Polymorphisms in *XRCC5, XRCC4, NFKB2,* and *BIRC5* genes: Influence in risk and prognosis of monoclonal gammopathies

Letícia Balanco[1], Ana Cristina Gonçalves[2,3,4], Luisa Mota–Vieira[5,6,7*], Ana Bela Sarmento–Ribeiro[2,3,4,8*]

[1]Faculty of Medicine, University of Coimbra (FMUC), Coimbra, Portugal; [2]Laboratory of Oncobiology and Hematology (LOH), University Clinic of Hematology and Applied Molecular Biology Unit, FMUC, Coimbra, Portugal; [3]Center of Investigation in Environment, Genetics and Oncobiology (CIMAGO), FMUC, Coimbra, Portugal; [4]Center for Neuroscience and Cell Biology and Institute for Biomedical Imaging and Life Sciences (CNC.IBILI), Coimbra, Portugal; [5]Molecular Genetics and Pathology Unit, Hospital of Divino Espírito Santo of Ponta Delgada, EPER, Ponta Delgada, São Miguel Island, Azores, Portugal; [6]Azores Genetics Research Group, Instituto Gulbenkian de Ciência, Oeiras, Portugal; [7]BioISI-Byosystems & Integrative Sciences Institute, University of Lisboa, Faculty of Sciences, Lisbon, Portugal; [8]Clinical Hematology Department, Centro Hospitalar Universitário de Coimbra, EPE (CHUC, EPE), 3000‑548 Coimbra, Portugal

[*]Both authors contributed equally to this work.

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LIST OF ABBREVIATIONS AND ACRONYMS

BIRC5	Baculoviral IAP repeat containing 5 (survivin)
BM	Bone marrow
CHUC	Centro Hospitalar e Universitário de Coimbra
CI	Confidence interval
CRAB	Hipercalcemia, renal insufficiency, anemia and lytic bone lesions
DNA	Deoxyribonucleic acid
DSB	DNA double strand break
EPE	Entidade Pública Empresarial
EPER	Entidade Pública Empresarial Regional
GM	Gamapatias monoclonais
HDFF	Hospital Distrital da Figueira da Foz
HR	Hazzard ratio
HWE	Hardy–Weinberg equilibrium
IC	Intervalo de confiança
IMWG	International Myeloma Working Group
ISS	International Staging System
MAF	Minor allele frequency
MG	Monoclonal gammopathies
MGUS	Monoclonal gammopathy of undetermined significance
MM	Multiple myeloma/ mieloma múltiplo
NFKB	Nuclear factor–kappa B
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B–cells 2
NHEJ	Non–homologous end–joining
NSCLC	Non–small cell lung cancer
OMIM	Online Mendelian Inheritance in Man
OR	Odds ratio
PCR	Polymerase chain reaction
SMM	Smoldering multiple myeloma
SNP	Single nucleotide polymorphism
XRCC4	X–ray repair complementing defective repair in Chinese hamster cells 4
XRCC5	X–ray repair complementing defective repair in Chinese hamster cells 5

RESUMO

Introdução: As gamapatias monoclonais (GM) são um conjunto de entidades nosológicas, caracterizadas pela proliferação clonal de células B, que incluem uma condição maligna – o mieloma múltiplo (MM). Diversas vias moleculares, nomeadamente a via da reparação por junção de extremidades não–homólogas, a via do fator nuclear–κB e a apoptose, desempenham um papel fundamental na patogénese do MM. Assim, admitimos a hipótese de que polimorfismos nos genes envolvidos nestas vias influenciam a suscetibilidade e o prognóstico das GM.

Métodos: No presente estudo caso-controlo de base hospitalar, analisámos oito polimorfismos em quatro genes (*XRCC5*, *XRCC4*, *NFKB2* e *BIRC5*), através da genotipagem de 189 indivíduos (63 doentes com GM e 126 controlos) com recurso à técnica TaqMan de PCR em tempo real. Os resultados são expressos em termos de frequências alélicas, genotípicas, haplotípicas e de perfis genéticos, e a sua correlação com a suscetibilidade para desenvolver GM. Investigámos, ainda, a associação dos referidos SNPs com a sobrevivência global.

Resultados: A análise dos dados revelou duas associações com GM. Primeiramente, a análise após estratificação por género sugeriu uma menor predisposição para GM nos indivíduos do género masculino portadores dos genótipos *NFKB2* rs12769316 GA e AA (OR 0.346, IC 95% 0.124–0.965, $p = 0.043$). Em segundo lugar, observámos que doentes com o genótipo *BIRC5* rs9904341 CC apresentaram uma sobrevivência global significativamente reduzida (modelo recessivo: HR 4.89, IC 95% 5.06–199.70, $p < 0.01$). O haplótipo *BIRC5* (rs4789551, rs9904341, rs8073069) GGC foi encontrado apenas num doente, sendo inexistente nos controlos.

Conclusão: O presente estudo sugere que a variante genética *NFKB2* c.-1853A pode estar associada com uma menor suscetibilidade para desenvolver GM nos indivíduos do género masculino, e que o genótipo *BIRC5* c.-31CC pode influenciar negativamente o prognóstico das GM. Todavia, são necessários mais estudos para que as associações encontradas possam ser validadas e para que o papel dos polimorfismos genéticos na suscetibilidade e no prognóstico das GM seja esclarecido.

Palavras–chave

Gamapatias monoclonais, mieloma múltiplo, reparação por junção de extremidades não–homólogas, fator nuclear–κB e *BIRC5*.

ABSTRACT

Background: Monoclonal gammopathies (MG) are a group of clonal B–cell proliferation disorders, which includes a malignant condition – multiple myeloma (MM). Several molecular pathways, namely non homologous end–joining (NHEJ) repair pathway, nuclear factor–κB pathway, and apoptosis, play a well–established role in MM pathogenesis. Here, we hypothesized that polymorphisms of genes involved in these pathways impact MG susceptibility and prognosis.

