GCAP2; it reacted only with one protein (marked as “+”).

identified reactive 23-kDa molecular weight protein antigens investigated in this study are shown in [Supplementary Figure S1](#).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA) using the Fisher’s exact test, χ^2 test, and 2-tailed Mann-Whitney U test. Differences with a P value <0.05 were considered significant.

Results

Identification of Novel Antigens

All identified proteins of the apparent 23-kDa molecular weight migrated in the SDS-PAGE gel similarly to recoverin and could be taken as recoverin without following confirmation with a purified protein or subsequent sequencing. We identified 173 patients with anti-23-kDa AAbs, including 68 (39%) individuals having anti-recoverin AAbs. However, the remaining 105 sera had not reacted with purified recoverin. Hence, our goal was to identify the reactive 23-kDa retinal protein(s) for those patients’ sera.

To aid the identification of these 23-kDa antigens, we separated the lanes of an SDS-PAGE gel of human retinal proteins into 30 equal sections, produced in-gel trypsin digestions, and analyzed peptides by LC-MS/MS. This resulted in the identification of 1033 human retinal proteins with a protein

false discovery rate of less than 2%. The results of this analysis are in [Supplementary Figure S1](#), which provides the list of 23-kDa identified proteins and the numbers of MS/MS spectra assigned to each protein. Since the numbers of assigned MS/MS spectra are related to the proteins abundance, the results can be used to assign the gel slices containing the highest abundance of each protein.¹⁸ The analysis of the 23-kDa region of SDS-PAGE gel revealed potential autoantigen candidates, such as GCAP1, GCAP2, HSP27, a series of Ras-related proteins, Rab GTPases, glutathione S-transferase P, ATP synthase O subunit, and others ([Supplementary Figure S1](#)). We selected 4 proteins (based on an availability of purified proteins for experiments) for further examination with the recoverin-negative sera to identify possible autoantigens in CAR/AR, namely HSP27, Rab6A, and GCAP1/2. Representative immunoblots with these purified proteins and incubated with seropositive patient sera are shown in [Figure 1](#). Results showed that of a pool of 105 sera, 53 (50%) sera reacted with Rab6A, 45 (43%) with HSP27, 6 (6%) with GCAP1, and 1 (1%) with GCAP2. Two proteins, such as recoverin and GCAPs, are photoreceptor-specific proteins and members of the Ca^{2+} -binding EF-hand protein superfamily.¹⁹

To further characterize these serum AAbs, we performed the confocal immunostaining studies of human retinas with representative only monospecific human sera, containing AAbs against 23-kDa proteins. The monospecific sera within the group (recoverin, HSP27, Rab6A, and GCAP1) showed a similar pattern of staining ($n = 4$).

As is presented in [Figures 2A](#) and [2B](#), anti-recoverin AAbs showed typical immunostaining of the whole photoreceptors cells (red color) and some bipolar cells. Double labeling studies showed overlapped staining of photoreceptor cells (yellow color).

The other anti-23-kDa sera showed a different pattern of staining, which further confirmed that those AAbs did not recognize recoverin in the tissue but some other antigen(s). Immunolabeling with anti-GCAP1-specific antibodies (red color) or patient’s serum (green color) showed stained rod and cone outer segments, synaptic terminals, and some cone somata ([Figs. 2C, 2D](#)). The colocalization experiments with a patient serum and anti-GCAP1 revealed a positive signal in cone photoreceptor cells (yellow color). Anti-GCAP2 serum was not used because it was represented by only 1 patient.

Antibodies against Rab6A (red color) showed a strong and uniform immunolabeling in human retina

