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A Genetic Case-Control Study Confirms the Implication of *SMAD7* and *TNF Locus* in the Development of Proliferative Vitreoretinopathy

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Purpose. Proliferative vitreoretinopathy (PVR) is still the major cause of failure of retinal detachment (RD) surgery and although the risk for developing this complication is associated with some clinical characteristics, the correlation is far from absolute, raising the possibility of genetic susceptibility. The objective of this study was to analyze the genetic contribution to PVR in patients undergoing RD surgery, the Retina 4 Project.

Methods. A candidate gene association study was conducted in 2006 in a Spanish population of 450 patients suffering from primary rhegmatogenous RD. Replication was carried out in a larger population undergoing RD surgery at several European centers among 546 new patients. Single nucleotide polymorphism (SNP) of 30 genes known to be involved with inflammation were analyzed. For replication stage, those genes previously detected as significantly associated with PVR were genotyped. Distribution of allelic and haplotypic frequencies in case and control group were analyzed. Single and haplotypic analysis were assessed. The Rosenberg two-stage method was used to correct for single and multiple analyses.

Results. After correction for multiple comparisons, four genes were significantly associated with PVR: *SMAD7* ($P = 0.004$), *PIK3CG* ($P = 0.009$), *TNF locus* ($P = 0.0005$), and *TNFR2* ($P = 0.019$) In the European sample, replication was observed in *SMAD7* ($P = 0.047$) and the *TNF locus* ($P = 0.044$).

Conclusions. These results confirm the genetic contribution to PVR and the implication of *SMAD7* and *TNF locus* in the development of PVR. This finding may have implications for understanding the mechanisms of PVR and could provide a potential new therapeutic target for PVR prophylaxis. (*Invest Ophthalmol Vis Sci.* 2013;54:1665–1678) DOI:10.1167/iovs.12-10931

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Proliferative vitreoretinopathy (PVR) is still in this era the major cause of failure of retinal detachment (RD) surgery with an incidence of 5% to 10%.[1] It is believed to represent an abnormal wound healing process induced by a retinal break, which allows egress of RPE cells into the vitreous cavity and there is strong evidence that inflammation plays an important role.[2,3] Following RD, the blood–ocular barrier breaks down, possibly due to disruption of the photoreceptor-RPE cell interface, and inflammatory cells are recruited together and increase in inflammatory mediators.[4–6] Growth factors and cytokines present in the vitreous cavity may be responsible for cell migration, metaplasia, and proliferation,[7–9] which can result in the development of glial scar tissue and retinal contraction.[10–13]

Most researches in this field have orientated their efforts to identify clinical factors responsible of this pathologic repairing process (such as, the method of retinopexy or tamponade used). However, it seems likely as a cell-based inflammatory response that genetic susceptibility may have a role, particularly as PVR can occur in patients with prompt and initially successful surgery following a primary rhegmatogenous RD.[14,15] Hence, we are still unable to predict the risk of PVR reliably and apart from surgery, there is currently no prevention or cure.[16–18]

We have learned that many diseases are the consequence of interaction between environmental factors (clinical variables) and the genetic profile of each subject.[19,20] Considering this concept of complex disease, we decided to investigate the genetic component of PVR. One preliminary study conducted by our group in a small sample had showed in 2005 an association between the *TGF-β1* and PVR.[21] We also developed three predictive models of PVR based on the analysis of genetic variables.[22]

The Retina 4 project, a candidate gene and replication study strategy, overcomes one of the major problems with association studies in generating false positives.[23,24] Associations need to be replicated in order to confirm their veracity. For that reason, this study comprises two stages: the discovery stage, where only samples coming from Spanish centers were analyzed; and the replication stage, where new samples coming from centers in Spain, Portugal, the Netherlands, and the United Kingdom were studied.

Methods

Setting and Design

A case-controlled, candidate gene association study was conducted in 2006 among patients from eight centers in Spain, the discovery stage (Table 1). For the replication stage, a European multicenter case-controlled association study in those genes found significantly associated to PVR in the discovery stage (*TNF locus*, mothers against decapentaplegic (*SMAD7*), phosphatidyl inositol 3-kinase, catalytic, gamma (*PIK3CG*), and *TNFR2*) was conducted between 2009 and 2010. All patients provided written informed consent, and the study was approved by the institutional research committees of each center and followed the tenets of the Declaration of Helsinki.

Study Population

Both samples were composed of patients who had undergone primary rhegmatogenous RD surgery. Careful ophthalmoscope examination by slit lamp and indirect ophthalmoscope were performed postoperatively to classify the patient as case or control.

Those who developed PVR grade C1 or higher[25] after surgery were included as a case. Those who did not develop PVR after 3 months of follow up were included in the control group. To achieve a stringent phenotype classification, other causes than a primary rhegmatogenous RD, such as traumatic, tractional, exudative, or iatrogenic RD were excluded. Exclusion criteria were also RD secondary to macular hole, giant retinal tears defined by more than 3 hours, patients with preoperative PVR grade C1 or higher,[25] and patients with RD in the affected eye and RD with PVR in the fellow eye. In addition, clinical characteristics that could affect the stringent phenotypification were investigated like race, family history of RD, status of the lens, and so on (Table 2). Surgeon experience was also valorated. Experts were considered to be those who had already performed greater than or equal to 100 pars plana vitrectomy (PPV) and no experts those with less than 100 PPV.

In the discovery stage only patients coming from Spanish centers were included, while in the replication stage participants were completely new patients coming from centers in Holland, Portugal, Spain, and the United Kingdom (Table 1). Inclusion of patients during the replication stage coming from centers that participated in the former stage was consecutive and prospective. Simulation-based power analysis by bootstrap was used for sample size determination of replication stage.

Measures

Blood Collection and DNA Extraction. A peripheral blood sample was used for DNA extraction, which was performed using the commercial REALpure Kit protocol SSS (DURVIZ SL, Valencia, Spain), or similar. Genotyping was performed by the Genotyping Center (CeGen-ISCIII, Santiago de Compostela, Spain). For the discovery stage, the SNPlex Genotyping System (Applied Biosystem, Foster City, CA) was used. For the replication sample the MassARRAY SNP Genotyping System (Sequenom Inc., San Diego, CA) was used following the manufacturer's instructions. The principles of this method are detailed in Buetow et al.[26]