Methods: In the present hospital-based case-control study, we analyzed eight polymorphism in four genes (*XRCC5*, *XRCC4*, *NFKB2*, and *BIRC5*), by genotyping 189 individuals (63 MG patients and 126 controls) using TaqMan real–time PCR technique. The results are expressed in terms of frequencies of allele, genotype, haplotype, and genotypic profiles, and their correlation with MG susceptibility. We also investigated the association of these SNPs with overall survival.

Results: The data analysis revealed two associations with MG. First, the analysis by gender stratification suggested decreased predisposition to MG in male carriers of *NFKB2* rs12769316 GA and AA genotypes (OR 0.346, 95% CI 0.124–0.965, $p = 0.043$). Second, we observed that patients with *BIRC5* rs9904341 CC genotype had a highly significant lower overall survival (recessive model: HR 4.89, 95% CI 5.06-199.70, $p < 0.01$). *BIRC5* GGC haplotype (rs4789551, rs9904341, and rs8073069) was found in one patient and absent in controls.

Conclusion: The present study suggests that *NFKB2* c.-1853A gene variant may be associated with MG susceptibility in males, and *BIRC5* c.-31CC genotype may negatively influence MG prognosis. Nonetheless, further studies are needed to validate

these findings, and enlighten the role of genetic polymorphisms in MG susceptibility and prognosis.

Keywords

Monoclonal gammopathies, multiple myeloma, non–homologous end–joining repair, nuclear factor–κB, and *BIRC5*.

INTRODUCTION

Monoclonal gammopathies (MG) are a spectrum of disorders that arise from B–cell proliferation in the bone marrow (BM) microenvironment and produce a monoclonal immunoglobulin. Monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM) represent the asymptomatic side of the spectrum, while multiple myeloma (MM) occupies the symptomatic counterpart[1].

In MM patients, the overproduction of plasma cells and its products, along with the host response to them, results in a myriad of end-organ damage related signs and symptoms, namely hipercalcemia, renal insufficiency, anemia, and lytic bone lesions (CRAB)[2]. The diagnosis of MM requires three criteria: (1) at least 10% of monoclonal plasma cells in the BM and/or biopsy–proven plasmacytoma, (2) monoclonal protein (M-component) in serum or urine, and (3) CRAB features[3, 4]. Interestingly, 15% of all patients with newly diagnosed MM have an asymptomatic and indolent disease form, the SMM[5]. This form of the disease is defined by a serum M–component greater than 3 g/dL and/or 10% to 60% of BM plasmacytosis with no evidence of CRAB criteria or myeloma-defining events[5, 6]. SMM patients have an annual probability of progression to active MM of 10% during the first 5 years after diagnosis, 3% for the next 5 years, and 1% for the following 10 years; at 15 years, the cumulative risk of progression is 73%[7]. Moreover, MM consistently evolves from MGUS or SMM[8].

Even though little is known about MM risk factors, a substantial fraction is expected to be ascribable to genetic variants. While it has been estimated that 15.2% (±2.8%) of the heritability of developing myeloma can be explained by SNPs, current known risk SNPs explain only 2.9% (±2.4%) of MM heritability[9]. Therefore, there is a strong rationale for pursuing the search for new susceptibility genetic variants associated with MM. A

good strategy for finding these unknown variants might be the screening of polymorphisms in candidate genes that are involved in key biological pathways related with MM pathogenesis, such as non–homologous end–joining (NHEJ) repair pathway, nuclear factor–κB (NFKB) pathway, and apoptosis.

NHEJ is an essential pathway that monitors and repairs DNA double strand breaks (DSBs), which is one of the most deleterious DNA lesions[10]. During B–cells development, the processes of V(D)J recombination and class switch recombination intentionally generate DSBs[11, 12]. Unrepaired or misrepaired DSBs are associated with genomic instability and oncogenesis, increasing the risk of cancers such as MM[11, 13]. Thus, variants in genes implicated in NHEJ repair, like *XRCC5* and *XRCC4*, may correlate with MM predisposition.

The NFKB is a critical signaling pathway that regulates the expression of proteins relevant for cell proliferation, inflammation, angiogenesis, and suppression of apoptosis[14]. Mutations involving NFKB pathway are implicated in more than 17% of MM cases and at least 40% of MM cell lines[15]. *NFKB2* is one of the main genes of this pathway. Du *et al.* found that *NFKB2* gene polymorphisms influence MM prognosis in Chinese Han subjects[14]. Further studies are needed to analyze the impact of *NFKB2* polymorphisms in other populations.

Another important mechanism related with myelomagenesis is apoptosis deregulation[16]. Survivin, an antiapoptotic protein encoded by *BIRC5* gene, was reported as one of the most tumor-specific genes[17]. Romagnoli *et al.* found that survivin is overexpressed in MM cells and the level of its expression is correlated with clinical course[18]. However, there are no previous studies investigating the relationship between survivin polymorphisms and MM susceptibility.

Aims

The purpose of the present study was to test the hypothesis that several SNPs in genes, namely *XRCC5*, *XRCC4*, *NFKB2* and *BIRC5*, of key pathways could have an impact in inherited susceptibility to develop MG, in particular MM and SMM. We also hypothesized that these polymorphisms may influence the prognosis, particularly overall survival, in patients with MG.

MATERIALS AND METHODS

Ethical statement

The present study was conducted in accordance with the ethical principles contained in the amended Declaration of Helsinki and the European Convention on Human Rights and Biomedicine of 1997 ("Oviedo Convention"). The Ethics Committee of Faculty of Medicine of University of Coimbra (Portugal) approved the research protocol. All individuals gave their written informed consent. The international ethical guidelines of anonymity of personal data, confidentiality, and abandonment option in the case of expressed will were followed.

