Polymorphisms of nuclear factor-κB family genes are associated with development of multiple myeloma and treatment outcome in patients receiving bortezomib-based regimens

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Acknowledgments: the authors would like to thank Benegen Services Inc. for technical support, and Alison Woo for editorial assistance.

Funding: this study was supported in part by the National Natural Science Foundation of China (NFSC30670884, 30900643) and sponsored by Shanghai Pujiang Program (09PJ1400400).

Manuscript received on July 13, 2010. Revised version arrived on December 6, 2010. Manuscript accepted on January 4, 2011.

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

The nuclear factor-κB pathway is an important signaling pathway activated in multiple myeloma cells. Bortezomib inhibits nuclear factor-κB activation and is an important antimyeloma agent. Nevertheless, patients treated with this drug eventually relapse. We hypothesized that the nuclear factor-κB pathway may be associated with multiple myeloma and patients' responses to bortezomib.

Design and Methods

In this study we analyzed 26 polymorphism sites of nuclear factor-κB family member genes, *IKBα*, *NFKB2*, and *TRAF3*, in 527 unrelated Chinese Han subjects (252 with multiple myeloma and 275 controls) using a Sequenom MassARRAY genotyping assay, and examined the outcome of 83 patients treated with a bortezomib-based regimen.

Results

Single nucleotide polymorphisms in the *TRAF3* rs12147254 A allele and a specific haplotype 1 of *TRAF3* [GAACAG] are associated with a decreased risk of multiple myeloma (odds ratio 0.709, $P<0.001$, and odds ratio 0.543, $P<0.0001$), while *TRAF3* haplotype 4 [GGACAG] was associated with an increased risk of development of multiple myeloma (odds ratio 2.099, $P=0.001$). Moreover, the *TRAF3* rs11160707 GA+AA genotype was significantly associated with a better progression-free survival ($P=0.018$). Patients with the *NFKB2* rs12769316 GA+AA genotype had a superior overall survival ($P=0.020$), while those with the rs1056890 CT+TT genotype had an inferior overall survival ($P=0.037$). In an exploratory analysis, patients with the GA+AA/CC/GG genotype at the rs12769316, rs1056890, and rs11160707 sites had a significantly superior overall survival compared to patients with a wild-type genotype ($P=0.007$). In the multivariable analysis, *TRAF3* rs11160707 was found to be an independent favorable factor for progression-free survival (hazard ratio 0.428, $P=0.028$).

Conclusions

Nuclear factor-κB family member gene polymorphisms play a role in the development of multiple myeloma and in the response to bortezomib therapy.

Key words: NF-κB, SNP, multiple myeloma.

Citation: Du J, Huo J, Shi J, Yuan Z, Zhang C, Fu W, Jiang H, Yi Q, and Hou J. 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(5):729-737. doi:10.3324/haematol.2010.030577

Introduction

Multiple myeloma (MM) accounts for approximately 10% of hematologic malignancies.[1] Despite new drug treatments and advances in stem cell transplantation, which have improved survival rates, MM remains an incurable disease. Of all the different signaling pathways constitutively activated in primary MM cells, the nuclear factor-κB (NF-κB) pathway has recently emerged as one of the most important drivers of the tumor-promoting machinery.[2]

NF-κB is a member of the Rel family of proteins, including RelA, RelB, cRel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100), which regulate the expression of proteins involved in cell proliferation, angiogenesis, metastasis, tumor promotion, inflammation, and suppression of apoptosis.[3-8] Activation of NF-κB proceeds along canonical and non-canonical pathways, and proteasome inhibitors specific to each of these pathways have been investigated in myeloma.[9] The canonical pathway is mostly dependent on NF-κB, through blocking the degradation of IKBα, which blocks the translocation of activated NF-κB into the nucleus and its binding to transcription factor motifs, leading to the transcription of genes.[10] The non-canonical NF-κB pathway is predominantly regulated by the control of NF-κB 2 (p100) processing to the active p52 isoform in MM.[11,12] *NFKB2* is a critical mediator in the development and function of a variety of organs and cell lineages, as shown by studies of genetically modified mice in which NF-κB2 function was ablated or modified.[13,14] Tumor necrosis factor-receptor-associated factor 3 (*TRAF3*), a putative ubiquitin ligase, can inhibit signaling in both the canonical and non-canonical NF-κB pathways by mediating NF-κB-inducing kinase degradation through direct binding to this kinase or by affecting other TRAF family members.[15,16] *TRAF3* is a tumor suppressor gene that is inactivated more frequently than any other known tumor suppressor in MM.[11,12] Because tumor cells often use NF-κB to achieve resistance to anticancer drugs and radiation,[17] the critical molecules of the NF-κB signaling pathways are molecular targets for the rational development of inhibitors that could be of therapeutic promise in MM.

