BAX and BCL-2 polymorphisms, as predictors of proliferative vitreoretinopathy development in patients suffering retinal detachment: the Retina 4 project

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ABSTRACT.
Purpose: **To compare the distribution of BCL-2 -938C>A (rs2279115) and BAX -248G>A (rs4645878) genotypes among European subjects undergoing rhegmatogenous retinal detachment (RRD) surgery in relation to the further development of proliferative vitreoretinopathy (PVR).**
Methods: **A case–control gene association study, as a part of Retina 4 project, was designed. rs2279115 and rs4645878 polymorphisms were analysed in 555 samples from patients with RRD (134 with PVR secondary to surgery). Proportions of genotypes and AA homozygous groups of BCL-2 and BAX polymorphisms between subsamples were analysed in two phases. Genotypic and allelic frequencies were compared in global sample and in subsamples.**
Results: **BAX: Differences were observed in the genotype frequencies and in AA carriers between controls and cases in the global series. The odds ratio (OR) of A carriers in the global sample was 1.7 (95% CI: 1.23–2.51). Proportions of genotypes in Spain + Portugal were significant different. The OR of A carriers from Spain and Portugal was 1.8 (95% CI: 1.11–2.95). BCL-2: No significant differences were observed in genotype frequencies. However, proportions of genotypes in Spain + Portugal were significant. A protective effect (OR: 0.6 95% CI: 0.43–0.96) was found in A carriers from Spain and Portugal.**
Conclusions: **Results suggest that A allele of rs4645878 could be a biomarker of high risk of developing PVR in patients undergoing RD surgery. The possible role of BCL-2 (inhibitor of necroptosis pathway) as a possible new target in PVR prophylaxis should be investigated.**

Key words: apoptosis – bax – Bcl-2 – necroptosis – proliferative vitreoretinopathy – retinal detachment

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Introduction

Proliferative vitreoretinopathy (PVR) is still the major cause of failure in retinal detachment (RD) surgery (Machemer et al. 1991), affecting 5–10% of RD and accounting for approximately 75% of all primary failures after RD surgery (Machemer et al. 1991; de la Rua et al. 2008).

PVR is a complex process, involving not only ischaemic tissue damage but also inflammation and proliferation of several types of intraretinal cells. Currently, it is considered a complex disease (Sanabria Ruiz-Colmenares et al. 2006; Rojas et al. 2010, 2013; Pastor-Idoate et al. 2013a,b), in which there is an interaction between environmental factors (clinical variables) and the genetic profile of each subject (Brennan 2002; Hinton et al. 2002). Despite the facts that the exact mechanisms responsible of PVR are not completely understood, it is widely accepted that inflammation plays a crucial role in its pathogenesis (Delyfer et al. 2011).

PVR is characterized not only by uncontrolled cell proliferation and migration into the retinal surface, the subretinal space and vitreous cavity but also by deep changes inside of the

retinal tissue with the disappearance of neurons and a reactive gliosis by Müller cells and astrocytes (Pastor et al. 2006). The RPE cell is thought to be one of the key cell types in this disease. And factors responsible for unwanted survival, migration and proliferation of RPE cells in this condition have not been clearly defined.

Over the recent years, some papers have highlighted that apoptosis and other cell death pathways, such as programmed necrosis, play an important role in the photoreceptor degeneration and subsequent visual loss (Arroyo et al. 2005; Trichonas et al. 2010; Lo et al. 2011; Murakami et al. 2011; Ricker et al. 2011a) and also in the development of PVR after RD (Charteris et al. 2007). It has been reported that levels of p53 (one important regulatory factor of apoptosis) expression could be a checkpoint in the development of RD and PVR. And how preventing decline in the level of p53 using inhibitors of mdm2 could be a promising approach as a prophylaxis in experimental RD and also in experimental PVR (Lei et al. 2012).

There are other mediators of apoptosis that are also involved in retinal cell death after retinal ischaemia, including endonucleases (Rosenbaum et al. 1997), caspases (Singh et al. 2001) and B-cell lymphoma 2 (Bcl-2) family (Kaneda et al. 1999; Hahn et al. 2003; Yang et al. 2004; Zhang et al. 2002), whose role in PVR development has not yet been completely studied.

The Bcl-2 family is divided into two classes of members that exert opposed effects on cell death. Anti-apoptotic members such as Bcl-2 and Bcl-x_L and pro-apoptotic members, such as Bax and Bak (Adams & Cory 2001). When Bax and/or Bak are activated, they trigger mitochondrial outer membrane permeabilization by a mechanism that has yet to be identified. This leads to the release of Cytochrome C and apoptotic regulatory proteins into the cytoplasm resulting in the activation of the executioner caspases. In contrast, Bcl-2 and/or Bcl-x_L block this process and thus inhibit programmed cell death. The pro- and anti-apoptotic members of Bcl-2 family can neutralize each other by heterodimerization or forming homodimers, but it remains unclear which complex of these serves as the functional moiety in regulating apoptosis (Knudson & Korsmeyer 1997).