Study design and population

We conducted a hospital-based case-control study that included 63 individuals with monoclonal gammopathies (53 multiple myeloma cases and 10 smoldering multiple myeloma cases) and 126 control individuals. Both cases and controls were recruited from two hospitals located in the central region of Portugal, "Centro Hospitalar e Universitário de Coimbra, EPE (CHUC, EPE)" and "Hospital Distrital da Figueira da Foz, EPE (HDFF, EPE)", from March 2010 to January 2016. Patient characteristics and follow–up information were collected and updated during hospital visits, and ended at November 2014.

Patients were diagnosed according to the International Myeloma Working Group (IMWG) criteria for the diagnosis of MG, and were classified according to the International Staging System (ISS). We used one variable as study endpoint: overall survival (OS). The OS was measured from the date of MM or SMM diagnosis. OS endpoints were deceased or alive at the date of last contact (patients who were still alive were censored). The control group was composed of healthy blood donors and

individuals with no known history of neoplasia attending the same hospitals. In order to minimize potential bias, cases and controls were matched based on gender and age (± 5 years). Characteristics of patients and controls are summarized in Table 1.

Table 1. Demographic and relevant clinical information of monoclonal gammopathy patients and controls.

Characteristics	Patients (total=63)		Controls (total=126)	
	n	%	*n*	%
Age[*] (years)	70.11 ± 10.25		69.90 ± 10.06	
Gender				
Male	31	(49)	61	(48)
Female	32	(51)	65	(52)
Condition				
SMM	10	(16)		
MM	53	(84)		
ISS				
I	17	(27)		
II	19	(30)		
III	27	(43)		
CRAB symptoms	(total=53)			
Hypercalcemia[†]	6	(11)		
Anemia[‡]	38	(72)		
Renal insufficiency[§]	9	(17)		
Bone disease[e]	35	(66)		

[*]Mean ± standard deviation. [†]Corrected serum calcium >11.5 mg/dL. [‡]Hemoglobin <10g/dL or >2 g/dl below normal lower limit. [§]Serum creatinine > 2mg/dL. [e]Lytic lesions, severe osteopenia and/ or pathologic fractures. SMM, smoldering multiple myeloma; MM, multiple myeloma; ISS, international staging system.

Genes and SNP selection

Since the focus of the present study was polymorphisms that might influence MM susceptibility, gene pathways related with MM pathogenesis were of particular interest. In order to identify potentially relevant pathways, we searched the medical literature querying two online databases (PubMed[1] and Cochrane[2]). For this purpose, first, we used widely known search strategies, such as the use of keywords, followed by the reading of abstracts and the evaluation of references of selected papers, as well as

[1] PubMed – http://www.ncbi.nlm.nih.gov/pubmed

[2] Cochrane – http://onlinelibrary.wiley.com/cochranelibrary/search

bibliographies provided by the supervisors of this work. During this stage, pathways associated with cell cycle control and DNA repair gained our attention, namely: NHEJ repair pathway, NFKB pathway, and apoptosis. Second, key genes involving these molecular processes were selected, using additionally OMIM[3], Ensembl[4], and Gene[5] databases. Finally, SNPs were chosen based on the following criteria: (1) known or promising pertinence to predisposition for MM or other human cancers, (2) reported association with tumorigenesis in humans, (3) minor allele frequency (MAF) ≥ 0.05 in Iberian population, according to 1000 Genomes[6] database, (4) validated allele substitutions, and/or (5) previously reported functional changes correlated with allele substitutions. Besides 1000 Genomes, we used other databases, like dbSNP[7] and SNPedia[8], for SNP evaluation and stratification in light of the above mentioned criteria. Noteworthy information of the eight selected SNPs is presented in Table 2.

DNA extraction

Human genomic DNA from cases and controls was extracted from blood samples and collected into EDTA tubes. DNA was quantified in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA).

SNP genotyping

The allelic discrimination of SNPs was performed by TaqMan MGB assays on a 7500 Fast real–time Polymerase Chain Reaction (PCR) equipment (Life Technologies,

[3] OMIM – http://www.omim.org/

[4] Ensembl – http://www.ensembl.org/index.html

[5] Gene database – http://www.ncbi.nlm.nih.gov/gene

[6] 1000 Genomes – http://www.1000genomes.org/home

[7] dbSNP – http://www.ncbi.nlm.nih.gov/SNP/

[8] SNPedia – http://www.snpedia.com/index.php/SNPedia

Table 2. Summary information for selected SNPs in candidate genes tested for association with monoclonal gammopathies (multiple myeloma and smoldering multiple myeloma).

Gene[*]	Pathway	dbSNP	Chromosomal position (GRCh38.p2) [†]	Variant	Most severe consequence[‡]	SNP functional effect			Iberian MAF[e]	Reported association					Ref
						FS[§]	Categ	Pathogenicity		Cancer type	Allele/ genotype	OR	HR	(95% CI)	
XRCC5	NHEJ	rs1051685	2:216205653	c.451A>G	3'-UTR	0.500	T reg	Changed	0.1262 (G)	MM[¶]	GG	[¥]	–	–	[19]
XRCC4	NHEJ	rs6869366	5:83075927	c.-1699T>G	Intron	0.000	T reg	Not changed	0.0701 (G)	NSCLC[¶]	TG+GG	1.86	–	(1.18-2.91)	[20]
		rs963248	5:83238075	c.746-20455T>C	Intron	0.500	T reg	Changed	0.2103 (C)	MM[¶]	A	1.51	–	(1.10-2.08)	[19]
NFKB2	Various	rs12769316	10:102392994	c.-1853G>A	Upstream gene	0.500	T reg	Changed	0.1916 (A)	MM[#]	GA+AA	–	0.26	(0.08-0.89)	[14]
		rs1056890	10:102403013	c.*637G>A	3'-UTR	0.215	T reg	Changed	0.3692 (A)	MM[#]	CT+TT	–	2.36	(1.02-5.46)	[14]
BIRC5	Apoptosis	rs8073069	17:78213692	c.-625G>C	Upstream gene	0.176	T reg	Changed	0.2804 (C)	NSCLC[#]	GG	–	1.76	(1.16-2.67)	[21]
		rs9904341	17:78214286	c.-31G>C	5'-UTR	0.268	T reg	Changed	0.2757 (C)	PC[¶]	CC	1.85	–	(1.12-3.01)	[22]
		rs4789551	17:78214998	c.221+209T>C	3'-UTR	0.176	T reg	Changed	0.0841 (C)	NSCLC[#]	CC	–	2.04	(1.08-3.86)	[21]