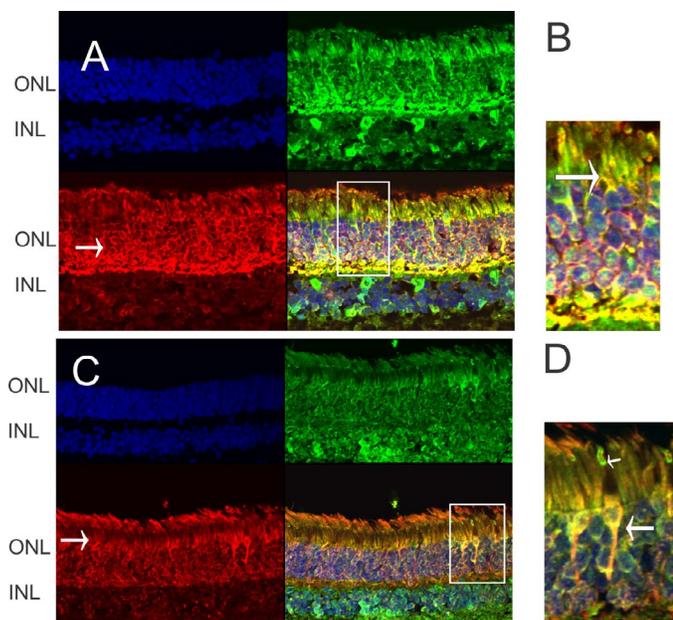


Figure 2. Representative double immunofluorescent labeling images of outer human retina incubated with monospecific serum against Recoverin (A, B) and GCAP1 (C, D). (A–D) Blue color represents DAPI-stained nuclei, green color represents Alexa Fluor 488-conjugated anti-human antibodies staining of photoreceptor cell layer recognized by the CAR patient AAbs; red color represents Alexa Fluor 594-conjugated antibodies staining of the photoreceptor cell layer by specific antibodies; the fourth image represents overlapping images—yellow color indicates common labeling. (B–D) Magnified overlapped images. An arrow in (A) points at photoreceptor cells and in (B) at outer segments; an arrow on (C) points at outer segments of photoreceptors and in (D) shows labeling of the cone cell, the green arrow shows tips of photoreceptors that were labeled by the patient serum (green color). Boxed areas indicated the enlarged area in (B) and (D). ONL, outer nuclear layer; INL, inner nuclear layer.

localized to the inner plexiform layer and cytoplasm of the ganglion cells (Figs. 3A, 3B). Also, punctate staining of the inner limiting membrane was present. Photoreceptor cells were not intensely labeled, although some immunofluorescent staining was observed in the inner segments (not shown). Colocalization studies showed that patient serum (green color) immunolabeled the same cellular structures of ganglion cells as did anti-Rab6A (yellow color) in human retina, confirming that AAbs recognize the same antigenic protein.

Autoantibodies specific to HSP27 also immunolabeled the ganglion cell layer and nerve fiber layer in a human retina (red color). The representative confocal image of double stained retinas (Figs. 3C, 3D) using specific anti-HSP27 antibody and a human patient

serum both produced a strong immunofluorescence in ganglion cells and nerve fibers, indicating similarity in immunolabeling of the same antigen (yellow color) in the retina.

Altogether, our findings revealed a discovery of the 4 novel CAR and AR autoantibody targets of similar molecular mass proteins, which we identified as a Rab6A, HSP27, GCAP1, and GCAP2. Sera specific for the above proteins showed a different immunostaining pattern relevant to their cellular location.

Frequency of Anti-Recoverin AAbs

In total, 62 of 173 (36%) patients had been diagnosed with various kinds of cancers, including 3 bladder, 10 breast, 6 colon, 10 gynecologic, 13 lung, 2 renal, 3 prostate, 5 melanomas, and other cancers. In 14 seropositive patients with anti-23-kDa AAbs, the detection of serum AAbs preceded a diagnosis of cancer. Of 62 patients with cancer, 34 had anti-recoverin AAbs (55%), 19/62 (31%) had anti-Rab6A AAbs, and 8/62 (13%) had anti-HSP27 AAbs. Patients with anti-GCAP1/2 AAbs did not have cancer at the time of testing. The statistical analysis (Fig. 4) shows that patients with anti-recoverin AAbs had a significantly higher likelihood of having cancer than those with other anti-23-kDa AAbs ($P = 0.0002$, Fisher's exact test).

Among our 173 patient cohort, 68 individuals (39%) were identified to be seropositive for anti-recoverin AAbs. Only 34 those 68 (50%) patients were reported to be diagnosed with cancer at the time of testing (Table 1). Patients with anti-recoverin AAbs and symptoms of CAR were older than the seropositive patients who had not been diagnosed with cancer by an average of 11 years. Seropositivity to recoverin was related to a rapid sudden onset of visual symptoms, and a progressive course. Table 2 compares the retinal phenotypes associated with anti-23-kDa AAbs, divided into 4 groups according to their antibody specificity and provides the frequencies of symptoms. Some patients had difficulty seeing in the dark (nyctalopia) and photopsia, and also peripheral vision loss, which may reflect the rod dysfunction more so than cone defects. One third of patients presented with reduced visual acuity from 20/70 to hand motion vision (HM), defects in visual fields, and severely impaired retinal function to unresponsiveness as determined by electroretinography (ERG), indicating loss of rod and/or cone function. Attenuated vessels, retinal atrophy, and vitritis were found in recoverin-seropositive patients.