Additionally, Bcl-2-related gene products have been shown to be critically involved in numerous central nervous system diseases and degeneration (Hetts 1998) and also in developmental and pathological retinal cell death processes (Mosinger Ogilvie et al. 1998).

After a RD, the outer retina layers can suffer from ischaemia. It has been reported that retinal ischaemia is one of the triggers to induce the expression of Bcl-2 proteins, but also is the responsible factor for the upregulation of other mediators such as p53 (Hinton et al. 2002) or tumour necrosis factor alpha (TNFA) (Campochiaro et al. 1996; El-Ghrably et al. 2001; Banerjee et al. 2007). And it is likely that Bcl-2 family could be also involved in RD-associated photoreceptor cell loss and may be in the development of PVR. This later idea has never been investigated. Bcl-2 and Bax not only are related with p53, as both are transcriptional targets for p53 protein, but also rs2279115 and rs4645878 in the *BCL-2* and *BAX* genes, respectively, are associated with a decrease in the apoptosis levels, being both involved in the control life or death of a cell, and in the cellular proliferative response.

Previous studies have highlighted the possible role of apoptosis in the PVR development (Charteris et al. 2007), and recent studies performed suggest that a deregulation in the apoptosis pathway could be one of the possible mechanisms in the pathogenesis of PVR (Pastor-Idoate et al. 2013a,b).

Thus, the purpose of this study has been to analyse the distribution of these 2 promoter polymorphisms (rs2279115 and rs4645878) in the *BCL-2* and *BAX* genes, respectively, in a sample of patients undergoing primary rhegmatogenous RD surgery, with and without postoperative PVR, recruited from several European clinical centres through the project named Retina 4.

Materials and Methods

Candidate gene association study

DNA samples from the Retina 4 project were analysed. The study was approved by the institutional research committee of each centre and followed the tenets of the Declaration of Helsinki. All patients gave a written informed consent before entering in the study.

Design and study population

The association studies were carried out among 555 patients from seven centres: 3 in Spain, 2 in Portugal, 1 in the United Kingdom (UK) and 1 in the Netherlands. The global sample was divided in subsamples, according to the country, for the analysis. This study was carried out in two phases. In the first one, subsamples from Spain and Portugal were analysed. After significant results were found in this first cohort, subsequent samples from the UK and the Netherlands were analysed (second phase). To compare if there were differences in the odds ratio with respect to geographical localization, Spain and Portugal were considered as southern countries and the UK and the Netherlands as northern countries. Genotypic and allelic frequencies were also compared between cases and controls in the global series.

Detailed explanation of the exclusion and inclusion criteria for classification of patients has been provided in previous publications (Pastor-Idoate et al. 2013a,b; Rojas et al. 2013). In brief, all participants were patients with a primary rhegmatogenous RD who underwent surgery (pars plana vitrectomy). Exclusion criteria were as follows: age under 16 years; traumatic, tractional, exudative or iatrogenic RD; RD secondary to macular hole or giant retinal tears (larger than 3 clock hours) and preoperative PVR grade higher than B. Those who did not develop clinical signs of PVR after 3 months of follow-up were included in the control group. Those who developed PVR grade C1 or higher, according to Machemer classification (Machemer et al. 1991), were included as cases.

Genotyping

BCL-2 -938C>A (rs2279115) and BAX -248G>A (rs4645878) polymorphisms were assessed at the Molecular Medicine Unit, at the University of Salamanca, (Salamanca, Spain) blinded to the clinical status of patients, by TaqMan 5′-exonuclease allelic discrimination assays (Applied Biosystems, Foster City, CA, USA) using a

Step-One Plus Real-time PCR system according to the manufacturer protocol (Applied Biosystems). Briefly, PCR was carried out with mixes of 15 ng of genomic DNA, 5 μl of TaqMan®SNP genotyping Mastermix (Applied Biosystems) and 0.25 μl of TaqMan® SNP Genotyping Assay (SNP ID C_3044428 for rs2279115 BCL-2 polymorphism and SNP ID C_27848291 for rs4645878 BAX polymorphism, Applied Biosystems) in a final volume of 10 μl. PCR conditions were 95°C for 10 min followed by 40 cycles at 95°C for 15 seconds, and 60°C for 1 min and finally 60°C for 1 min. For quality control purposes, each sample was processed by duplicate for each SNP.