[*]According to HUGO Gene Nomenclature Committee (HGNC). [†]According to Genome Reference Consortium Human Build 38 patch release 2. [‡]According to Ensembl Variant Effect Predictor (VEP). [§]According to F-SNP database. Statistical analysis of SNPs at splicing, transcriptional, translational and post-translational levels are used to predict the F-SNP functional score (FS). [e]Minor Allele Frequency source: 1000 Genomes. [¶]Associated with disease susceptibility. [#]Associated with overall survival. [¥]The GG genotype was identified in 10 MM patients and only one control; thus, a recessive model was statistically tested using Fisher's exact test ($P = 0.015$). OR, odds ratio. HR, hazzard ratio; CI, confidence interval; NHEJ, Non-homologous end joining; MM, Multiple myeloma; PC, Pancreatic cancer; NSCLC, Non-small cell lung cancer; *NFKB2*, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2; *BIRC5*, Baculoviral IAP Repeat Containing 5 (survivin); *XRCC4*, X-ray repair complementing defective repair in Chinese hamster cells 4; *XRCC5*, X-ray repair complementing defective repair in Chinese hamster cells 5; Categ, category; T reg, transcriptional regulation; Ref, reference; A, adenine; G, guanine; T, thymine; C, cytosine; MM, multiple myeloma; NSCLC, Non-small Cell Lung Cancer; PC, pancreatic cancer.

USA), according to manufacturer's instructions. These pre–designed assays use two primers and two dually labeled fluorescent probes that are allele–specific. Succinctly, we used 25.00 ng of DNA for PCR amplification. Each PCR (12.40 µL) contained 6.00 µL of TaqMan Genotyping Master Mix, 0.16 µL primers and probes, 5.24µL of water and 1.00 µL DNA (25.00 ng/µL). Reactions were run on 96–well plates. In every plate, a negative control containing water instead of genomic DNA was simultaneously tested. The DNA amplification was performed under the following cycling conditions: pre–PCR holding at 60ºC for 1 minute, followed by enzyme activation at 95ºC for 10 minutes, a two–stage polymerase run of 40 cycles at 95ºC for 15 seconds, then 60ºC for 1 minute, and a final post–PCR holding at 60ºC for 1 minute. In approximately 10% of the samples, genotyping was repeated by virtue of accuracy.

Statistical analysis

We calculated allele and genotype frequencies by direct counting. The Hardy–Weinberg equilibrium (HWE) in the control group was tested using Arlequin software (version 3.5.2.2). Statistical analysis of the data was performed on IBM SPSS Statistics (version 23.0). The mean data of patients and controls were subjected to normality test (Kolmogorov–Smirnov test). If data obeyed the normal distribution, we would use the parametric Student's *t*–test; otherwise, we would perform the nonparametric Mann-Whitney U test. Gender differences were tested using Pearson's Chi–square test. Haplotypes were indirectly inferred using a maximum likelihood method on Arlequin software. The odds ratio (OR) and the respective 95% confidence interval (CI) were determined as a measurement of association between genotypes and risk for MM or SMM, by applying the Fisher's exact test with GraphPad Prism (version 6.0) or by unconditioned logistic regression with SPSS. In order to test the disease association with SNPs, we compared the minor allele of control group with the major allele as

reference. Genotype association was analyzed according to three genetic models: (1) codominant model (each genotype was compared with homozygous of major allele of control group), (2) dominant model (minor allele carriers were compared with homozygous of major allele of control group), and (3) recessive model (homozygous of minor allele were compared with major allele carriers of control group). We applied the Kaplan-Meier method to estimate overall survival of patients stratified according to their genotypes using SPSS. Differences in survival were tested with log rank statistic. The hazard ratio (HR) and its 95% CI were calculated using Cox proportional hazard model. All statistical analyses were two–sided: $p < 0.05$ was set as statistically significant, and $p < 0.01$ was considered to be highly significant.

RESULTS

Characteristics of MG patients and controls

A total of 189 individuals were enrolled in the present hospital-based case-control study, being 63 monoclonal gammopathy (MG) patients and 126 controls. In the patient group, 51% (32/63) of the individuals were females and 49% (31/63) were males; the mean age was 70.11 ± 10.25 years old. Among the control group, 52% (65/126) of the individuals were females and 48% (61/126) were males, and the mean age was 69.90 ± 10.06 years old. We assessed differences in age and gender to eliminate potentially confounding variables and confirm proper matching. No age– or gender–related differences were found between MG patients and controls (p = 0.785 and p = 0.918, respectively). These results ensure the adequacy of group matching.

Most of patients were diagnosed with multiple myeloma (84%, 53/63) and the remaining ones (16%, 10/63) were diagnosed with smoldering multiple myeloma. According to the ISS classification, 43% (27/63) of patients had a stage III disease. As far as CRAB criteria were concerned, anemia was detected as the most prevalent of these features, being present in 72% (38/53) of MM cases, followed by bone lesions in 66% (35/53), while renal insufficiency and hypercalcemia were detected in only 17% (9/53) and 11% (6/53) of MM patients, respectively. Characteristics of patients (including clinical features) and controls are described in Table 1.