Genes and Single Nucleotide Polymorphism Selection. According the inflammatory nature of PVR,[2,27] the following 30 candidate genes were investigated in the discovery stage: connective tissue growth factor (*CTGF*), epidermal growth factor (*EGF*), fibroblast growth factor (*FGF*), hepatocyte growth factor (*HGF*), insulin-like growth factor 1 (*IGF-1*), interferon gamma (*IFNG*), insulin-like growth factor 2 (*IGF-2*), insulin-like growth factor I receptor (*IGF1R*), interleukin 1 alpha (*IL1A*), interleukin 1 beta (*IL1B*), interleukin 1 receptor antagonist (*IL1RN*), interleukin 6 (*IL6*), interleukin 8 (*IL8*), interleukin 10 (*IL10*), monocyte chemotactic protein 1 (*MCP1*), macrophage migration inhibitory factor (*MIF*), matrix metalloproteinase (*MMP*)*-2*, matrix metalloproteinase 7 (*MMP7*), nuclear factor kappa-b subunit 1 (*NFKB1*), nuclear factor of kappa light chain gene enhacer in B cells inhibitor alpha (*NFKBIA*), nuclear factor of kappa light chain gene enhacer in B cells inhibitor beta (*NFKBIB*), platelet-derived growth factor (*PDGF*), platelet-derived growth factor receptor alpha (*PDGFRα*), *PI3K*, *SMAD3*, *SMAD7*, transforming growth fator beta 1 (*TGF-β1*), transforming growth fatcor beta 2 (*TGF-β2*), tumor necrosis factor alpha (*TNF-α*), and tumor necrosis factor receptor 2 (*TNFR2*). Common tag SNPs with correlation coefficients greater than or equal to 0.85 and a minor allelic frequency greater than or equal to 10% were studied to explain as much as possible the known genetic variation for each gene. The Tagger method implemented in the Haploview program (provided in the public domain by Broad Institute; http://www.broad.mit.edu/mpg/haploview/) was used for this purpose.[28] According to the linkage disequilibrium observed in the *TNF-α* region, the following four genes were necessary to be investigated in the *TNF locus*: lymphotoxin alpha (*LTα*), *TNF-α*, leucocyte specific transcript 1 (*LST1*), and the activating natural killer receptor p30 (*NCR3*). Genic and extragenic regions 10 kb upstream and downstream were considered for each gene. Functional SNPs or ones previously described in association with other inflammatory diseases were added for analysis.[29–33] In the replication stage same SNPs previously studied in *PIK3CG*, *SMAD7*, *TNF locus*, and *TNFR2* (30 SNPs) were investigated.

Statistical Analysis

Preliminary and Descriptive Analysis. The data quality were evaluated by Hardy-Weinberg equilibrium using a Pearson goodness-of-fit test. When there was a low genotype count, a Fisher exact test was used. Data quality were also evaluated by differential missingness between cases and controls. Allelic and genotypic frequencies were estimated.

Test of Association: Single SNPs. Single associations were established using the χ^2 and Fisher tests. Five inheritance models were defined: (1) In the codominant model, every genotype gave a different and nonadditive risk, (2) in the dominant model, a single copy of the variant allele was enough to modify the risk, (3) in the recessive model, two copies of the variant allele were necessary to change the risk, (4) in the over dominant model, heterozygosity was compared with a pool of each allele homozygosity, and (5) in the additive model, each copy of the variant allele modified the risk in an additive form. The Akaike Information Criteria (AIC) was used to choose the inheritance model that best fit the data.

Test of Association: Haplotypic SNPs. Haplotypes consistent with the sample were analyzed taking into consideration blocks from as few as two consecutive markers (subhaplotypes) to as much as all of the markers included in each gene (haplotypes). Haplotypic frequencies were estimated using the expectation-maximization algorithm.[34] To evaluate the association between haplotypes and disease, general-

Table 1. Centers Involved in the Study

Country	Center	Discovery Stage			Replication Stage		
		Cases PVR	Controls RD	Total	Cases PVR	Controls RD	Total
England							
Moorfields Eye Hospital, London	*N*	-	-	-	32	118	150
	% Total	-	-	-	5.9	21.6	27.5
Holland							
Rotterdam Eye Hospital, Rotterdam	*N*	-	-	-	39	89	128
	% Total	-	-	-	7.1	16.3	23.4
Portugal							
Coimbra University Hospital, Coimbra	*N*	-	-	-	6	1	7
	% Total	-	-	-	1.1	0.2	1.3
Sao Joao Hospital, Oporto	*N*	-	-	-	6	52	58
	% Total	-	-	-	1.1	9.5	10.6
Spain							
Germans Trias i Pujol University Hospital, Badalona	*N*	-	-	-	11	11	22
	% Total	-	-	-	2.0	2.0	4.0
Jiménez Díaz Foundation, Madrid	*N*	-	-	-	2	2	4
	% Total	-	-	-	0.4	0.4	0.7
Ophthalmological Foundation of Mediterranean, Valencia	*N*	-	-	5	2	7	
	% Total	-	-	0.9	0.4	1.3	
San Millan San Pedro Hospital, Logroño	*N*	-	-	-	9	1	10
	% Total	-	-	-	1.6	0.2	1.8
Universitary Hospital La Fe, Valencia	*N*	-	-	-	10	25	35
	% Total	-	-	-	1.8	4.6	6.4
University Hospital of Navarra	*N*	-	-	-	11	19	30
	% Total	-	-	-	2.0	3.5	5.5
Universitay Hospital Ramón y Cajal, Madrid	*N*	-	-	-	2	3	5
	% Total	-	-	-	0.4	0.5	0.9
Barraquer Ophthalmology Centre	*N*	8	35	43	2	6	8
	% Total	1.8	7.8	9.5	0.4	1.1	1.5
Donostia Hospital, San Sebastian	*N*	7	35	42	2	0	2
	% Total	1.5	7.8	9.3	0.4	0	0.4
IOBA (University Eye Institute), Valladolid	*N*	28	22	50	10	15	25
	% Total	6.2	4.9	11.1	1.8	2.7	4.6
Reina Sofia Universitary Hospital, Cordoba	*N*	5	9	14	-	-	-
	% Total	1.1	2	3.1	-	-	-
Universitary Hospital Pio del Rio Hortega	*N*	7	10	17	-	-	-
	% Total	1.5	2.2	3.8	-	-	-
University Hospital Vall d'Hebrón	*N*	20	75	95	1	25	26
	% Total	4.4	16.7	21.1	0.2	4.6	4.8
University Hospital of Valladolid	*N*	32	28	60	2	8	10
	% Total	7.1	6.2	13.3	0.4	1.5	1.8
Vissum, Alicante	*N*	31	98	129	1	18	19
	% Total	6.9	21.8	28.7	0.2	3.3	3.5
TOTAL	*N*	138	312	450	151	395	546
	% Center	30.7	69.3	100.0	27.7	72.3	100.0

N, number of patients from each center; % Center, distribution of patients coming from each center in percent; % Type, distribution of case control for each center in percent; % Total, percentage of patients from each center to the total amount of patients included.

ized linear models were used. The effects of haplotypes were modeled as additive, dominant, and recessive. The log-likelihood criterion was used to select the best model.

Multiple Testing and False Discovery Rate Analysis. To correct for multiple analyses, the Rosenberg two-stage method was used.[35] For the first stage, single-SNP association tests and haplotype-SNP association tests within one gene were considered. An omnibus test that combined SNP and haplotype analysis was then constructed. This omnibus test used the test statistic

$$\text{omni} = \min\{p^{SNP}, p^{HAP}\} \quad (1)$$

where p^{SNP} was the Simes-adjusted *P* value[36] for the most significant SNP, and p^{HAP} was the Simes-adjusted *P* value for the most significant haplotype. The distribution of the omnibus test statistic under the independence hypothesis was computed from the permutation distribution obtained by shuffling case and control indicators. In the second stage, summary gene *P* values were adjusted for multiplicity by controlling the expected false discovery rate with the Benjamini-Hochberg procedure (q-value).[37]

All statistical analyses were conducted using R software including SNPassoc and haplo.stat packages.[38–40]

Results

Discovery Stage

Four hundred fifty patients were analyzed, including 138 cases and 312 controls. A total of 230 SNPs distributed along the 30