Statistical analysis

Genotypes of the SNPs were analysed in the subsamples and in the global sample separately. Also, the characteristics of the patients were explored. The quality of data was evaluated in control subsamples by Hardy–Weinberg equilibrium using the chi-square test. Genotypic frequencies were estimated in each subsample for each SNP. The proportions of genotypes and the AA homozygous group of BCL-2 and AA homozygous group of the BAX polymorphisms between subsamples were analysed for each SNP. Also, the genotypic and allelic frequencies were compared between cases and controls in the global sample and in the subsamples for each SNP.

Association was assessed using the chi-Square and the Fisher's tests. The strength of association was measured using odds ratio (OR) and 95% confidence intervals (CIs).

Two inheritance models were considered in the BAX analysis: dominant model, in which the heterozygous (GA) and homozygous (AA) genotypes have the similar risk, as a single copy of A is sufficient to alter the risk. Hence, these two possible genotype G/A+A/A together in combination is compared to the homozygous G/G. And the additive model, in which the risk conferred by an allele is increased r-fold for heterozygotes (G/A) and 2r-fold for homozygous (A/A). In this model, each copy of A allele alters the risk in an additive form.

Dominant and overdominant models were considered in the BCL-2 analysis. In the dominant model, heterozygous (CA) and homozygous (AA) genotypes have similar risk, because a single copy of A is sufficient to alter the risk. Hence, these two possible genotypes C/A+A/A together in combination were compared to the homozygous C/C. In the overdominant model, heterozygous (CA) was compared to a pool of both allele homozygous (AA and CC). The C/A was compared with A/A+C/C).

The Akaike information criterion (AIC) was used to choose the inheritance model that best fitted the data. The statistical analyses were performed using SPSS 16.0 (IBM Inc, Armonk, NY, USA) for Macintosh and R software (Software Foundation's GNU project).

All the statistical analysis has been made by Itziar Fernández (Sct, PhD) from the statistical unit of IOBA, the Eye Institute of the University of Valladolid, Valladolid, Spain.

Results

Candidate gene association study

A total of 555 peripheral DNA blood samples including 134 cases and 421 controls were analysed, 203 from Spain (36.57%), 68 from Portugal (12.25%), 121 from the Netherlands (21.80%) and 163 from the UK (29.36%).

Regarding clinical information, some significant associations were observed as follows: control group was significantly older than cases ($p < 0.0001$) with a difference between median of 6 years (95% CI: 3.39–8.31). A significant association in patients with history of PVR in the fellow eye was also found in cases. The status of the lens was determined because aphakia has been related to a higher incidence of developing PVR after RD (Pastor 1998; Ricker et al. 2012) (Table 1). There were no significant associations with sex, race, affected eye or history of cataract surgery. There were no differences regarding the geographical localization.

There were no relevant failures for the genotyping process, with a global call rate of 96.21% for the BCL-2 and 97.13% for the BAX. Additionally, to ensure accuracy of allele-specific results, a randomized selection of PCR samples was assessed by an independent researcher unaware of the condition of the patient. All control subsamples verified the Hardy–Weinberg equilibrium.

Phase I

Genotypic distribution of rs2279115 and rs4645878 polymorphisms in Spain and Portugal.

1 rs2279115-BCL-2 polymorphism

The frequencies of the genotypes in each country for this polymorphism are shown in Table 2A. The comparison of proportions of genotypes between subsamples showed no significant differences ($p > 0.05$) between cases and controls in Spain and Portugal. Also, no significant differences in AA homozygous carriers between subsamples in controls (CI AA homozygous: Spain [34.9–46.5], Portugal [45.7–65.2]) and cases in groups (CI AA homozygous: Spain [24.0–41.5], Portugal [25.9–62.3]) were found.

Regarding geographical localization, a significant difference ($p < 0.05$) in the comparison of proportions of genotypes between cases and controls was found in southern countries. Also, a significant difference in AA homozygous carriers between cases and controls was found in southern countries. Control group (CI AA homozygous: Spain plus Portugal [39.7–49.7]) and the case group (CI AA homozygous: Spain plus Portugal [26.9–42.7]).

The OR of A carriers from Spain and Portugal together considering a dominant model (A/A, C/A and C/C) (AIC = 315.4 versus 317.3 of a codominant model) was 0.50 (95% CI: 0.29–0.86) (Table 3).