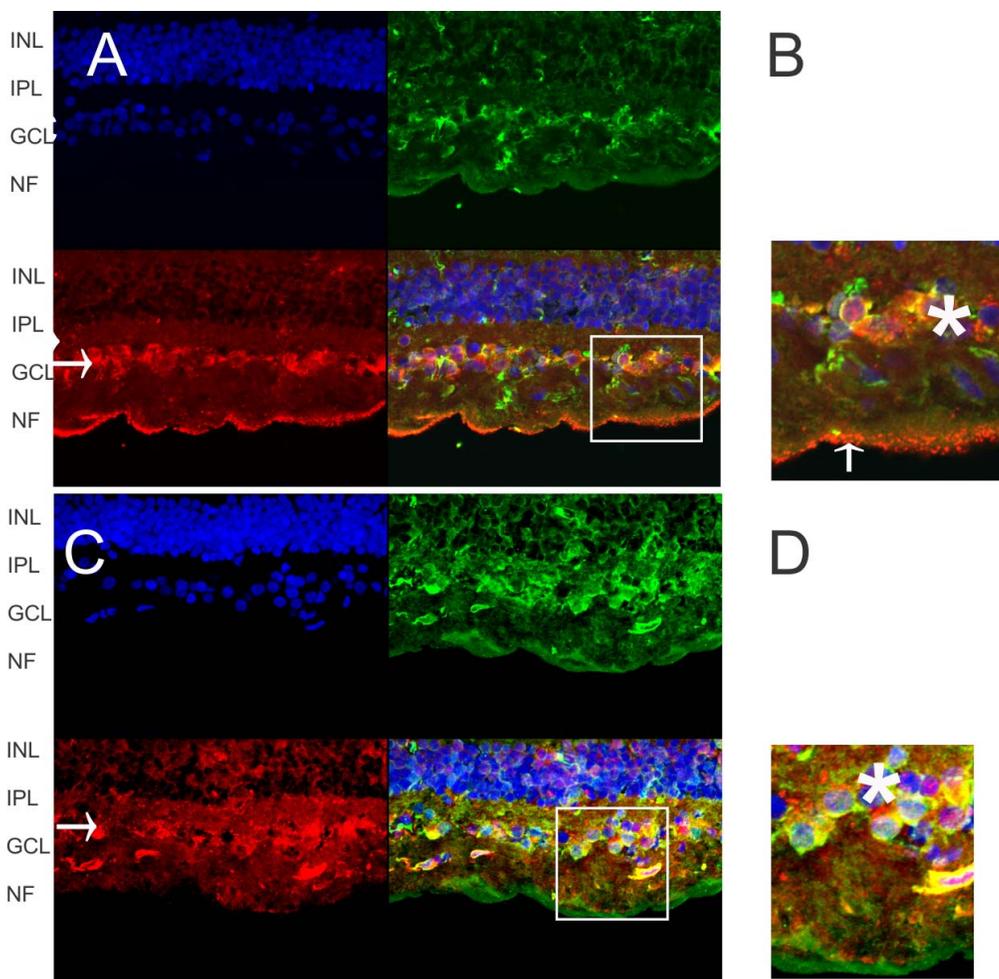


Figure 3. Representative double immunofluorescent labeling images of inner human retina incubated with monospecific serum against Rab6A (A, B) and HSP27 (C, D). (A–D) Blue color represents DAPI stained nuclei, green color represents Alexa Fluor 488-conjugated anti-human antibodies staining with the CAR patient AAbs; red color represents Alexa Fluor 594-conjugated antibodies staining with specific antibodies; the fourth image represents overlapping images—yellow color indicates common labeling. An arrow in (A) points at the ganglion cells and in (B) at the punctuated staining of the nerve fiber layer; a star in (B) shows intense staining of the ganglion cells stroma; an arrow in (C) points at the ganglion cells and in (D) shows intense staining of the nerve fiber layer, the star shows the ganglion cell body labeled yellow; Boxed areas indicated the enlarged area in (B) and (D). (B, C) magnified overlapped images. IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer.