Frequencies and association analyses of alleles and genotypes with MG risk

In order to evaluate the contribution of polymorphisms in genes involved in non-homologous end–joining repair (NHEJ) pathway, nuclear factor–κB pathway, and apoptosis to MG development, we estimated odds ratios (95% CI) using logistic regression. In the present study, all tested SNPs were in Hardy–Weinberg equilibrium in

patients and controls. Allele frequencies of patients were compared against controls (Table 3). Minor allele frequencies were similar in both groups and no statistically significant association was found (all $p > 0.05$). Table 4 presents genotype frequencies in these groups, along with the respective OR. We found no statistically significant association between any of the SNPs and MG susceptibility ($p > 0.05$, all comparisons). However, gender and age (≥70 and < 70) stratification was performed (data not shown), and the analysis by gender revealed decreased predisposition for MG in male carriers of *NFKB2* rs12769316 GA and AA genotypes (dominant model: OR 0.346, 95% CI 0.124–0.965, $p = 0.043$). There were no age–related differences in genotypes (all $p > 0.05$).

Table 3. Allele frequencies of selected SNPs in patients and controls, and its association with risk of monoclonal gammopathies (multiple myeloma or smoldering multiple myeloma)

Gene	dbSNP	Minor allele*	Patients	Controls	Patients *vs* controls		
			MAF	MAF	OR	(95% CI)	*p*-value
XRCC5	rs1051685	G	0.103	0.099	1.045	(0.485-2.226)	1.000
XRCC4	rs6869366	G	0.016	0.056	0.274	(0.042-1.292)	0.102
	rs963248	C	0.198	0.202	0.976	(0.551-1.719)	1.000
NFKB2	rs12769316	A	0.151	0.170	0.863	(0.459-1.612)	0.661
	rs4789551	A	0.389	0.353	1.165	(0.731-1.856)	0.499
BIRC5	rs8073069	C	0.175	0.214	0.776	(0.431-1.388)	0.415
	rs9904341	C	0.349	0.417	0.751	(0.470-1.199)	0.221
	rs4789551	C	0.056	0.075	0.721	(0.267-1.879)	0.526

*Minor allele of controls and database (1000 Genomes Iberian population). MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; G, guanine; C, cytosine; A, adenine.

Association analysis of haplotypes with MG risk

In pursuance of potential associations between the three *BIRC5* polymorphisms (rs4789551, rs9904341, and rs8073069) with MG susceptibility, we performed a haplotype analysis. We detected five haplotypes and compared their frequencies in cases *versus* controls. No significant haplotype association was found (all $p > 0.05$). All haplotypes were common (frequency > 1%) in the patient group; however, H4 (GGC) was present in one patient and was not found in the control group (Table 5).

Table 4. Genotype frequencies of selected SNPs in multiple myeloma patients and controls, and its association with risk of monoclonal gammopathies (multiple myeloma or smoldering multiple myeloma).

Gene	dbSNP	Genotype	Patients		Controls		Patients *vs* controls		
			n	%	*n*	%	OR	(95% CI)	*p*-value
XRCC5									
	rs1051685	AA	50	(79.4)	103	(81.7)	Ref.		
		AG	13	(20.6)	20	(15.9)	1.423	(0.651-3.111)	0.376
		GG	0	(0.0)	3	(2.4)	–	–	–
		Dominant model					1.229	(0.572-2.639)	0.597
		Recessive model					–	–	–
XRCC4									
	rs6869366	TT	0	(0.0)	0	(0.0)	Ref.		
		TG	2	(3.2)	14	(11.1)	0.262	(0.058-1.192)	0.083
		GG	61	(96.8)	112	(88.9)	–	–	–
		Dominant model					0.262	(0.058-1.192)	0.083
		Recessive model					–	–	–
	rs963248	TT	40	(63.5)	84	(66.7)	Ref.		
		TC	21	(33.3)	33	(26.2)	1.336	(0.688-2.596)	0.392
		CC	2	(3.2)	9	(7.1)	0.467	(0.096-2.261)	0.344
		Dominant model					1.150	(0.611-2.165)	0.665
		Recessive model					0.426	(0.089-2.035)	0.285
NFKB2									
	rs12769316	GG	47	(74.6)	90	(71.4)	Ref.		
		GA	13	(20.6)	29	(23.0)	0.858	(0.408-1.805)	0.687
		AA	3	(4.8)	7	(5.6)	0.821	(0.203-3.320)	0.782
		Dominant model					0.851	(0.428-1.691)	0.645
		Recessive model					0.850	(0.212-3.405)	0.818
	rs1056890	GG	24	(38.1)	54	(42.9)	Ref.		
		GA	29	(46.0)	55	(43.7)	1.186	(0.614-2.292)	0.611
		AA	10	(15.9)	17	(13.5)	1.324	(0.529-3.312)	0.549
		Dominant model					1.219	(0.656-2.263)	0.531
		Recessive model					1.210	(0.518-2.823)	0.660
BIRC5									
	rs8073069	GG	42	(66.7)	79	(62.7)	Ref.		
		GC	20	(31.7)	40	(31.7)	0.940	(0.489-1.809)	0.854
		CC	1	(1.6)	7	(5.6)	0.269	(0.032-2.258)	0.226
		Dominant model					0.695	(0.356-1.359)	0.288
		Recessive model					0.291	(0.035-2.418)	0.253
	rs9904341	GG	25	(38.7)	45	(35.7)	Ref.		
		GC	32	(50.8)	57	(45.2)	1.000	(0.516-1.939)	1.000
		CC	6	(9.5)	24	(19.0)	0.489	(0.175-1.365)	0.172
		Dominant model					0.903	(0.477-1.711)	0.754
		Recessive model					0.467	(0.180-1.213)	0.118
	rs4789551	TT	56	(88.9)	107	(84.9)	Ref.		
		TC	7	(11.1)	19	(15.1)	0.704	(0.279-1.775)	0.457
		CC	0	(0.0)	0	(0.0)	–	–	–
		Dominant model					0.704	(0.279-1.775)	0.457
		Recessive model					–	–	–