2 rs4645878-BAX polymorphism

The frequencies of the genotypes in each country for this SNP are shown in Table 2B. The comparison of proportions of genotypes between subsamples did not show significant differences ($p > 0.05$) between cases and controls neither in Spain nor in Portugal. Also, no significant differences in AA homozygous carriers between subsamples in the control group (CI AA homozygous: Spain [10.0–18.1]) and the case group (CI AA homozygous: Spain [15.4–30.8) were found. However, regarding geographical comparison, a significant difference ($p < 0.05$) in the proportions of genotypes between cases and controls was found in southern countries. Also, a significant difference in AA homozygous carriers between cases and controls was found in southern countries. Control group (CI AA homozygous: Spain plus Portugal [10.1–17.0]) and the case group (CI AA homozygous: Spain plus

Table 1. Clinical characteristics of the whole sample.

		Controls		Cases						
Characteristics		*n*	%	*n*	%	Total	% Total	p-Value	OR	CI 95% OR
Race	Unknown	27	4.86%	2	0.36%	29	5.23%	0.2368	0.6	0.314–1.336
	Caucasian	370	66.67%	120	21.62%	490	88.29%		2.5	0.740–5.998
	Hispano-American	6	1.08%	5	0.90%	11	1.98%		1.7	0.599–6.145
	Hindu	7	1.26%	4	0.72%	11	1.98%		0.2	0.012–4.031
	Arabic-North-African	6	1.08%	0	0%	6	1.08%		1.4	0.134–16.63
	Sub-Saharan	2	0.36%	1	0.18%	3	0.54%		2.0	0.331–12.13
	Asian	3	0.54%	2	0.36%	5	0.90%			
	Total	421	75.86%	134	24.14%	555	100%			
Sex	Unknown	20	3.60%	7	1.26%	27	4.87%	0.4866	1.1	0.760–1.777
	Male	258	46.49%	86	15.50%	344	61.98%			
	Female	143	25.77%	41	7.39%	184	33.15%			
	Total	421	75.86%	134	24.14%	555	100%			
Status of the Lens (Phakia)	Unknown	23	4.14%	7	1.26%	30	5.41%	0.2419	1.2	0.846–1.937
	Yes	267	48.11%	78	14.05%	345	62.16%			
	No	131	23.60%	49	8.83%	180	32.43%			
	Total	421	75.86%	134	24.14%	555	100%			
RD in fellow eye	Unknown	17	3.06%	5	0.90%	22	3.96%	0.9506	0.9	0.466–2.046
	Yes	32	5.77%	10	1.80%	42	7.57%			
	No	372	67.03%	119	21.44%	491	88.47%			
	Total	421	75.86%	134	24.14%	555	100%			
PVR in fellow eye	Yes	0	0%	5	0.90%	5	0.90%	0.0157	35	1.96–651.86
	No	4211	75.86%	129	23.24%	550	99.10%			
	Total	421	75.86%	134	24.14%	555	100%			
Geographical location	Southern	197	35.50%	74	13.33%	271	48.83%	0.0926	1.4	0.948–2.072
	Northern	224	40.36%	60	10.81%	284	51.17%			
	Total	421	75.86%	134	24.14%	555	100%			

OR = odds ratio, RD = retinal detachment, PVR = proliferative vitreoretinopathy.

Portugal [15.4–29.2]).

The OR of A carriers from Spain and Portugal together considering an additive model (A/A double risk than G/A) (AIC = 316.6 versus 316.7 of a dominant model) was 1.75 (95% CI: 1.09–2.83) (Table 3).

Phase II

Genotypic distribution of rs2279115 and rs4645878 polymorphisms in the UK and the Netherlands.

1 rs2279115-BCL-2 polymorphism

The frequencies of the genotypes in each country for this SNP are shown in Table 2A. The comparison of proportions of genotypes between subsamples did not show significant differences ($p > 0.05$) between cases and controls neither in the UK nor in the Netherlands. Also, no significant differences in AA homozygous carriers between subsamples in the control group (CI AA homozygous: UK [34.9–47.1], Netherlands [28.0–42.1]) and the case group (CI AA homozygous: UK [26.8–51.9], Netherlands [27.3–53.3]) were found. Regarding geographical comparison, no significant differences in the proportions of genotypes and in AA homozygous carriers analysis between cases and controls were found.

The OR of A carriers from the UK and the Netherlands together considering a dominant model (A/A, C/A and C/C) (AIC = 296.8 versus 298.4 of a codominant model) was 0.95 (95% CI: 0.53–1.68) (Table 3).

2 rs4645878-BAX polymorphism

The frequencies of the genotypes in each country for this SNP are shown in Table 2B. The comparison of proportions of genotypes between subsamples did not show significant differences ($p > 0.05$) between cases and controls in the UK and the Netherlands. Also, no significant differences in AA homozygous carriers between subsamples in the control group (CI AA homozygous: UK [8.5–16.8], Netherlands [9.6–20.2]) and the case group (CI AA homozygous: UK [9.6–29.9], Netherlands [12.9–35.6]) were found. Also, in the geographical comparison, no significant differences in the proportions of genotypes and in AA homozygous carriers analysis between cases and controls were found.