Table 2. Clinical Characteristics of the Replication Sample

	Replication Stage					
	Case—PVR		Control—RD			
Characteristic	***N***	**% Type**	***N***	**% Type**	**Total**	***P* Value**
Sex						
Male	100	28.74	248	71.26	348	0.362
Female	45	25.00	135	75.00	180	
Total	145 (6)°	27.46	383 (12)°	72.54	528	
Race						
North-African	0	0	6	100	6	0.0644
Asian	2	40.00	3	60.00	5	
European	133	26.44	370	73.56	503	
Hispanic	7	53.85	6	46.15	13	
Indian	5	41.67	7	58.33	12	
Subsaharian	2	50.00	2	50.00	4	
Total	149 (2)°	27.44	394 (1)°	72.56	543	
RD family history						
No	142	27.52	374	72.48	516	0.932
Yes	9	30	21	70	30	
Total	151	27.66	395	72.34	546	
PVR family history						
No	149	27.49	393	72.51	542	0.4758*
Yes	1	50	1	50	2	
Total	150 (1)°	27.57	394 (1)°	72.43	544	
RD in fellow eye						
Unknown	5	38.46	8	61.54	13	0.5289*
No	136	27.7	355	72.3	491	
Yes	10	23.81	32	76.19	42	
Total	151	27.66	395	72.34	546	
PVR in fellow eye						
Unknown	5	38.46	8	61.54	13	**0.0057***
No	141	26.70	387	73.30	528	
Yes	5	100	0	0	5	
Total	151	27.66	395	72.34	546	
Phakia/aphakia						
Unknown	7	33.33	14	66.67	21	0.2333
Aphakia	19	38.78	30	61.22	49	
Pseudophakia	40	28.37	101	71.63	141	
Phakia	85	25.37	250	74.63	335	
Total	151	27.66	395	72.34	546	
Retinopexy						
No	147	27.63	385	72.37	532	0.8268*
Yes	4	28.57	10	71.43	14	
Total	151	27.66	395	72.34	546	
Scleral surgery (SS)						
No	116	25.55	338	74.45	454	**0.0281***
Yes	34	38.2	55	61.8	89	
Total	150 (1)°	27.62	393 (2)°	72.38	543	
SS + drainage						
No	131	29.18	318	70.82	449	**0.0383***
Yes	20	20.62	77	79.38	97	
Total	151	27.66	395	72.34	546	
PPV						
No	15	10.87	123	89.13	138	**<0.0001**
Yes	136	33.33	272	66.67	408	
Total	151	27.66	395	72.34	546	
Air						
No	138	26.59	381	73.41	519	**0.0153***
Yes	13	48.15	14	51.85	27	
Total	151	27.66	395	72.34	546	
SF6						
No	87	30.21	201	69.79	288	**0.0077***
Yes	64	24.81	194	75.19	258	
Total	151	27.66	395	72.34	546	

Table 2. Continued

	Replication Stage					
	Case—PVR		Control—RD			
Characteristic	***N***	**% Type**	***N***	**% Type**	**Total**	***P* Value**
C3F8						
No	116	26.54	321	73.46	437	0.3041*
Yes	35	32.11	74	67.89	109	
Total	151	27.66	395	72.34	546	
Silicone						
No	55	13.03	367	86.97	422	**<0.0001***
Yes	96	77.42	28	22.58	124	
Total	151	27.66	395	72.34	546	
Laser						
No	35	13.41	226	86.59	261	**<0.0001***
Yes	116	40.7	169	59.3	285	
Total	151	27.66	395	72.34	546	
Cryotherapy						
No	99	31.03	220	68.97	319	**0.0481***
Yes	52	22.91	175	77.09	227	
Total	151	27.66	395	72.34	546	

N, number of patients. (N)° Missing information is indicated for each variable in parentheses. In bold, statistical significant values.
* χ^2 or Fisher exact test.

candidate genes were investigated. Six SNPs did not pass the design pipeline, and 27 failed during the genotyping process. All informative SNPs verified the Hardy-Weinberg equilibrium except one. It was located in the *PDGFα* gene and was eliminated because it exhibited poor performance during the genotyping process. Thus, a total of 196 SNPs were analyzed, yielding a conversion rate of 85.2%.

Single and Haplotypic Associations. Twenty two single significant associations were observed in 16 genes: *EGF*, *IGF1*, *IL1RN*, *MIF*, *MMP-2*, *NFKB1*, *NFKBIA*, *NFKBIB*, *PDGFA*, *PDGFRA*, *SMAD3*, *SMAD7*, *TGFB1*, *TGFB2*, *TNF locus*, and *TNFR2* (Table 3). Following permutation, only three single significant associations in three genes were detected: the rs243845 in *MMP-2* ($P = 0.050$), the rs7226855 in *SMAD7* ($P = 0.0015$), and the rs2229094 in *TNF locus* ($P = 0.0283$) (Table 3).

In multiple analyses, significant haplotypic and subhaplotypic associations were detected in 13 genes: *IGF-IR*, *IL-10*, *IL6*, *MIF*, *MMP-2*, *NFKB1*, *NFKBIA*, *NFKBIB*, *PIK3CG*, *SMAD3*, *TGF-β2*, *TNF locus*, and *TNFR2* (Table 4). Following permutation, five genes maintained their significant association: *NFKB1* ($P = 0.0460$), *NFKBIA* ($P = 0.0460$), *PIK3CG* ($P = 0.0010$), *TNF locus* ($P = 0.0050$), and *TNFR2* ($P = 0.0130$) (Table 4).

Taking into consideration now the total amount of genes, following the second stage of the Rosenberg method, four genes maintained significant association with PVR (Table 5): *PIK3CG* ($P = 0.009$), *SMAD7* ($P = 0.004$), *TNF locus* ($P = 0.005$), and *TNFR2* ($P = 0.019$).

Replication Stage

A total of 546 peripheral DNA blood samples (151 cases and 395 controls) from 17 European centers were included (Table 1). Seventy-one SNPs were included. There was one failure in the design of the primers, the one corresponding to the rs1982073. There were no failures for the genotyping process, with a global call rate of 97.59%. The genotypic data quality was evaluated, and all informative markers verified the Hardy-Weinberg equilibrium.

Regarding clinical information some significant associations were observed. The control group was significantly older than cases ($P < 0.0001$) with a median of 6 years (95% confidence interval [CI]: 3.39–8.31). Patients with history of PVR in the fellow eye were more frequently cases (83%) than controls (17%). Pneumatic retinopexy was more frequently performed on patients that did not developed PVR (71%); scleral surgery ($P = 0.0281$), PPV ($P < 0.0001$), and tamponade-like air ($P = 0.0153$) or silicone oil (SO) ($P < 0.0001$) were more frequent in cases, while scleral surgery plus drainage (SS + d) ($P = 0.0383$), SF6 as tamponade ($P = 0.0077$), and cryotherapy ($P = 0.0481$) were significantly less frequent in cases. Regarding systemic and ocular diseases and treatment occurring simultaneously with the RD, very few patients reported inherited ocular disease or connective tissue disease (1 case versus 11 controls and 1 case versus 13 controls, respectively). Seven cases were receiving systemic nonsteroidal anti-inflammatory drugs (NSAIDs), 2 steroids, and 1 antiproliferant treatment when suffered from RD versus 21, 6, and 2 controls, respectively. One case was receiving topic steroids, while 7 controls were receiving ocular NSAIDs, and 15 steroids. In the same way as in the discovery stage, none of the patients received intravitreal Triamcinolone Acetonide (TA) in any surgery. Fifty one percent of patients who suffered from PVR following a RD surgery were operated on by an experienced surgeon (nontraining grade). There were no significant associations with sex, race, affected eye, family history of PVR, or phakic/aphakic status. There were no differences regarding the geographical localization or center where the patients came from.