Frequency of Anti-GCAP1 AAbs

The occurrence of anti-GCAP AAbs was rare. Autoantibodies binding to human GCAP1 were found only in 6 of 173 patients (3%) and 1 patient showed a reaction with GCAP2 on Western blots. The average age of anti-GCAP seropositive patients was 57 years, with an equal number of men and women. Of the GCAP1-seropositive patients, 67% reported loss of visual acuity and central vision loss, and 50% presented with maculopathy and cone dysfunction confirmed by ERG. Loss of vision was progressive. No association with cancer was reported; however, the small number

of patients with such AAbs prevents us from drawing definitive conclusions.

Frequency of Anti-Rab6A AAbs

Of the patients, 31% had AAbs against a newly identified target antigen Rab6A. Over 35% of those patients presented with cancer, including lung (4 patients), colon (3), breast (2), renal (2), lymphomas (2), and others. The average latency time between cancer, ocular symptom manifestation, and detection of circulating AAbs was 2.7 years, with 1 exception of a patient whose serum antibody preceded diagnosis of lung cancer by 3 years. The patients complained

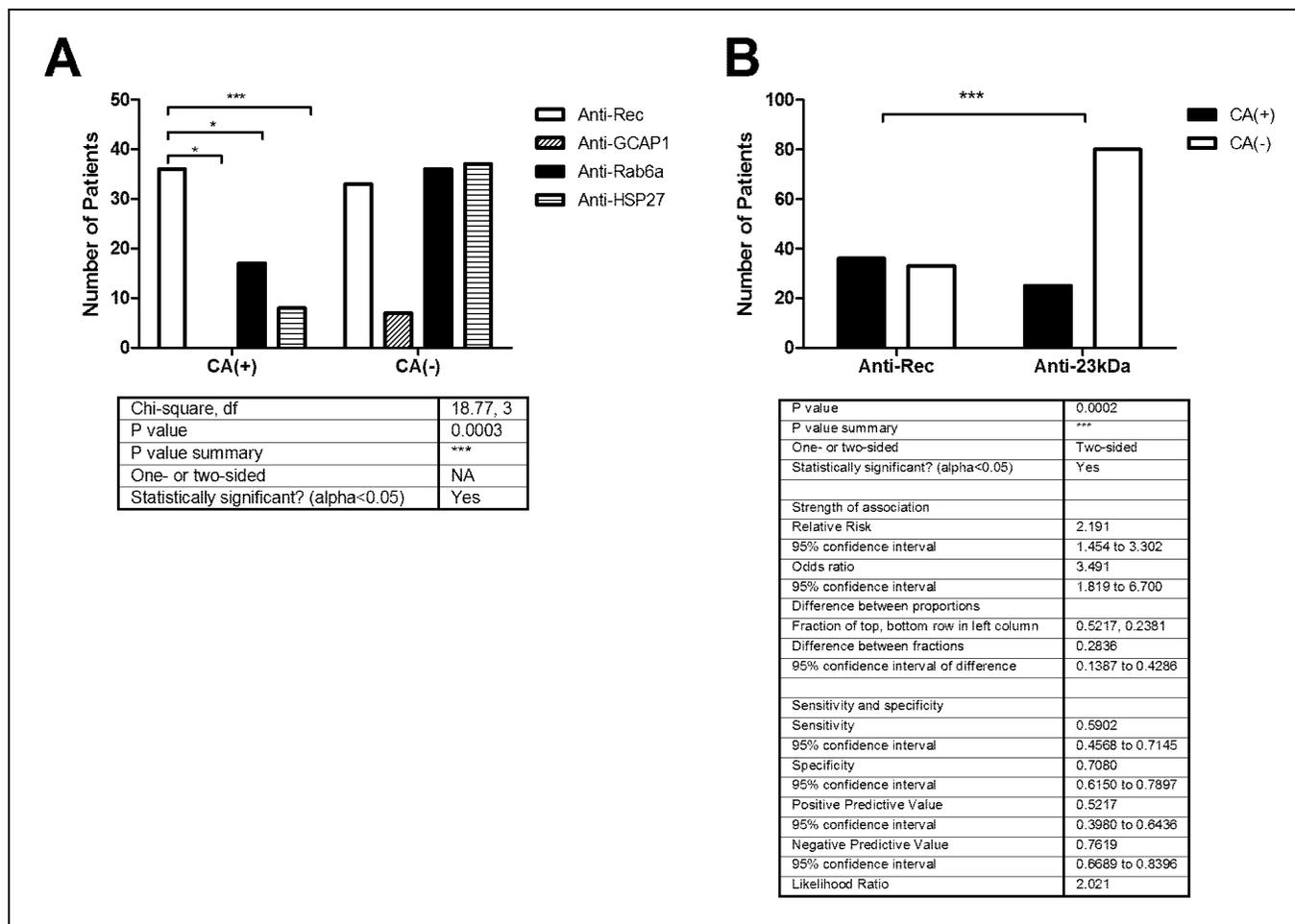


Figure 4. Statistical analysis of seropositive patients with and without cancer having anti-recoverin and other anti-23-kDa AAbs. (A) Four groups of seropositive patients with and without cancer were analyzed by χ^2 analysis ($***P = 0.0003$, χ^2 test) and *t* Fisher’s exact test (significance denoted by *asterisks*). (B) Patients with anti-recoverin antibodies had higher likelihood of having cancer than with other anti-23-kDa AAbs ($***P = 0.0002$, Fisher’s exact test). CA(+), patients with cancer; CA(–), patients without cancer.

about progressive vision loss with defects in visual acuity ranging from 20/80 to HM. Visual field defect (34%), central and peripheral loss (11%), and loss of rod and cone function (23%) was documented. Retinal dystrophy and outer retinal thinning was reported in 43% patients.

Frequency of Anti-HSP27 AAbs

Autoantibodies against HSP27 have been reported previously in patients with glaucoma,²⁰ and animal experiments revealed their pathogenic potential.²¹ We identified 45 (26%) sera that did not react with recoverin but bound strongly to human HSP27 on the blot (Fig. 1). Seropositive patients presented with slowly progressive central vision loss (20%), photophobia, photopsia, and nyctalopia, constriction of visual fields (central scotoma, enlarged blind spot) along with