The OR of A carriers from UK and the Netherlands together considering an additive model (A/A double risk than G/A) (AIC = 293.4 versus 294.1 of a dominant model) was 1.66 (95% CI: 0.98–2.80) (Table 3).

3 rs2279115 and rs4645878 polymorphisms in the global sample

When all samples were grouped, (Table 4A), significant difference in the distribution of genotypes between the controls and the cases ($p < 0.05$) was found in BAX polymorphism analysis, but not in the BCL-2 polymorphism analysis. Also, homozygous carriers of the A variant were more frequent in PVR cases (CI: 16.3–26.4) than in controls (CI: 10.9–15.6) in the BAX analysis, but not in the BCL-2 analysis. The OR of the A variant in the global sample in the BAX analysis using an additive model (AIC = 608.8 versus 609.7 of a dominant model) was 1.72 (CI: 1.21–2.44) (Table 3). Whereas the OR of the A variant in the global sample in the BCL-2 using a dominant model (AIC = 614.1 versus 615.7 of a codominant model) was 0.69 (CI: 0.47–1.02) (Table 3).

Allelic frequencies comparison

1 rs2279115-BCL-2 polymorphism

Only significant differences in the BCL-2 analysis of the allelic frequencies were

Table 2. (A) Distribution of genotypes and allelic frequencies of BCL-2 in subsamples and in southern (Spain + Portugal) and northern (UK + Netherlands) countries. (B) Distribution of genotypes and allelic frequencies of BAX in subsamples and in southern (Spain + Portugal) and northern (UK + Netherlands) countries.

Countries	Genotype	Cases		Controls		p-Value Fisher′s test	Alleles	Cases		Controls		95% CI Alleles		p-Value Chi Square test	OR	CI OR 95%
												Cases	Controls			
Spain	A/A	9	15.2%	24	16.6%	0.1126*	AA	38	32.2%	117	40.6%	24.0–41.5	34.9–46.5	0.1305†	0.7	0.44–1.09
	C/A	20	33.9%	69	47.9%		CC	80	67.8%	171	59.4%	69.2–84.8	81.8–90.1			
	C/C	30	50.8%	51	35.4%											
Portugal	A/A	4	26.6%	18	33.9%	0.4376*	AA	13	43.3%	59	55.6%	25.9–62.3	45.7–65.2	0.3438†	0.6	0.26–1.37
	C/A	5	33.3%	23	43.4%		CC	17	56.7%	47	44.4%	37.6–74.0	34.8–54.2			
	C/C	6	40.0%	12	22.6%											
UK	A/A	7	22.5%	27	20.4%	0.6670*	AA	24	38.7%	108	40.9%	26.8–51.9	34.9–47.1	0.7943†	0.9	0.51–1.60
	C/A	10	32.3%	54	41.0%		CC	38	61.3%	156	59.1%	48.0–73.1	52.8–65.0			
	C/C	14	45.2%	51	38.6%											
Netherlands	A/A	6	20.7%	15	16.3%	0.7822*	AA	23	39.6%	64	34.7%	27.3–53.3	28.0–42.1	0.5689†	1.2	0.67–2.26
	C/A	11	38.0%	34	37.0%		CC	35	60.4%	120	65.3%	46.6–72.6	57.8–71.9			
	C/C	12	41.3%	43	46.7%											
Spain + Portugal	A/A	13	17.6%	42	21.3%	0.0409*	AA	51	34.4%	176	44.7%	26.9–42.7	39.7–49.7	0.0405†	0.6	0.43–0.96
	C/A	25	33.8%	92	46.7%		CC	97	65.6%	218	55.3%	57.2–73.0	50.2–60.2			
	C/C	36	48.6%	63	31.9%											
UK + Netherlands	A/A	13	21.6%	42	18.7%	0.7834*	AA	47	39.1%	172	38.4%	30.5–48.5	33.9–43.0	0.9232†	1.0	0.68–1.56
	C/A	21	35.0%	88	39.3%		CC	73	60.9%	276	61.6%	51.4–69.4	56.9–66.1			
	C/C	26	43.4%	94	41.2%											
(B)																
Spain	A/A	3	5.08%	5	3.47%	0.0659*	AA	26	22.0%	39	13.5%	15.4–30.8	10.0–18.1	0.0479†	1.8	1.04–3.13
	G/A	20	33.9%	29	20.1%		GG	92	78.0%	249	86.5%	69.2–84.8	81.8–90.1			
	G/G	36	61.0%	110	76.3%											
Portugal	A/A	1	6.67%	0	0.00%	0.2343*	AA	6	20.0%	13	12.2%	8.4–39.1	6.9–20.4	0.3622†	1.7	0.61–5.19
	G/A	4	26.6%	13	24.5%		GG	24	80.0%	93	87.8%	60.8–91.6	79.6–93.0			
	G/G	10	66.7%	40	75.5%											
UK	A/A	1	3.23%	2	1.52%	0.3459*	AA	11	17.7%	32	12.1%	9.6–29.9	8.5–16.8	0.2989†	1.5	0.73–3.30
	G/A	9	29.0%	28	21.2%		GG	51	82.2%	232	87.8%	70.0–90.4	83.1–91.4			
	G/G	21	67.7%	102	77.2%											
Netherlands	A/A	2	6.9%	2	2.1%	0.2411*	AA	13	22.4%	26	14.1%	12.9–35.6	9.6–20.2	0.1645†	1.7	0.83–3.69
	G/A	9	31.0%	22	23.9%		GG	45	77.6%	158	85.9%	64.4–87.0	79.8–90.4			
	G/G	18	62.0%	68	73.9%											
Spain + Portugal	A/A	4	5.41%	5	2.54%	0.0492*	AA	32	21.6%	52	13.2%	15.4–29.3	10.1–17.0	0.0212†	1.8	1.11–2.95
	G/A	24	32.4%	42	21.3%		GG	116	78.4%	342	86.8%	70.7–84.5	82.9–89.9			
	G/G	46	62.2%	150	76.2%											
UK + Netherlands	A/A	3	5.0%	4	1.79%	0.1414	AA	47	39.2%	172	38.4%	15.4–29.3	10.1–17.0	0.9232†	1.6	0.99–2.84
	G/A	18	30.0%	50	22.2%		GG	73	60.8%	276	61.6%	70.7–84.5	82.9–89.9			
	G/G	39	65.0%	170	75.9%											