Single and Haplotypic Associations. In the single analysis new significant associations were observed in *PIK3CG* (rs4460309, $P = 0.0457$), *SMAD7* (rs6507877, $P = 0.0345$), and *TNF locus* (rs2256974, $P = 0.02615$; rs909253, $P = 0.005917$; rs1799964, $P = 0.03152$; and rs1800629, $P = 0.04563$) (Table 3). No associations were observed in *TNFR2*. One replication was detected, the rs7226855 in *SMAD7* ($P = 0.0064$) (Table 3). Following permutation, all genes maintained their association: *PIK3CG* ($P = 0.0470$), *SMAD7* ($P = 0.070$), and *TNF locus* ($P = 0.0060$) (Table 3).

Table 3. Single Associations in the Discovery and Replication Stages

			Discovery Stage								Replication Stage							
			Frequency, %				CI 95% OR				Frequency, %				CI 95% OR			
Gene	SNP	Genotype	Control	Case	Inheritance Model*	OR	Lower	Upper	*P* Value	Corrected *P* Value	Control	Case	Inheritance Model*	OR	Lower	Upper	*P* Value	Corrected *P* Value
EGF	rs1024600	CC/CG	92.7	85.7	Recessive				**0.02846**	0.6870	-	-	-	-	-	-	-	-
	C→G	GG	7.3	14.3		2.12	1.09	4.11			-	-	-	-	-	-	-	-
IGF1	rs2195240	TT/CT	93.4	98.4	Recessive	0.23	0.05	0.98	**0.017**	0.1816	-	-	-	-	-	-	-	-
	T→C	CC	6.6	1.6							-	-	-	-	-	-	-	-
	rs5742629	AA/AG	90.4	97.5	Recessive	0.25	0.07	0.84	**0.0087**	0.1816	-	-	-	-	-	-	-	-
	A→G	GG	9.4	2.5							-	-	-	-	-	-	-	-
IL1RN	rs3087270	AA	47.6	56.8	Additive	0.72	0.53	0.99	**0.0420**	0.9132	-	-	-	-	-	-	-	-
	A→G	AG	38.8	35.2							-	-	-	-	-	-	-	-
		GG	13.6	8							-	-	-	-	-	-	-	-
MIF	rs1007888	AA	30	19	Dominant				**0.0201**	0.2306	-	-	-	-	-	-	-	-
	A→G	AG/GG	70	81		1.82	1.08	3.07			-	-	-	-	-	-	-	-
MMP2	***rs243845***	***CC***	***45.4***	***29.6***	***Dominant***				***0.0023***	***0.0500***	-	-	-	-	-	-	-	-
	C→T	***CT/TT***	***54.6***	***70.4***		***1.98***	***1.26***	***3.09***			-	-	-	-	-	-	-	-
	rs243864	TT/GT	92.2	97.5	Recessive	0.30	0.09	1.01	**0.026**	0.3446	-	-	-	-	-	-	-	-
	T→G	GG	7.8	2.5							-	-	-	-	-	-	-	-
	rs243866	GG/AG	92.4	97.5	Recessive	0.31	0.09	1.05	**0.0317**	0.3446	-	-	-	-	-	-	-	-
	G→A	AA	7.6	2.5							-	-	-	-	-	-	-	-
NFKB1	rs4698858	CC	45.1	32.3	Dominant	1.73	1.11	2.69	**0.01435**	0.2614	-	-	-	-	-	-	-	-
	C→G	CG/GG	54.9	67.7							-	-	-	-	-	-	-	-
NFKBIA	rs17103274	TT	84.9	72.1	Dominant				**0.0034**	0.0515	-	-	-	-	-	-	-	-
	T→C	CT/CC	15.1	27.9		2.17	1.3	3.61			-	-	-	-	-	-	-	-
NFKBIB	rs3136640	GG/CC	51.3	65.1	Overdominant				**0.0087**	0.0993	-	-	-	-	-	-	-	-
	G→C	CG	48.7	34.9		0.57	0.37	0.87			-	-	-	-	-	-	-	-
PDGFA	rs7806249	TT/CC	52.4	62.9	Overdominant				**0.04696**	0.3917	-	-	-	-	-	-	-	-
	T→C	CT	47.6	37.1		0.65	0.42	1.00			-	-	-	-	-	-	-	-
PDGFRA	rs7656613	TT/CC	65.1	51.4	Overdominant				**0.1654**	0.1884	-	-	-	-	-	-	-	-
	T→C	CT	34.9	48.6		1.76	1.11	2.8			-	-	-	-	-	-	-	-
SMAD3	rs6494634	CC	40.8	50.8	Additive	0.71	0.50	0.99	**0.04016**	0.8741	-	-	-	-	-	-	-	-
	C→T	CT	49.3	43							-	-	-	-	-	-	-	-
		TT	9.9	6.2							-	-	-	-	-	-	-	-
PIK3CG	**rs4460309**	CC/CT	-	-	-	-	-	-	-	-	95.1	90.2	Recessive				**0.04566**	**0.047**
	C→T	TT	-	-	-	-	-	-	-	-	4.9	9.8		2.12	1.03	4.35		
SMAD7	***rs7226855***	***AA***	***56.3***	***36.2***	***Overdominant***	***2.27***	***1.48***	***3.48***	***0.00014***	***0.0015***	***34.8***	***22.8***	***Dominant***				***0.006382***	***0.0070***
	A→G	***GG***									***65.2***	***77.2***						
		AG	***43.7***	***63.8***										***1.8***	***1.17***	***2.79***		
	rs6507877	GG/AA									***57.2***	***46.9***	***Overdominant***				***0.03446***	***0.0070***
	A→G	AG									***42.8***	***53.1***		***1.51***	***1.03***	***2.22***		
TGFB1	rs2241713	GG/CG	79.2	87.6	Recessive						-	-	-	-	-	-	-	-
	G→C	CC	20.8	12.4		0.54	0.29	0.99	**0.03810**	0.3175	-	-		-	-	-		
TGFB2	rs1891467	AA	53.5	45	Additive	1.44	1.03	2.02	**0.03447**	0.25	-	-	-	-	-	-	-	-
	A→G	AG	42.2	45.7							-	-		-	-	-		
		GG	4.3	9.3							-	-		-	-	-		

Table 3. Continued

			Discovery Stage								Replication Stage							
			Frequency, %				CI 95% OR				Frequency, %				CI 95% OR			
Gene	SNP	Genotype	Control	Case	Inheritance Model*	OR	Lower	Upper	*P* Value	Corrected *P* Value	Control	Case	Inheritance Model*	OR	Lower	Upper	*P* Value	Corrected *P* Value
	rs2000220	AA	48.1	39.1							-	-	-	-	-	-	-	-
	A→G	AG	41.8	43.8	Addictive						-	-		-	-	-		
		GG	10.1	17.2		1.41	1.04	1.90	**0.02595**	0.25	-	-		-	-	-		
	rs947712	GG	58.6	47.6							-	-	-	-	-	-	-	-
	G→A	AG	35.4	38.9	Addictive						-	-		-	-	-		
		AA	6.1	13.5		1.54	1.12	2.11	**0.00787**	0.1718	-	-		-	-	-		
TNF locus	rs2857706	AA/GG	75.2	62.2	Overdominant				**0.0077**	0.0837	-	-	-	-	-	-	-	-
	G→A	AG	24.83	37.80		1.84	1.18	2.87			-	-		-	-	-		
	rs2229094	***CT/CC***	***6.33***	***8.94***	***Dominant***	***2.02***	***1.31***	***3.11***	***0.0013***	***0.0283***	-	-	-	-	-	-	-	-
	T→C	***TT***	***52.00***	***34.96***							-	-		-	-	-		
	rs2256974	GG	64.55	74.59	Additive	0.64	0.42	0.97	**0.0315**	0.2783	***64.7***	***74.7***	***Dominant***				***0.02615***	***0.0060***
	G→T	GT	31.34	23.77							***35.3***	***25.3***						
		TT	4.10	1.64										***0.62***	***0.40***	***0.95***		
	rs909253	TT/CT	-	-	-	-	-	-	-	-	***87.4***	***95.1***	***Recessive***				***0.0059***	***0.0060***
	T→C	CC	-	-		-	-	-	-	-	***12.6***	***4.9***		***0.36***	***0.16***	***0.81***		
	rs199964	TT/CT	-	-	-	-	-	-	-	-	***93.6***	***97.9***	***Recessive***				***0.03152***	***0.0060***
	T→C	CC	-	-		-	-	-	-	-	***6.4***	***2.1***		***0.31***	***0.09***	***1.06***		
	rs1800629	GG/AA	-	-	-	-	-	-	-	-	***76.3***	***67.8***	***Overdominant***				***0.04563***	***0.0060***
	G→A	GA	-	-		-	-	-	-	-	***23.7***	***32.2***		***1.53***	***1.01***	***2.32***		