generalized loss of rod and cone function as confirmed by the ERG (36%). Optic nerve defect was not a predominant feature in this group, although 3 patients had been diagnosed with low tension glaucoma. Some patients presented with intraocular inflammation (18%). Associated infectious and autoimmune diseases (lupus) were reported in 8 (18%) patients. The majority of persons (37/45, 82%) in this group were without cancer at the time of testing.

Retinal Phenotypes

Table 2 summarizes the clinical phenotypes of the 173 patients with anti-23-kDa protein AAbs based on seropositivity, symptoms, and findings using the following criteria: visual manifestation, clinical findings, ERGs, and presence of cancer. The clinical data were obtained from the summary information submit-

Table 2. Retinal Phenotypes Based on Seropositivity with Recoverin, Rab6A, HSP27, and GCAP1

Features	Anti-Recoverin	Anti-Rab6	Anti-HSP27	Anti-GCAP1
Average age	60+	60	58	57
F:M ratio	2:1	2:1	3:1	1:1
Onset	Sudden, unexplained (40%)	Sudden, unexplained (21%)	Subacute, progressive (36%)	Subacute, chronic (17%)
Ocular symmetry	Symmetric (97%)	Often symmetric (89%)	Mostly symmetric	Symmetric
Symptoms	Photopsia, nyctalopia, photophobia (40%)	Photopsia, nyctalopia (22%)	Photopsia, nyctalopia (29%)	Photophobia (28%)
Presentation	Severe central and peripheral vision loss; ring scotoma (51%)	Central and peripheral vision loss (54%)	Constriction of visual fields, central scotoma, blind spot (61%)	Maculopathy, loss of visual acuity and color vision (50%)
Course	Rapidly progressive severe cone and rods loss (38%)	Mildly progressive over wks/mos (25%)	Slowly progressive over years (36%)	Progressive loss (17%)
ffERG	Generalized reduced dysfunction of rods and cones to unresponsiveness (40%)	Reduction of rod and cone function (21%)	Generalized loss rod/cone function (39%)	Cone dysfunction (29%)
Frequency of cancer with seropositivity	50%	Over 32%	18%	0%
Cancer latency time to retinopathy onset	2.8 y Often precedes diagnosis of cancer	2.7 y Often coincided with diagnosis of cancer	5 y Years after diagnosis of cancer	ND ND
Typical cancers	Lung, breast, gynecological	lung, colon, breast	Variety of cancers	None reported

ND, not determined.

ted with the specimen for antibody testing. Seropositivity to Rab6A, HSP27, and GCAP1 was compared with anti-recoverin phenotypes. The important difference compared to the prior findings is the incidence of cancer in patients with anti-recoverin AAbs. Until now, we and others reported almost 100% association of anti-recoverin AAbs with cancer. However, the current study identified only 50% recoverin-seropositive patients with cancer at the time of testing.