Bortezomib, a proteasome inhibitor currently used in the treatment of MM and an inhibitor of activation of NF-κB, was approved by the USA Food and Drug Administration (FDA) in 2003, 2005, and 2008 for the treatment of relapsed/refractory, relapsed, and newly diagnosed MM, respectively.[18-20] Bortezomib-based regimens, including bortezomib-dexamethasone, bortezomib-thalidomide-dexamethasone (VTD), bortezomib-doxorubicin-dexamethasone (PAD), and bortezomib-thalidomide-dexamethasone - cisplatin-doxorubicin-cyclophosphamide-etoposide (VDT-PACE), have made a substantial contribution to high response rates and improvement in long-term outcomes in MM.[21-23] Bortezomib does, however, have several dose-limiting side effects, and all patients eventually relapse. The discovery and application of biomarkers of the NF-κB pathway should, therefore, help to improve the care and prognosis of patients with MM.

With the completion of the Human Genome Project, millions of single nucleotide polymorphisms (SNP) have been identified, which are thought to be attractive biomarkers in cancer risk assessment, screening, staging, and grading.[17] Although polymorphisms within the *IKBα* gene have been described to be associated with MM,[24,25] Crohn's disease,[26] trachoma,[27] sarcoidosis,[28] and invasive pneumococcal disease,[29] the role of genetic variation within critical components of the NF-κB pathway, including *NFKB2* and *TRAF3*, remains unexplored in myeloma. Moreover, whether polymorphisms in NF-κB pathway-related genes influence the efficacy of bortezomib-based treatment in MM patients is unknown.

In this study we investigated the association of polymorphisms in the major NF-κB pathway-related genes *IKBα*, *NFKB2*, and *TRAF3* with MM, and evaluated the outcome of patients receiving bortezomib-based regimens in relation to the polymorphisms.

Design and Methods

Study subjects and treatment

Two hundred and fifty-two Chinese Han (161 males, 91 females) treated for MM between May 2001 and February 2010 at our Institution, Changzheng Hospital, were included in this study. The median age of these patients was 58 years (range, 28-82 years). According to the Durie-Salmon staging system,[30] 2 patients had stage I MM, 16 patients had stage II, and the remaining 234 patients had stage III disease. Among these patients, 83 with relapsed/refractory MM received bortezomib-based treatment at tolerated doses (1.0 or 1.3 mg/m^2) on days 1, 4, 8, and 11 for a maximum of eight 21-day cycles, together with dexamethasone on days 1-4 and doxorubicin (PAD, n = 32) or cyclophosphamide (VCD, n = 20) on days 1-4, or thalidomide on days 1-21 (VTD, n = 31).

A control group was formed of 275 age- and sex-matched, Han nationality Chinese, living in China, who were selected from subjects undergoing regular physical check-ups. The study was approved by the Institutional Review Board at Changzheng Hospital. All participants provided written informed consent.

Selection of single nucleotide poplymorphisms

Haplotype-tagging SNP were selected from the International Haplotype Mapping (HapMap) (*www.hapmap.org*). Additional informative SNP were selected from the National Center for Biotechnology Information SNP database (*http://www.ncbi.nlm.nih.gov/SNP*). SNP with a minor allele frequency of greater than 5% in the Han Chinese population were selected. Overall, 26 SNP were selected.

Genotype analysis

Genomic DNA was isolated from peripheral blood mononuclear cells using DNAzol reagent according to the manufacturer's instructions (Invitrogen; Carlsbad, CA, USA). Genotyping was performed using the SEQUENOM MassARRAY matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry platform (Sequenom; San Diego, CA, USA). Primers for the polymerase chain reaction and single base extension were designed using the Assay Designer software package (Sequenom) (*Online Supplementary Table S1*). Multiplex polymerase chain reaction was performed in 5 μL volumes containing 0.1 units of HotStar Taq polymerase (Qiagen, Hilden, Germany), 5 ng of whole-genome-amplified genomic DNA, 2.5 pmol of each primer, and 2.5 μmol of dNTP. Thermocycling was carried out at 94°C for 15 min followed by 45 cycles at 94°C for 20 s, 56°C for 30 s, and 72°C for 1 min, with a final incubation at 72°C for 3 min. Unincorporated dNTP were deactivated using 0.3 units of shrimp alkaline phosphatase