In bold, statistical significant values. In bold and italic, those SNPs that maintained their significant association following the correction for multiple comparison. CI, confidence interval; OR, odds ratio.

* Inheritance models: Additive, each copy of the rare variant modify the risk; dominant, a single copy of the frequent variant is enough to modify the risk; recessive, two copies of the variant allele are necessary to change the risk; overdominant, heterozygosity modifies the risk.

Table 4. Haplotypic Associations in the Discovery and Replication Stages

				Discovery Stage								Replication Stage								
				Frequency, %			CI 95% OR		Multiple Association				Frequency, %			CI 95% OR		Multiple Association		
Gene	*N* SNP	Initial SNP	Inheritance Model*	Control	Case	OR	Inferior	Superior	*P* Value	Corrected *P* Value	*P* Value Corrected by Gene	Inheritance Model*	Control	Case	OR	Inferior	Superior	*P* Value	Corrected *P* Value	*P* Value Corrected by Gene
IGF-IR	2	rs10794486	Additive	9.72	16.98	1.78	1.12	2.85	**0.0125**	**0.0150**	0.1760	-	-	-	-	-	-	-	-	-
	3	rs7166287	Additive	6.69	14.42	2.49	1.37	4.52	**0.0031**	**0.0050**		-	-	-	-	-	-	-	-	-
	4	rs7166287	Additive	2.74	7.78	3.19	1.36	7.49	**0.0054**	**0.0110**		-	-	-	-	-	-	-	-	-
		rs10794486	Additive	2.10	7.94	4.15	1.61	10.70	**0.0032**	**0.0043**		-	-	-	-	-	-	-	-	-
	5	rs7166287	Additive	2.54	6.90	4.19	1.37	12.83	**0.0168**	**0.0180**		-	-	-	-	-	-	-	-	-
		rs10794486	Additive	2.01	7.82	4.18	1.59	10.98	**0.0010**	**0.0020**		-	-	-	-	-	-	-	-	-
	6	rs7166287	Additive	2.51	6.72	3.88	1.27	11.86	**0.0072**	**0.0080**		-	-	-	-	-	-	-	-	-
		rs10794486	Additive	2.0	7.27	3.36	1.30	8.66	**0.0013**	**0.0030**		-	-	-	-	-	-	-	-	-
IL-10	3	rs1800890	Additive	4.6	3.09	9.17	1.87	45.05	**0.0015**	**0.0023**	0.1700	-	-	-	-	-	-	-	-	-
IL-6	6	rs4719713	Additive	1.48	3.80	3.43	1.16	10.14	**0.0533**	**0.0529**	0.1100	-	-	-	-	-	-	-	-	-
	8	rs22725336	Additive	1.06	3.29	3.75	1.02	13.75	**0.0127**	**0.0112**		-	-	-	-	-	-	-	-	-
				0.46	3.18	9.98	1.99	49.95	**0.0025**	**0.0010**		-	-	-	-	-	-	-	-	-
				1.42	3.81	3.49	1.17	10.42	**0.0468**	**0.0360**		-	-	-	-	-	-	-	-	-
MIF	2	rs1007888	Additive	7.07	11.60	1.90	1.12	3.24	**0.0391**	**0.0430**	0.1850	-	-	-	-	-	-	-	-	-
MMP2	2	rs9928731	Additive	33.88	42.54	1.43	1.02	1.99	**0.0185**	**0.0180**	0.2050	-	-	-	-	-	-	-	-	-
		rs243845	Dominant	34.27	43.36	1.95	1.20	3.14	**0.0127**	**0.0092**		-	-	-	-	-	-	-	-	-
	3	rs9928731	Additive	0.15	2.07	14.13	**12.59**	15.86	**0.0267**	**0.0170**		-	-	-	-	-	-	-	-	-
NFKB1	***3***	***rs3774932***	***Dominant***	***2.36***	***5.81***	***2.69***	***1.19***	***6.07***	***0.0173***	***0.0230***	***0.0460***	-	-	-	-	-	-	-	-	-
		rs4698858	***Dominant***	***18.68***	***10.01***	***0.49***	***0.28***	***0.87***	***0.0033***	***0.0030***		-	-	-	-	-	-	-	-	-
		rs11722146	***Dominant***	***1.80***	***5.90***	***3.87***	***1.61***	***9.30***	***0.0026***	***0.0023***		-	-	-	-	-	-	-	-	-
	4	***rs4698858***	***Dominant***	***18.46***	***10.82***	***0.55***	***0.32***	***0.95***	***0.0097***	***0.0063***		-	-	-	-	-	-	-	-	-
				0.84	***3.27***	***4.89***	***1.32***	***18.11***	***0.0021***	***0.0047***		-	-	-	-	-	-	-	-	-
		rs11722146	***Dominant***	***1.80***	***5.93***	***3.83***	***1.59***	***9.21***	***0.0025***	***<0.0001***		-	-	-	-	-	-	-	-	-
	5	***rs2300540***	***Dominant***	***1.05***	***3.08***	***3.43***	***1.07***	***10.93***	***0.0326***	***0.0230***		-	-	-	-	-	-	-	-	-
				0.67	***2.80***	***4.76***	***1.25***	***18.17***	***0.0081***	***0.0100***		-	-	-	-	-	-	-	-	-
		rs4698858	***Dominant***	***0.82***	***3.45***	***5.25***	***1.48***	***18.67***	***0.0019***	***0.0022***		-	-	-	-	-	-	-	-	-
	6	***rs3774932***	***Dominant***	***0.17***	***2.10***	***12.85***	***1.42***	***116.34***	***0.0020***	***0.0050***		-	-	-	-	-	-	-	-	-
		rs2300540	***Dominant***	***1.05***	***3.17***	***3.37***	***1.06***	***10.67***	***0.0293***	***0.0320***		-	-	-	-	-	-	-	-	-
				0.67	***2.71***	***4.46***	***1.16***	***17.16***	***0.0090***	***0.0070***		-	-	-	-	-	-	-	-	-
NFKBIA	***2***	***rs2007960***	***Dominant***	***8.04***	***14.48***	***2.47***	***1.44***	***4.24***	***0.0054***	***0.0037***	***0.0460***	-	-	-	-	-	-	-	-	-
	3	***rs3138045***	***Dominant***	***8.02***	***13.64***	***2.59***	***1.49***	***4.52***	***0.0054***	***0.0020***		-	-	-	-	-	-	-	-	-
	6	***rs7152826***	***Dominant***	***5.75***	***8.24***	***0.27***	***0.21***	***0.35***	***0.0493***	***0.0500***		-	-	-	-	-	-	-	-	-
NFKBIB	2	rs11879872	Dominant	0.32	2.49	11.88	1.28	110.62	**0.0333**	**0.0360**	0.0830	-	-	-	-	-	-	-	-	-
PIK3CG	***3***	***rs4460309***	***Dominant***	***0.01***	***1.14***	***15.8 × 10⁴***	***15.8 × 10⁴***	***15.8 × 10⁴***	***0.0010***	***0.0410***	***0.0010***	-	-	-	-	-	-	-	-	-
SMAD7	2	rs4939826	-	-	-	-	-	-	-	-	-	Additive	41.85	49.69	1.40	1.06	1.86	0.0207	**0.0330**	0.3110
TGFB2	2	rs947712	Dominant	13.41	19.20	1.99	1.22	3.24	**0.0205**	**0.0230**	0.3180	-	-	-	-	-	-	-	-	-
		rs2796821	Dominant	14.04	20.49	1.83	1.15	2.89	**0.0189**	**0.0120**		-	-	-	-	-	-	-	-	-