Discussion

Our studies showed, for the first time to our knowledge, new autoantigens in CAR and AR. Among the cohort of 173 patients seropositive for

23-kDa protein, only 68 had reacted with recoverin and the remaining 105 had 4 AAbs targeting novel antigens of similar molecular mass, which we identified as a Rab6A, HSP27, GCAP1, and GCAP2. This discovery has an important implication for anti-recoverin antibody testing, and we always recommend a verification of antibody reactivity with purified recoverin protein before calling it recoverin-positive. Confocal images confirmed that these autoantigens were expressed in different types of cells in the retina, consistent with their respective cellular functions.

The question is whether the newly identified specific AAbs have a pathogenic role in retinal diseases. To understand how AAbs could affect vision, one must appreciate the function of the target

antigen. Historically, recoverin was considered as a sole autoantigen in CAR, however, as our findings showed only in 2% total patients studied ($n = 2955$) had anti-recoverin AAbs. Recoverin serves as an important calcium-binding protein in vision and regulates rhodopsin phosphorylation in a calcium-dependent manner by inhibiting rhodopsin kinase from phosphorylating rhodopsin.²² Among retina-specific proteins, recoverin was found in rods, cones, some bipolar cells, and a rare population of cells in the ganglion cell layer. The pathogenic role of recoverin was confirmed in in vitro and in vivo studies.^{9,10,23–25} We showed that an epitope for anti-recoverin antibodies located within residues 64 to 70 and was uniquely pathogenic, causing photoreceptor degeneration upon immunization of Lewis rats with this peptide.²⁶ Moreover, recoverin was found in cells of resected tumors from CAR patients and has been speculated that aberrantly expressed recoverin may be involved in the GRK-dependent cellular regulation in cancer cells.^{26,27}

Retinal guanylyl cyclase (RetGC)-activating proteins (GCAPs) are calcium-sensitive proteins that have an essential physiologic role in photoreceptors by accelerating the recovery of rods and cones following excitation by light²⁸ and they can trigger congenital retinal diseases in humans.^{29–31} Several mutations in the *GCAP1* gene have been associated with autosomal dominant cone dystrophy in human patients. The loss of *GCAP1* alters the recovery kinetics in *GCAP1*^{-/-} mice but does not initiate a degenerative disorder.³² Therefore, the presence of the GCAP1 AAbs may more likely contribute to photoreceptor apoptosis rather than affecting normal photoreceptor function at the signaling level. Three of our patients seropositive for GCAP1 presented with cone dystrophy although their genetic status presently is unknown. So far, we identified only a single patient with anti-GCAP2 AAbs so we cannot presume their role in retinal disease.

Autoantibodies against Rab6A have not been reported before in disease. Rab proteins are small (21–25 kDa) monomeric GTPases/GTP-binding proteins, and form the largest branch of the Ras superfamily.^{33,34} There are at least 70 Rab genes in the human genome, and a number of Rab GTPases are conserved from yeast to humans.^{34–37} The different Rab GTPases are localized to the cytosolic surface of intracellular membranes, where they function as regulators of distinct steps in membrane traffic pathways.³⁸ Rab1 and Rab6 GTPases have been found in the outer retina and likely have an

important role in maintaining the structure and function of photoreceptors.³⁹ In photoreceptors, rhodopsin is believed to be transported via a Rab6 regulated pathway and, thus, defects in trafficking pathways may lead to retinal degeneration.⁴⁰ Our study also showed Rab6A to be present in the outer segment, and also in bipolar and the cytoplasm of the ganglion cells. Its role in autoimmunity has not been studied previously to our knowledge, but AAbs could potentially block those important cellular functions.