OR = odds ratio, UK = United Kingdom.

* Fisher′s test. Ho. Independence between genotype case/control group. Significant differences were observed between cases and controls in the southern (Spain + Portugal)countries.

† AA homozygous carriers analysis between different countries revealed differences in southern (Spain + Portugal) countries.

Table 3. Models of inheritance in the global sample. Results of odds ratio using a dominant model for *BCL-2* and additive model for *BAX* in Spain plus Portugal and Netherlands plus United Kingdom (UK).

Global sample		*BCL-2*				*BAX*			
Model	Genotype	OR	95% CI OR	p-Value	AIC	OR	95% CI OR	p-Value	AIC
Co-dominant	C/C	1.00	–	0.1452	615.7	1.00	–	0.0124	610.8
	C/A	0.65	0.42–1.00			1.72	1.11–2.66		
	A/A	0.78	0.46–1.33			2.93	1.06–8.09		
Dominant	C/C	1.00	–	0.0655	614.1	1.00	–	0.0052	609.7
	C/A-A/A	0.69	0.47–1.02			1.83	1.20–2.77		
Recessive	C/C-C/A	1.00	–	0.8893	617.5	1.00	–	0.0812	614.5
	A/A	0.97	0.59–1.58			2.52	0.92–6.91		
Over-dominant	C/C-A/A	1.00	–	0.0817	614.5	1.00	–	0.0283	612.7
	C/A	0.70	0.47–1.05			1.63	1.06–2.51		
Additive	–	0.84	0.64–1.10	0.1951	6.15.9	1.72	1.21–2.44	0.0030	6.08.8
Spain + Portugal		1.00	–	0.0119	315.4	1.75	1.09–2.83	0.0229	316.6
		0.50	0.29–0.86						
Netherlands + UK		1.00	–	0.8489	296.8	1.66	0.98–2.80	0.0626	293.4
		0.95	0.53–1.68						

OR = odds ratio, AIC = Akaike information criterion.
The AIC is a measure of the relative goodness of fit of a statistical model. It can generally be used for the identification of an optimum model in a class of competing models.
Given a set of candidate models for the data, the preferred model is the one with the minimum AIC value.

found between cases and controls in the Spain plus Portugal group (Table 2A).

2 rs4645878-BAX polymorphism
Significant differences in the BAX analysis of the allelic frequencies were found between cases and controls in Spain as a subsample, in Spain plus Portugal in the geographical comparison (Table 2B) and in the global sample (Table 4).

Discussion

Inappropriate apoptosis is an important factor in many human pathologic conditions including neurodegenerative diseases, ischaemic damage, autoimmune disorders and many types of cancer (Hetts 1998; Elmore 2007). In addition, it has been reported that a deregulation of apoptosis during wound-healing process can lead to pathologic forms of healing such as excessive scarring and fibrosis (Elmore 2007).