Table 4. Continued

				Discovery Stage								Replication Stage								
				Frequency, %			CI 95% OR		Multiple Association				Frequency, %			CI 95% OR		Multiple Association		
Gene	*N* SNP	Initial SNP	Inheritance Model*	Control	Case	OR	Inferior	Superior	*P* Value	Corrected *P* Value	*P* Value Corrected by Gene	Inheritance Model*	Control	Case	OR	Inferior	Superior	*P* Value	Corrected *P* Value	*P* Value Corrected by Gene
TNF locus	2	***rs909253***	***Dominant***	***27.14***	***37.18***	***1.98***	***1.27***	***3.08***	***0.0024***	***0.0046***	***0.0050***	-	-	-	-	-	-	-	-	-
		rs2229094	***Dominant***	***2.98***	***9.49***	***3.65***	***1.82***	***7.31***	***0.0001***	***<0.0001***		-	-	-	-	-	-	-	-	-
	3	***rs2857706***	***Dominant***	***15.16***	***20.52***	***1.87***	***1.18***	***2.96***	***0.0484***	***0.0430***		-	-	-	-	-	-	-	-	-
		rs909253	***Dominant***	***2.97***	***9.57***	***3.64***	***1.80***	***7.37***	***0.0001***	***0.0001***		-	-	-	-	-	-	-	-	-
		rs2229094	***Dominant***	***2.97***	***9.53***	***4.08***	***2.01***	***8.28***	***0.0001***	***0.0001***		-	-	-	-	-	-	-	-	-
	4	***rs2857706***	***Dominant***	***3.07***	***9.54***	***3.36***	***1.66***	***6.79***	***0.0002***	***0.0005***		-	-	-	-	-	-	-	-	-
		rs909253	***Dominant***	***2.97***	***9.57***	***3.91***	***1.92***	***7.99***	***0.0001***	***0.0001***		-	-	-	-	-	-	-	-	-
		rs2229094	***Dominant***	***2.98***	***9.53***	***4.15***	***2.04***	***8.45***	***0.0001***	***0.0002***		-	-	-	-	-	-	-	-	-
	5	***rs2857602***	***Dominant***	***3.06***	***9.50***	***3.35***	***1.66***	***6.77***	***0.0002***	***0.0004***		-	-	-	-	-	-	-	-	-
		rs2857706	***Additive***	***3.07***	***9.54***	***3.47***	***1.76***	***6.85***	***0.0002***	***0.0001***		-	-	-	-	-	-	-	-	-
		rs909253	***Dominant***	***2.98***	***9.57***	***3.97***	***1.94***	***8.12***	***0.0001***	***0.0002***		-	-	-	-	-	-	-	-	-
		rs2229094	***Dominant***	***3.20***	***9.47***	***1.60***	***1.02***	***2.50***	***0.0002***	***0.0003***		-	-	-	-	-	-	-	-	-
	6	***rs2857602***	***Additive***	***3.06***	***9.50***	***3.45***	***1.75***	***6.81***	***0.0002***	***0.0001***		-	-	-	-	-	-	-	-	-
		rs2857706	***Dominant***	***3.07***	***9.54***	***3.65***	***1.79***	***7.46***	***0.0002***	***0.0002***		-	-	-	-	-	-	-	-	-
		rs909253	***Dominant***	***3.05***	***9.48***	***3.89***	***1.90***	***7.99***	***0.0002***	***<0.0001***		-	-	-	-	-	-	-	-	-
		rs2229094	***Dominant***	***3.22***	***9.47***	***3.44***	***1.69***	***6.98***	***0.0003***	***0.0004***		-	-	-	-	-	-	-	-	-
	7	***rs2857602***	***Dominant***	***3.06***	***9.50***	***3.61***	***1.77***	***7.38***	***0.0002***	***0.0001***		-	-	-	-	-	-	-	-	-
		rs2857706	***Dominant***	***1.46***	***2.02***	***1.89***	***1.17***	***3.05***	***0.0406***	***0.0375***		-	-	-	-	-	-	-	-	-
				3.07	***9.48***	***3.54***	***1.74***	***7.18***	***0.0002***	***0.0002***		-	-	-	-	-	-	-	-	-
		rs909253	***Dominant***	***2.95***	***9.48***	***3.81***	***1.83***	***7.92***	***0.0002***	***<0.0001***		-	-	-	-	-	-	-	-	-
	8	***rs2857602***	***Dominant***	***3.18***	***9.34***	***3.48***	***1.72***	***7.06***	***0.0002***	***0.0007***		-	-	-	-	-	-	-	-	-
		rs2857706	***Dominant***	***14.67***	***20.31***	***1.83***	***1.14***	***2.94***	***0.0403***	***0.0391***		-	-	-	-	-	-	-	-	-
				2.97	***9.48***	***3.68***	***1.80***	***7.52***	***0.0002***	***0.0005***		-	-	-	-	-	-	-	-	-
	9	***rs2857602***	***Dominant***	***3.19***	***9.34***	***3.63***	***1.78***	***7.40***	***0.0002***	***0.0008***		-	-	-	-	-	-	-	-	-
TNFR2	6	rs12141399	Additive	NA	1.04	NA	NA	NA	**0.0093**	**<0.0001**	**0.0130**	-	-	-	-	-	-	-	-	-

In bold, statistical significant values. In bold and italic, those genes that maintained their significant association following the correction for multiple comparison. OR, odds ratio.

* Inheritance models: additive, each copy of the rare variant modify the risk; dominant, a single copy of the frequent variant is enough to modify the risk.