The OR (95% CI) and *P*-value were calculated using logistic regression according to the following genetic models: codominant model (AA vs. AA, AA vs. Aa, and AA vs. aa, i.e. each genotype was compared with major allele homozygous genotype as reference); dominant model (AA vs. Aa + aa); and recessive model (AA + Aa vs. aa). Bold indicates statistically significant association. A, major allele; a, minor allele; OR, odds ratio; CI, confidence interval; Ref., reference; A, adenine; G, guanine; T, thymine; C, cytosine.

Table 5. Inferred *BIRC5* (survivin) haplotype frequencies in monoclonal gammopathy (multiple myeloma or smoldering multiple myeloma) patients and controls.

Haplotype*				Patients		Controls		Patients *vs* controls		
ID	rs4789551	rs9904341	rs8073069	*n*	%	*n*	%	OR	(95% CI)	*p*-value
H1	C	G	C	2	(3.6)	9	(7.5)	0.426	(0.061-2.213)	0.342
H2	C	G	T	9	(13.8)	18	(13.9)	1.000	(0.385-2.550)	1.000
H3	G	C	T	22	(34.9)	53	(41.7)	0.739	(0.376-1.448)	0.431
H4	G	G	C	1	(1.9)	0	(0.0)	ND	ND	
H5	G	G	T	29	(45.7)	46	(36.9)	1.483	(0.767-2.870)	0.212

*Haplotypes were inferred according to SNPs physical position. C, cytosine; G, guanine; T, thymine; ND, not determined.

Association analysis of genotypic profiles involved in NHEJ repair with MG risk

We also determined the genotypic profiles of gene SNPs involved in NHEJ repair pathway, *i.e. XRCC5* and *XRCC4* polymorphisms, with the purpose of detecting possible associations with risk for MG development. These profiles were compared between MG patients and controls (Table 6). We observed a total of seven genotypic profiles in the patient group and five additional profiles in controls. There were no significant differences regarding genotypic profiles between both groups (all $p > 0.05$).

Table 6. Genotypic profiles frequencies of *XRCC5* (rs1051685) and *XRCC4* (rs6869366, rs963248) in patients and controls, and its association with susceptibility for monoclonal gammopathies (multiple myeloma and smoldering multiple myeloma).

Genotypic profile*				Patients	Controls	Patients *vs* controls		
ID	*XRCC5*	*XRCC4*		*n* (%)	*n* (%)	OR	(95% CI)	*p*-value
	rs1051685	rs6869366	rs963248					
GP1	AA	TG	CC	1(1.6)	3 (2.4)	0.661	(0.026-7.343)	1.000
GP2	AA	TG	CT	1(1.6)	4 (3.2)	0.492	(0.020-4.822)	0.666
GP3	AA	TG	TT	0(0.0)	3 (2.4)	ND	ND	ND
GP4	AA	TT	CC	0(0.0)	1 (0.8)	ND	ND	ND
GP5	AA	TT	CT	16(25.4)	25 (19.8)	1.375	(0.632-2.983)	0.454
GP6	AA	TT	TT	32(58.8)	8 (53.9)	0.880	(0.459-1.687)	0.758
GP7	AG	TG	CC	0(0.0)	3 (2.4)	ND	ND	ND
GP8	AG	TG	CT	0(0.0)	1 (0.8)	ND	ND	ND
GP9	AG	TT	TT	1(1.6)	1 (0.8)	2.016	(0.054-75.184)	1.000
GP10	GG	TT	CC	4(6.3)	3 (2.4)	2.780	(0.505-16.262)	0.224
GP11	CC	TT	TT	8(12.7)	11 (8.7)	1.521	(0.522-4.374)	0.444
GP12	AG	TT	CC	0(0.0)	1 (0.8)	ND	ND	ND
GP13	AG	TT	CT	0(0.0)	2 (1.6)	ND	ND	ND

*Genotypic profiles are according to SNPs physical order. OR, odds ratio; CI, confidence interval; GP, genotypic profile; *XRCC5*, X-ray repair complementing defective repair in Chinese hamster cells 5; *XRCC4*, X-ray repair complementing defective repair in Chinese hamster cells 4; ND, not determined.

Prognostic impact of selected SNPs in MG overall survival

Finally, we assessed the impact of selected SNPs on MG prognosis, by analyzing the overall survival in patients whose follow–up information was available (n = 54). Overall survival was analyzed using the Kaplan–Meier method. Patients were stratified according to their SNP genotypes. As shown in Figure 1, we observed that patients with *BIRC5* rs9904341 CC genotype had a highly significant lower overall survival when compared to G carriers (recessive model: HR 4.89, 95% CI 5.06–199.70, $p < 0.01$). Differences in overall survival were tested with log rank test, and the HR with 95% CI was calculated using Cox proportional hazard model.