Current studies have highlighted the involvement of extrinsic and intrinsic pathways of apoptosis in retinal cells after RD, and also, the existence of other death pathways, such as programmed necrosis (which are more inflammatory pathways) and is enhanced when apoptosis is inhibited (Lo et al. 2011; Murakami et al. 2011).

PVR, as a multifactorial disease (Sanabria Ruiz-Colmenares et al. 2006; Rojas et al. 2010, 2013; Pastor-Idoate et al. 2013a,b), shows many similarities to the wound-healing response in other tissues where inflammation plays an important role (Pastor et al. 2002; Ricker et al. 2011b). However, what exactly initiates the development of PVR still remains speculative.

Bax is a death-promoting protein shown to be a tumour suppressor that stimulates cellular apoptosis *in vivo* (Zhang et al. 2000; Bellosillo et al. 2002). The *BAX* gene is located on chromosome 19 and consists of six exons and a promoter region with four p53-binding sites (Saxena et al. 2002). Sequence variations in the promoter region and coding sequence can abolish its pro-apoptotic function. The G(-248) A BAX promoter polymorphism (rs4645878) is associated with decreased cell Bax expression (Saxena et al. 2002; Starczynski et al. 2005).

Our results show that Spanish carriers of the homozygous AA genotype at position (-248) in the *BAX* gene (which is associated with a decrease in apoptotic function) have a 1.8-fold increased risk of PVR after RD than those carrying the GG genotype. This observation was confirmed also in the analysis of the southern countries (Spain plus Portugal). Results also showed a significant association between PVR risk and G(-248)A BAX promoter polymorphism when the global sample was analysed. The OR in the global sample was 1.72 (CI: 1.21–2.44).

Pro-cell-death bcl-2 proteins such as Bax are required for mitochondrial dysfunction in response to apoptotic and necroptotic agonists (Irrinki et al. 2011). It has been reported that reduced expression of *BAX* or even its deficiency, in *BAK* and *BAX* in knockout models, protects against apoptosis (Janssen et al. 2009). However, it does not compromise necrosis induction or the activation of other non-apoptotic pathways such as endoplasmic reticulum stress-induced cell death or autophagy increased as well in stress oxidative and hypoxia situations (Janssen et al. 2009).

The *BCL-2* gene consists of three exons and two promoters. The SNP rs2279115 is located in the inhibitory P2 promoter of *BCL-2* gene (Park et al. 2004). The second promoter, P2, is located 1400-bp upstream of the translation initiation site and decreases the activity of the P1 promoter, thus functioning as a negative regulatory element (Nuckel et al. 2007). The -938C allele in comparison with the A allele displayed significantly increased inhibition of BCL-2 promoter activity and binding of nuclear proteins (Nuckel et al. 2007). Thus, the BCL-2-938 AA genotype is associated with an increase in Bcl-2 expression.

This SNP (rs2279115) has been associated with an improved survival rate in some type of tumours such as breast or renal cancer (Faderl et al. 2002; Masago et al. 2013). In addition, it has

Table 4. Distribution of rs2279115 and rs4645878 in the whole sample. Fisher's test. Allelic frequencies comparison and analysis of AA homozygous carriers between case and control group in *BCL-2* and *BAX*. Chi square test.

Global sample	Genotype	Cases		Controls		p-Value Fisher's test	Alleles	Cases		Controls		95% CI Alleles Cases	95% CI Alleles Controls	p-Value Chi Square test	OR	CI OR 95%
BCL-2	A/A	26	19.4%	84	19.9%	0.1455*	AA	98	36.6%	348	41.3%	30.8–42.6	37.9–44.7	0.2064†	0.8	0.61–1.08
	G/A	46	34.3%	180	42.7%		GG	170	63.4%	494	58.7%	57.3–69.1	55.2–62.0			
	G/G	62	37.7%	157	37.3%											
BAX	A/A	7	5.2%	9	2.1%	0.0084*	AA	56	20.9%	110	13.0%	16.2–26.3	10.9–15.5	0.0029†	1.7	1.23–2.51
	G/A	42	31.3%	92	21.8%		GG	212	79.1%	732	86.9%	73.6–83.7	84.4–89.1			
	G/G	85	63.4%	320	76.0%											

OR = odds ratio.

* Fisher's test. Ho. Independence between genotype case/control group. Significant difference was observed between cases and controls in BAX in global sample.