Table 5. Associations following the Correction for Single and Multiple Comparisons

				Discovery Stage							Replication Stage						
				Single Contrasts		Haplotypic Contrasts		Omnibus Test			Single Contrasts		Haplotypic Contrasts		Omnibus Test		
Gene	Chr	Extension	*n*	Min*P* Test, WYZ*	*P* Value, P^{WYZ}†	Min*P* Test, HFT‡	*P* Value, P^{HFT}§	Min*P* Test, OMNI||	*P* Value, P^{OMNI}#	q-Value	Min*P* Test, WYZ*	*P* Value, P^{WYZ}†	Min*P* Test, HFT‡	*P* Value, P^{HFT}§	Min*P* Test, OMNI||	*P* Value, P^{OMNI}#	q-Value
CTGF	6q23.1	3.13 kpb (132311017–132314146)	6	0.0484	0.2096	0.1374	0.5432	0.2096	0.53	0.2409	-	-	-	-	-	-	-
EGF	4q25	99.37 kbp (111053499–111152860)	9	0.0214	0.1476	0.1686	0.749	0.328	0.47	0.2409	-	-	-	-	-	-	-
FGF2	4q26	71.53 kbp (123967313–124038840)	10	0.0674	0.68	0.1218	0.599	0.599	0.8	0.2667	-	-	-	-	-	-	-
HGF	7q21.1	68.01 kbp (81166258–81237388)	5	0.1326	0.605	0.1444	0.372	0.372	0.519	0.2409	-	-	-	-	-	-	-
IFNγ	12q14	4.972 kbp (66834816–66839787)	6	0.221	0.868	0.23	0.551	0.551	0.682	0.2526	-	-	-	-	-	-	-
IGF1	12q22-q23	84.65 kbp (101313809–101398471)	10	0.0087	0.154	0.1759	0.716	0.154	0.236	0.1854	-	-	-	-	-	-	-
IGF2	11p15.5	6.047 kbp (2106918–2125616)	5	0.0739	0.536	0.2921	0.735	0.536	0.734	0.2576	-	-	-	-	-	-	-
IGF-IR	15q26.3	308.7 kbp (97010302–97319034)	12	0.0574	0.706	0.0004	0.176	0.176	0.329	0.2056	-	-	-	-	-	-	-
IL10	1q31-q32	4.892 kbp (205007570–205012462)	9	0.0735	0.665	0.0233	0.17	0.17	0.238	0.1854	-	-	-	-	-	-	-
IL1α	2q12-q21	11.48 kbp (113247966–113259442)	7	0.2414	0.912	0.2356	0.48	0.48	0.619	0.2526	-	-	-	-	-	-	-
IL1β	2q13-q21	7.02 kbp (113303567–113310586)	5	0.1298	0.735	0.1649	0.494	0.494	0.641	0.2526	-	-	-	-	-	-	-
IL1RN	2q14.2	16.12 kbp (113591941–113608063)	9	0.042	0.47	0.1095	0.447	0.447	0.59	0.2526	-	-	-	-	-	-	-
IL-6	7p21	4.797 kbp (22732028–22738091)	8	0.1806	0.891	0.0151	0.11	0.11	0.162	0.1800	-	-	-	-	-	-	-
IL-8	4q13-q21	3.157 kbp (74825139–74828295)	5	0.054	0.25	0.5179	0.8	0.25	0.363	0.2135	-	-	-	-	-	-	-
MCP1	17q11.2-q21.1	1.923 kbp (29606409–29608329)	6	0.1538	0.805	0.2292	0.507	0.507	0.666	0.2526	-	-	-	-	-	-	-
MIF	22q11.2	845 bp (22369647–22567417)	7	0.0202	0.223	0.0434	0.185	0.185	0.284	0.2029	-	-	-	-	-	-	-
MMP2	16q13-q21	27.52 kbp (54070589–54098101)	8	**0.0023**	**0.048**	0.0185	0.205	0.048	0.113	0.1425	-	-	-	-	-	-	-
MMP9	20q12-q13	7.653 kbp (44070954–44078607)	7	0.0549	0.421	0.2193	0.579	0.421	0.528	0.2409	-	-	-	-	-	-	-
NFκB1	4q24	116 kbp (103641518–10357506)	9	0.0144	0.162	**0.0088**	**0.046**	0.046	0.079	0.1425	-	-	-	-	-	-	-
NFκBIA	14q13	3.228 kbp (34940467–34943694)	6	0.0035	0.059	**0.0036**	**0.046**	0.046	0.097	0.1425	-	-	-	-	-	-	-
NFκBIB	19q13.1	8.919 kbp (44082454–44091372)	7	0.0087	0.13	0.023	0.083	0.083	0.114	0.1425	-	-	-	-	-	-	-
PDGFα	7p22	21 bp (179285–179305)	5	0.047	0.367	0.0637	0.148	0.148	0.241	0.1854	-	-	-	-	-	-	-
PDGFRα	4q11-q13	68.97 kbp (54790204–54859171)	8	0.0165	0.197	0.5423	0.913	0.197	0.313	0.2056	-	-	-	-	-	-	-
PIK3	7q	41.67 kbp (106292977–106334801)	10	0.0551	0.611	**<0.0001**	**0.001**	**0.001**	**0.009**	**0.0300**	**0.0457**	**0.0470**	0.5491	0.982	**0.0456**	0.331	0.1866
SMAD3	15q21-q22	129.3 kbp (65145249–65274586)	10	0.0402	0.551	0.0188	0.371	0.371	0.747	0.2576	-	-	-	-	-	-	-
SMAD7	18q21.1	30.86 kbp (44700222–44731079)	7	**0.0001**	**0.002**	0.0603	0.313	**0.002**	***0.004***	***0.0250***	**0.0064**	**0.0070**	0.0554	0.311	**0.0064**	***0.047***	***0.0397***
TGFβ1	19q13.1	23.17 kbp (46528490–46551655)	6	0.0381	0.3	0.1671	0.397	0.3	0.434	0.2409	-	-	-	-	-	-	-
TGFβ2	1q41	95.1 kbp (216586200–216684584)	9	0.0079	0.151	0.035	0.318	0.151	0.237	0.1854	-	-	-	-	-	-	-
TNF	6p21.3	2.763 kbp (31651328–31654090)	11	**0.0013**	**0.034**	**0.0004**	**0.005**	**0.005**	***0.005***	***0.0250***	**0.0059**	**0.0060**	0.0870	0.416	**0.0059**	***0.044***	***0.0397***
TNFR2	1p36.22	42.22 kbp (12149647–12191872)	8	0.099	0.716	**<0.0001**	**0.013**	**0.013**	**0.019**	**0.0475**	0.1052	0.2140	0.2213	0.777	0.1052	0.649	0.2706

Chr, Chromosome; FR%, Failure rate; *n*, number of SNP studied in each gene; Single contrasts, single SNP associations. * Minimum *P* value obtained following single association analysis. † Minimum *P* value obtained following permutations. Haplotypic contrasts, haplotypic SNP associations. ‡ Minimum *P* value obtained following multiple association analysis. § Minimum *P* value obtained following permutations; Omnibus test, summary of *P* values from single associations and multiple associations. || Minimum *P* value obtained following the analysis of single and multiple associations. # Minimum *P* value obtained following permutations. q-value, second step of FDR procedure, where the set of genes were tested. In bold, statistical significant values. In bold and italic, those genes in which replication was observed.

In multiple analyses, no haplotype was associated to PVR. One subhaplotype was significantly associated in the *SMAD7*, the one which implies the two first markers: rs4939826-rs7226855 ($P = 0.0330$; odds ratio [OR] = 1.4062; CI 95%: 1.0637–1.8590). However, this association was lost following permutation (Table 4).

Finally, after corrections for multiple comparisons, replication was observed in two genes: *SMAD7* ($P = 0.047$) and *TNF locus* ($P = 0.044$) (Table 5).

Discussion

In the present study, we analyzed common genetic variants in genes implicated in the inflammatory cascade in order to investigate their role in the susceptibility to developing PVR following primary rhegmatogenous RD surgery.

After the discovery stage, four genes maintained their significant association to the disease following a very stringent statistical analysis. This finding warranted investigation whether these associations were genuine or not, as is recommended in the literature.[23,24,41,42] In the replication stage, studying a new larger sample of DNA from patients across different countries in Europe, two of these four genes continued to be significant: the *SMAD7* and the *TNF locus*.