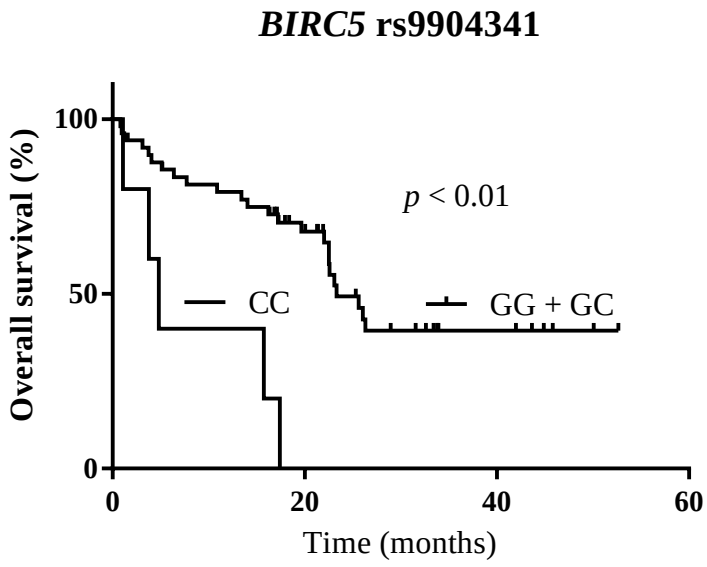

Figure 1. Overall survival curve of monoclonal gammopathy patients (MG), according to *BIRC5* rs9904341 genotypes (recessive model). Survival analysis was performed by Kaplan–Meier method.

DISCUSSION AND CONCLUSION

A comprehensive knowledge of the genetic scenario underlying monoclonal gammopathies is of the great relevance, since it can allow the identification of polymorphisms that predispose to MG and promote the development of new targeted therapies. The repercussions for patient care may go even further, as the understanding of these molecular mechanisms gives a better insight of disease prognosis and prediction of treatment response. In the present study, current results identified a polymorphism in *NFKB2* (c.-1853A) that is associated with MG susceptibility in male individuals, and in *BIRC5* (c.-31C) that is associated with overall survival, being a potential prognostic marker.

A considerable number of studies have stated the role of NHEJ pathway on maintaining genome stability and protecting cells from carcinogenesis[10, 23]. This DNA repair pathway has been implicated in the pathogenesis of many cancers, namely breast, pancreatic, non–small cell lung cancer (NSCLC), and hematologic neoplasias like chronic myeloid leukemia[20, 23-25]. Here, we detected no differences in NHEJ-related genotype frequencies between MG cases and controls. However, a Chinese study, based on 507 NSCLC patients, suggested the association of *XRCC4* rs6869366 TG and GG genotypes with an increased risk of NSCLC (OR 1.86, 95% CI 1.18–2.91). Moreover, Hayden *et al.* reported an association of *XRCC5* rs1051685 GG genotype and *XRCC4* rs963248 A allele with the risk of developing MM in individuals from six European countries (Germany, Italy, Spain, Ireland, France and Czech Republic)[19]. These diverge findings may be related to sample size and/or different population's genetic makeup.

Several studies provide genetic evidence that *NFKB2* signaling pathway plays a critical part in the pathogenesis of human MM[26, 27]. Du *et al.* reported that MM patients with *NFKB2* rs12769316 GA and AA genotypes had an increased overall survival ($p = 0.020$), while those with *NFKB2* rs1056890 CT and TT genotypes had a decreased overall survival ($p = 0.037$)[14]. Polymorphisms in this gene may also be implicated in treatment response and drug resistance[28]. In the present study, we found that only male carriers of *NFKB2* rs12769316 GA and AA genotypes had a decreased predisposition for MG; however, no association was observed between *NFKB2* polymorphisms and overall survival. These diverse results may be explained by differences in population ethnicities, since Du and colleagues conducted a study in 527 Chinese Han individuals (252 MM patients and 275 controls), and/or by the reduced sample size of our study, which is nearly three times smaller[14]. Given the importance of NFKB pathway on MG pathogenesis, more extensive studies with larger samples are needed in order to clarify the influence of *NFKB2* polymorphisms in MG susceptibility.

BIRC5 encodes survivin, an apoptotic inhibitor, which has been reported to confer genetic susceptibility and influence cancer prognosis[29-31]. Previous studies have shown that survivin is overexpressed in cancer cells of MM patients, and the expression level of this protein is significantly correlated with MM clinical course[18]. However, polymorphisms of *BIRC5* in patients with MM or SMM had not been investigated. In the present study, we observed that the *BIRC5* rs9904341 CC genotype is associated with overall survival (recessive model: HR 4.89, 95% CI 5.06–199.70, $p < 0.01$). No other significant association was found in *BIRC5* genotypes or haplotypes as regard to MG susceptibility or prognosis. Haplotype H4 (GGC) was present in only one patient and was not identified in the control group. Since this haplotype can be related to

disease susceptibility, further studies with a larger sample size are needed to test this association.

The present study has a few strengths and limitations. MG is a multifaceted group of plasma cell disorders which encompasses different clinical entities. Therefore, one of the main strengths of our work is that we only included MM and SMM cases, which may mitigate potential bias, as patients diagnosed with a milder condition like MGUS may be less prone to have susceptibility polymorphisms. Additionally, an important strength of this work was the proper matching, since both cases and controls were very similar in terms of gender and age. On the other hand, the greatest study limitation is the small sample size. Similar design studies usually enroll 200 to 500 patients. The sample size may limit the identification of meaningful differences. Besides, less common susceptibility SNPs, genotypes, haplotypes, and genotypic profiles are unlikely to be detected. A larger sample size could also narrow the confidence interval of significant associations. Even though all SNPs were in HWE, a quality control genotyping of some randomly selected samples using a sequencing technique should have been done in order to validate the genotypes obtained by real time – PCR and exclude potential genotyping errors.