† AA homozygous carriers analysis revealed significant difference in BAX in global sample analysis.

been reported that Bcl-2 overexpression significantly improved neuron survival in cerebral ischaemia models (Zhao et al. 2003). And also, previous studies in retinal degeneration models showed that in pathologic photoreceptor apoptosis, the bcl-2 overexpression mediates a transient protection (Adams & Cory 2001). Moreover, bcl-2 overexpression has been associated to a substantial reduction in the elimination of ganglion cells in cell death induced by optic nerve axotomy (Zhao et al. 2003).

Our results showed no significant differences in the analysis of subsamples and in the global one. However, the results showed a protective effect in the analysis of homozygous AA carriers in the southern countries. The OR of AA carriers (which is associated with an increase in Bcl-2 expression) from Spain and Portugal was 0.50 (95% CI: 0.29–0.86).

Although an overexpression of bcl-2 is also related with a decreased apoptotic response, unlikebax, the overexpression in bcl-2 is able to induce an inhibitory effect in the programmed necrosis cell death and other non-apoptotic pathways such as autophagy. In fact, some studies have suggested the use of inhibitors of bcl-2 as a new target in cancer therapy (Kang & Reynolds 2009). In addition, it has been reported that an increase of bcl-2 attenuates the TNFA-induced necroptosis pathway (Irrinki et al. 2011).

These two promoter SNPs in the *BAX* and *BCL-2* genes are particularly interesting because they are located within 100 bases from the TP53-binding element in the BAX promoter region and Tp53 responsive element in the BCL-2 promoter region, respectively. Thus, these SNPs may affect the interaction between the Tp53 protein and the Tp53-regulated sequences in the promoters (Chen et al. 2007). An association between the Tp53 Arg72-Pro polymorphism and the PVR has been already reported by our group (Pastor-Idoate et al. 2013a,b).

Besides, it has been reported that in many cells, as RPE cells, the activation of different anti-apoptotic factors such as Bcl-2 family induces a cell proliferation and transdifferentiation (Yang et al. 2005); however, the role of anti-apoptotic factors such as MDM2 (Pastor-Idoate et al. 2013a,b) or Bcl-2 family in RPE transdifferentiation in PVR after RD has not yet been completely studied.

Thus, deregulation in the apoptosis during wound healing and the activation of other cell death pathways could lead to pathologic forms of healing, such as excessive scarring and fibrosis. It can be speculate that the reduction in the levels of apoptosis in retinal cells may activate other cell death pathways, such as programmed necrosis, which would increase the intra-ocular inflammation after RD, thus generating a cascade of tissue responses that generate and amplify the hostile microenvironment in which activated RPE can transdifferentiate.

This study had some limitations. One important issue in any association study is the sample size (Dempfle et al. 2008). Unlike other association studies, our sample could be too small and the power sample could be not enough to draw absolute conclusions. Nevertheless, the results found in this study are strongly consistent with the previous reported findings by our group. And the sample collection to achieve greater power would be an extremely challenging for a low prevalence condition such as PVR.

It is important to point out that functional SNPs are considered of interest because they allow a better understanding of the molecular basis of different pathologies. They also could help to identify new targets in the development of new therapeutic strategies. In this case–control study, we have identified the rs4645878 SNP within the *BAX* gene that shows an association with PVR in Spain, in Spain plus Portugal and in global sample analysis. Although we have carried out the study in two phases, these findings must be interpreted with caution until these results are confirmed with further replication studies to confirm its association, because one of the major pitfalls of genetic association studies are the false positives (Crawford & Nickerson 2005; Dempfle et al. 2008).

Regarding clinical information, recent studies have highlighted that there is no clear association between retinal detachment and gender (Ho et al. 2009; Day et al. 2010; Hajari et al. 2014). Our results showed a predominance of males in the ratio of retinal detachments (approximately 1.8:1). However, we have considered

that this fact should not affect to the genetic analysis, as there were no differences in the percentage between cases and controls regarding distribution between males and females (75% and 77%, respectively), and there was no significant association between gender and group ($p = 0.4866$).

In the present data, we found no association between rs2279115 BCL-2 polymorphism (which is associated with an increase in anti-apoptotic Bcl-2 expression) and PVR in the whole sample, or when results were subdivided into subsamples. However, we found that BCL-2 has a protective effect in the southern countries probably because it has the ability, contrary to BAX, to inhibit both cell death signals (apoptosis and necrosis).

In summary, this study highlights the role of genetic factors as a useful tool in the identification of high-risk patients to suffer PVR and indicates that reduced apoptosis could be implicated as a significant risk factor for PVR after RD. Also, it highlights the role of the SNP rs4645878 as a possible marker of PVR risk or the role of SNP rs2279115 as a possible new target in the PVR prophylaxis. But further studies are necessary to analyse the role of these SNPs in PVR development.

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