Limitations of this work must be discussed before analyzing our results. Despite the fact that the exact mechanisms responsible of PVR are not completely understood, it is widely accepted that inflammation plays an important role in its pathogenesis.[3] Although not every gene participating in the inflammatory cascade could be included, genes encoding for the key mediators and their signaling pathway molecules were investigated. Genetic variation in each gene was widely covered, taking into consideration that in addition to the parameters used for the SNPs selection (TagSNPs with a $r^2 \geq 0.85$), previously described as functional SNPs and extragenic regions were also studied.

Other important issue in an association study is the sample size.[24] When we analyze markers with a very subtle effect on the entire population, there is a significant risk that the genes may be below the threshold for detection. The power sample was greater than 80% for most of genes. In the replication stage *TNFR2* did not reach 70%, as it would have been necessary to collect more than 800 samples to achieve this power. This would be extremely challenging for a low prevalence condition such as PVR. This could have been one of the reasons for not detecting replication in this gene.

An inadequate phenotyping and stratification are other factors that could invalidate these kinds of studies.[41] In order to prevent this, very stringent exclusion and inclusion criteria for classification of patients were defined (e.g., PVR patients were included only once they have been operated on by PPV, confirming preoperatively the diagnosis by the membrane peeling; complexity of RDs was homogeneous as those patients with PVR grade C1 or higher were excluded). Although not all clinical variables related with a high risk of developing PVR were recorded, the classification process exhaustively covered others that could induce any kind of error during the phenotyping process and that could also introduce biases such as history of trauma, status of the lens, and so on. In addition to phenotyping, location is the other main source of subpopulation in these kind of studies. No differences regarding geographical origin or center where the patients came from were found as well as race, sex, and so on. Methodology of collection of samples was also carefully defined for the replication stage in order to avoid including same patients in those centers that had participated in the discovery stage.

In the replication stage, there were some differences among clinical variables that worth to be discussed. Cases were younger than controls and this could be considered a confounding factor. However, this difference was only 6 years, making it unlikely that this is responsible for the differences found in the genetic profiles of cases and controls. Moreover, this difference should not be of concern as in another work carried out by our group we found that in people aged over 55 there was a risk for PVR (Pastor JC, Fernandez I, Rodriguez de la Rua E, et al., unpublished data, 2012). In younger patients (who had a higher rate of PVR), the posterior vitreous may be more frequently attached and is more difficult to peel. Although the difference in age between groups was not great, this could explain why non PVR controls are older than cases in our sample. A history of PVR in the fellow eye was more frequently observed in cases, as is expected since those patients with RD and history of PVR in the fellow eye were excluded. Regarding the systemic or ocular diseases and treatments receiving at the time of the RD, there were very few patients that reported these characteristics. Then it would not be appropriate to draw any conclusion about it. Regarding the differences observed in the intraoperative variables, it is important to point out that each procedure was recorded as positive or negative. In this way, one patient could have been operated on by more than one procedure (e.g., SS + d and PPV in a second time). This justifies that PPV or the usage of SO as tamponade are more frequent among cases. Cases could have required intravitreal procedures in order to peel membranes while scleral procedures could have been sufficient among controls. These differences could be considered a bias, as they could mean that we have included more complicated RDs in the case group, and then we could have an spurious association. However, there are some issues to emphasize. First, it is has been demonstrated that current tendency of retinal surgeons toward PPV for treating RDs is not based in a judgment of the degree of complexity of the RDs. In addition to that, if we take into account that PVR is per se a complication of the RD surgery, we could say that we did analyze more complicated RD in this group. The question that arises here is why these RDs are more complicated. Genetic profile could be one of the answers. In order to avoid this confounding factor, those procedures orientated to treat the RDs once the patient developed PVR should have probably been eliminated. In this way, only procedures in RDs clinically comparable would have been considered and this *intríngulis* would have been solved. Despite that, it worth to remark that phenotype was carefully defined, and well recognized experts on the vitreoretinal surgery were in charge of classifying the patients. Therefore, we consider that we can rely that patients were correctly classified as case or control. Since phenotyping was correctly performed, we consider that all mentioned above do not invalidate our work.

It is important to remark that half of patients who suffered from PVR following RD surgery were operated on by an experienced surgeon. This allows us to downplay the influence of the surgeon's skill in the susceptibility to develop PVR, stressing also the importance of the genetic component. Finally, one of the major pitfalls of genetic association studies is the problem of multiple comparisons, which may throw up anomalous connections purely by chance.[24] In order to limit the presence of these spurious associations we have used a multiple comparison procedure based on the estimation of the false discovery rate (FDR).[35] The estimation of this rate of "false positives" was used to correct the original *P* values. We have set the threshold of significance level, traditionally 0.05, and we have considered that a association is statistically significant when the adjusted *P* value, and not the original *P* value, is less than this threshold.

Threshold of significance is another important issue here. Ioannidis states that the threshold for declaring the presence or not of an effect can still be a subject of discussion of schools of statistics.[43] In our study, we observed *P* values well below the traditional 0.05 that Ioannidis admits for the replication data (0.0070 for *SMAD7* and 0.0060 for *TNF locus* in the single analysis of replication data). Following the FDR estimation, we observed that 95% of these associations can be genuine, which can be consider a reliable result to continue working on these genes in future researches.

Regarding the replication found in *SMAD7* gene and the TNF locus, it reinforces the idea that prevention or even treatment of PVR should probably be targeted to the inflammation mediators and their signaling pathway molecules.

High vitreous levels of *TNF-α* and its receptors (*TNFR1* and *2*) have been found in eyes with PVR[44] and local production of TNF-α has been suggested to occur in these eyes.[45] Keeping in mind the important role of *TNF-α* and its signaling pathway as early mediators in the inflammatory cascade, it makes sense that cytokines from the *TNF-family* have a protagonical role in the genesis of PVR. Our finding in the *TNF locus* reinforces this notion.

Apoptosis may be another important mechanism in PVR, where *TGF-β* has been strongly implicated.[46] Amongst other numerous biological functions of *TGF-β*, of significance may be the stimulation of epithelial-mesenchymal cells, fibroblast-myofibroblast conversion, and the enhanced expression of extracellular matrix proteins[47]; all key processes in the establishment of PVR. The *TGF-β* family members (*TGFβ*1–3) bind to their membrane receptors, and use the SMADs signaling pathway. *SMAD2* and *3* (known as receptor-regulated *SMADs*) play an important role in the activation of *TGF-β*–dependent gene targets, with *SMAD3* mediating the mainly profibrotic actions of *TGF-β*. *SMAD7* is known to be inhibitory, blocking phosphorylation of *SMAD2/3*.[47,48] These events are evidence of potentially important roles that SMAD proteins could have in the pathogenesis of PVR. In fact, over expression of *SMAD7* suppresses the fibrotic response of RPE cells to *TGF-β2* in mice, inhibiting the RPE cells transition to myofibroblasts.[49] Our results highlight the possible role of *SMAD7* in the development of PVR in humans.

One question that arises from the above is why *TGF-β* was not significantly associated to the disease. One explanation could be that there were three markers (two in the *TGF-β1* and one in the *TGF-β2*) without information either for failure in the design pipeline or during the genotyping process. That means that some regions of the gene were not exhaustively studied as there were some SNPs that could not be genotyped. In order to solve this problem, *TGF-β* should be studied by other method.

In summary, we have confirmed that two genes previously implicated in the establishment of PVR are indeed significantly associated to the disease in Europeans undergoing retinal detachment surgery. These results may help us to understand the molecular basis of this complication and could potentially guide us to develop new strategies in the prevention or treatment of PVR. Further experimental studies on these genes are now warranted.

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APPENDIX

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