In conclusion, the present study suggests that *NFKB2* rs12769316 GA and AA genotypes may be associated with MG susceptibility in male individuals and *BIRC5* rs9904341 CC genotype may influence overall survival of MG patients. Even though other polymorphisms failed to show a significant association, further research is needed to validate these findings. The understanding of gene variants implicated in NFKB pathway, NHEJ repair pathway and apoptosis can provide crucial information for targeted drug development and prediction of treatment outcome. Consequently, replication studies are important to confirm reported genetic associations, and large

scale genome wide association analysis can help uncover novel associations. In the near future, these more extensive studies will hopefully enlighten the role of genetic polymorphisms in MG.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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REFERENCES

1. Attaelmannan, M. and Levinson, S.S. Understanding and Identifying Monoclonal Gammopathies. *Clin Chem*, 2000. 46: 1230-1238.
2. Palumbo, A. and Anderson K. Multiple Myeloma. *N Engl J Med*, 2011. 364: 1046-1060.
3. Rajkumar, S.V. Evolving diagnostic criteria for multiple myeloma. *ASH Education Program Book*, 2015. 2015: 272-278.
4. Rajkumar, S.V., *et al*. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol*, 2014. 15: e538-e548.
5. Gao, M., *et al*. Smoldering multiple myeloma. *Biomed Res Int*, 2015. 2015: 7.
6. Rajkumar, S.V., *et al*. Smoldering multiple myeloma. *Blood*, 2015. 125: 3069-3075.
7. Kyle, R.A., *et al*. Clinical Course and Prognosis of Smoldering (Asymptomatic) Multiple Myeloma. *N Engl J Med*, 2007. 356: 2582-2590.
8. Bories, C. and Jagannath, S. Asymptomatic Monoclonal Gammopathies. *Clin Lymphoma Myeloma Leuk*, 2014. 14: S78-S86.
9. Mitchell, J.S., *et al*. Implementation of genome-wide complex trait analysis to quantify the heritability in multiple myeloma. *Scientific Reports*, 2015. 5: 12473.
10. Davis, A.J. and Chen, D.J. DNA double strand break repair via non-homologous end-joining. Transl Cancer Res, 2013. 2: 130-143.
11. Prochazkova, J. and Loizou, J.I. Programmed DNA breaks in lymphoid cells: repair mechanisms and consequences in human disease. *Immunology*, 2016. 147: 11-20.
12. Malu, S., *et al*. Role of non-homologous end joining in V(D)J recombination. *Immunol Res*, 2012. 54: 233-246.

13. Yang, C., *et al*. Impaired NHEJ function in multiple myeloma. *Mutat Res Fund Mol Mech Mut*, 2009. 660: 66-73.

14. Du, J., *et al*. Polymorphisms of nuclear factor-κB family genes are associated with development of multiple myeloma and treatment outcome in patients receiving bortezomib-based regimens. *Haematologica*, 2011. 96: 729-737.

15. Demchenko, Y.N., *et al*. Classical and/or alternative NF-κB pathway activation in multiple myeloma. *Blood*, 2010. 115: 3541-3552.

16. Unruhe, B., *et al*. An Old Flame Never Dies: Survivin in Cancer and Cellular Senescence. *Gerontology*, 2016. 62: 173-181.

17. Romagnoli, M., *et al*. The imbalance between Survivin and Bim mediates tumour growth and correlates with poor survival in patients with multiple myeloma. *Br J Haematol*, 2009. 145: 180-189.

18. Romagnoli, M., *et al*. Significant impact of survivin on myeloma cell growth. *Leukemia*, 2007. 21: 1070-1078.

19. Hayden, P.J., *et al*. Variation in DNA repair genes XRCC3, XRCC4, XRCC5 and susceptibility to myeloma. *Hum Mol Gen*, 2007. 16: 3117-3127.

20. He, F., *et al*. Association of XRCC3 and XRCC4 gene polymorphisms, family history of cancer and tobacco smoking with non-small-cell lung cancer in a Chinese population: a case–control study. *Am J Hum Genet*, 2013. 58: 679-685.

21. Dai, J., *et al*. Prognostic Significance of Survivin Polymorphisms on Non-small Cell Lung Cancer Survival. *J Thorac Oncol*, 2010. 5: 1748-1754.

22. Qin, L. and Yu, T. Association between rs9904341 G<C gene polymorphism and susceptibility to pancreatic cancer in a Chinese population. *Genet Mol Res*, 2015. 14: 5197-5202.

23. Pawlowska, E. and Blasiak, J. DNA Repair—A Double-Edged Sword in the Genomic Stability of Cancer Cells—The Case of Chronic Myeloid Leukemia. *Int J Mol Sci*, 2015. 16: 26049.

24. Willems, P., *et al*. Polymorphisms in nonhomologous end-joining genes associated with breast cancer risk and chromosomal radiosensitivity. *Genes Chromosom Cancer*, 2008. 47: 137-148.

25. Shen, Q., *et al*. Association of single nucleotide polymorphisms of DNA repair gene and susceptibility to pancreatic cancer. *Int J Clin Exp Pathol*, 2015. 8: 3180-3185.

26. McCarthy, B.A., *et al*. NF-κB2 mutation targets survival, proliferation and differentiation pathways in the pathogenesis of plasma cell tumors. *BMC Cancer*, 2012. 12: 203-203.

27. Demchenko, Y.N. and Kuehl, W.M. A critical role for the NFkB pathway in multiple myeloma. *Oncotarget*, 2010. 1: 59-68.

28. Abdi, J., *et al*. Drug resistance in multiple myeloma: latest findings and new concepts on molecular mechanisms. *Oncotarget*, 2013: 2186-2207.

29. Zhu, Y., *et al*. Association of Survivin Polymorphisms with Tumor Susceptibility: A Meta-Analysis. *PLoS ONE*, 2013. 8: e74778.

30. Lv, Y.G., *et al*. The role of survivin in diagnosis, prognosis and treatment of breast cancer. *J Thorac Dis*, 2010. 2: 100-110.

31. Jaiswal, P.K., *et al*. Survivin: A molecular biomarker in cancer. *Indian J Med Res*, 2015. 141: 389-397.