We have reported on AAbs specific for HSPs, including HSP60 that was a frequently recognized autoantigen by AAbs of patients with AR, irrespective of cancer status.⁴ In the current study, we present findings of AAbs against a small heat shock protein, HSP27, mostly in patients with AR and in 18% CAR patients diagnosed with different kinds of cancer. HSP27 is a protein that can act as a protective and harmful factor in pathologies, such as neurodegenerative diseases, myopathies, asthma, cataracts, and cancers.^{41,42} HSP27 has powerful neuroprotective effects, increasing the survival of cells subjected to cytotoxic stimuli.⁴² This is especially relevant to the study of the retina where cells die as a result of retinal disease and injury. The upregulation of this protein occurs in response to ischemia and oxidative stress, traumatic nerve injury, and elevated intraocular pressure and glaucoma.⁴³ We believe that AAbs act as a stress to photoreceptor cells, and multiple antibodies may promote antibody-mediated retinal degeneration by blocking their functions.

Several mechanisms have been proposed to explain the protective actions of HSP27 in cells, including its role as a molecular chaperone, a stabilizer of the cytoskeleton, and a regulator of apoptosis.^{44,45} However, the retinal expression of HSP27 coincides with induced neuronal injury in the rat glaucoma model.^{46,47} HSP27 was shown to be expressed in retinal ganglion cells (RGCs) and in retinal astrocytes and Müller cells,⁴⁶ which the current studies confirmed. Moreover, immunization of Lewis rat with HSP27 and HSP60 proteins induced ganglion cell degeneration and axonal loss 1 to 4 months later, mimicking human glaucoma.^{21,48} Also, AAbs against HSP27 were reported in sera of patients with glaucoma.^{49,50} Individuals with normal pressure glaucoma had a higher titer of AAbs to HSP proteins than did age-matched patients with high-pressure glaucoma or control subjects. The autoantibody pathogenic potential was shown in vitro when application of antibodies directly onto retinal tissue

or cultured retinal cells triggered cell death through an apoptotic mechanism.⁴⁹

The production of AABs may be triggered by the antigen accessibility to the immune system. In the case of heat shock proteins, a tumor can be a source of antigens because HSPs are highly expressed in many malignant human tumors, including tumors of the genital tract.^{51,52} HSP27 was shown to be associated strongly with the process of carcinogenesis and was found in high frequencies in patients with breast cancers and gynecologic cancers, including ovarian (50%), endometrial (38%), cervical and uterine (30%), vaginal and vulvar (60%), and other (23%) cancers.⁵³ It has been suggested that HSPs bind to tumor antigens and the exposure to the immune system of such complexes on the tumor cell surface or their release from disrupted cells may result in induction of an immune response to HSPs.⁵³ Also, a high incidence of anti-HSP27 serum AABs was reported in rheumatic diseases, such as Behçet's disease (57%), rheumatoid arthritis (72%), Sjögren's syndrome (36%), and systemic lupus erythematosus (24%), but none in healthy controls (0/91).⁵⁴ Moreover, serum anti-HSP27 AABs were associated with severe and progressive coronary artery disease compared to patients without the disease or control subjects.⁵⁵

In conclusion, testing for AABs against retinal antigens provides important information for molecular diagnosis. The identification of over 1000 human retinal proteins classified by their migration by SDS-PAGE will help support identification of new retinal autoantigens following detection by Western blotting. The relevance of newly identified autoantigens (Rab6A, GCAP1, and HSP27) as biomarkers to diagnose, monitor disease activity/severity, and predict response to therapy is limited but they are promising candidates. These AABs are associated with distinctive clinical subsets with different prognostic features. The mechanism of anti-Rab6A, anti-GCAP1, and anti-HSP27 autoantibody formation in patients with and without cancer is not fully understood, although immunity to recoverin and HSP27 has been shown to be pathogenic in animals. The fact that these AABs are associated with symptoms of CAR and AR suggests their pathogenic function alone or in combination with other pathogenic anti-retinal AABs. Circulating AABs consistently precede the clinical onset of the autoimmune diseases by years, therefore, the benefits of anti-retinal AABs testing include aiding with diagnosis of unique retinal presentation, in monitoring and prognosis of vision loss, treatment options, and also

helping with early diagnosis of cancer. Whether AABs are causative by reducing/extinguishing cellular functions (e.g., protective function of HSP27), leading to cell death, or are generated later as an effect of retinal degeneration, they also may enhance progression of undergoing retinal disease. An important observation in this large group of CAR and AR patients is that the association of anti-recoverin AABs with the presence or future development of cancer was substantially lower than in prior reports.

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