(Sequenom) followed by primer extension using 5.4 pmol of each primer extension probe, 50 μmol of the appropriate dNTP/ddNTP combination, and 0.5 units of iPLEX enzyme (Sequenom). The extension reactions were carried out at 94°C for 30 s and then 94°C for 5 s, followed by 5 cycles at 52°C for 5 s and 80°C for 5 s, for a total of 40 cycles, and then 72°C for 3 min. A cation exchange resin was used to remove residual salt from the reactions. Purified primer extension reaction products were spotted onto a 384-well spectroCHIP by a MassARRAY Nanodispenser and determined by MALDI-TOF mass spectrometry. Genotype calling was performed in real time with MassARRAY RT software version 3.0.0.4 and analyzed using MassARRAY Typer software version 3.4. To check the quality of the data, we sequenced more than 5% of the samples (17 cases and 19 controls) using an ABI 3100 DNA sequencer (Applied Biosystems). The genotype concordance rate between duplicate samples was 100%. Only those samples with a greater than 95% success rate and only those SNP with a genotype success rate of greater than 95% were included in the analysis.

Assessment

The response of patients was assessed according to the criteria of the European Group for Blood and Marrow Transplantation (EBMT)[31] plus an additional category of very good partial response.[32] Complete response was defined by the absence of monoclonal immunoglobulin (M-protein) in serum and urine confirmed by immunofixation, the absence of soft-tissue plasmacytomas, no increase in the size of lytic bone lesions, and the presence of less than 5% marrow plasma cells. Very good partial response was defined as a 90% or greater reduction in the serum M-protein plus a urinary M-protein level of less than 100 mg/24 h. Partial response was defined by a 50% or greater M-protein reduction in serum and 90% or greater reduction in urine. Minimal response was defined by a 25-49% M-protein reduction in serum and 50-89% reduction in urine. The response rate refers to the percentage of patients achieving at least a partial response. An increase in paraprotein of at least 25% or the development of new bone lesions indicated progressive disease.

Statistical analysis

The allelic and genotypic frequencies were compared by SHEsis software[33,34] and PLINK (version 1.07).[35] The haplotype was analyzed using both SHEsis and PHASE (version 2.1).[36,37] To assess significance, a permutation procedure (1,000 tests) was performed to correct the *P* value of single-locus association results by the SHEsis and PLINK software packages. For genotype-based analysis, 1,000 permutations were implemented using label-swapping in PLINK and corrected (EMP2) *P* values were used. Association of the various SNP, clinical characteristics, and response category subgroups were analyzed using the Mann-Whitney test for continuous variables and the χ^2 test or Fisher's exact test for categorical variables. A multivariate analysis to identify risk factors for achieving an overall response was performed by the logistic-regression model. Odds ratios (OR) and 95% confidence intervals (CI) were calculated by unconditional logistic regression, and further adjusted for age and sex. Progression-free survival and overall survival were estimated by the Kaplan-Meier method, and survival distributions were compared by using the log-rank test. The Cox proportional hazards model was used to assess the hazard ratio (HR) and the 95% CI of the polymorphisms with prognostic relevance for survival. A *P* value less than 0.05 is considered statistically significant. All statistical tests were two-sided. Statistical analyses were performed with SPSS version 15 software (SPSS Inc.; Chicago, IL, USA).

Results

Association between individual single nucleotide polymorphisms and risk of multiple myeloma

Of the 26 SNP selected for examination in this study, three sites (rs10131139, rs8023164, rs12435483) were either inconsistent with the Hardy-Weinberg equilibrium or non-polymorphic, leaving a total of 23 SNP across the *IKBα*, *NFKB2*, and *TRAF3* genes for analysis (Table 1). In the *IKBα* locus, the rs2233406 and rs2233409 T alleles were under-represented in patients (*P*=0.036 and *P*=0.043 *versus* the controls, respectively), with evidence of a protective effect in MM among carriers of the CT+TT genotypes in rs2233406 and rs2233409 (OR 0.693, 95% CI 0.499-0.961, *P*=0.024; and OR 0.702, 95% CI 0.479-1.029, *P*=0.067, respectively). In the *TRAF3* gene, the rs7143468 A allele was over-represented among patients (*P*=0.034), suggesting an increased risk effect in MM (OR 1.512, 95% CI, 0.995-2.298, *P*=0.066). After permutation tests, all *P* values for the above-mentioned comparisons increased beyond the level of significance of 0.05. However, the frequency of carriers of the rs12147254 A allele was significantly lower among patients than among controls (33.1% *versus* 51.7%, *P*<0.0001), even after 1,000 permutation tests (*P*<0.001), indicating a protective effect in MM (OR 0.709, 95% CI, 0.619-0.817, *P*<0.001). None of the SNP sites in *NFKB2* was associated with MM.

Association between haplotypes and the risk of multiple myeloma

A haplotype analysis was conducted of all SNP located in the *IKBα*, *NFKB2*, or *TRAF3* genes with a frequency of 1% in either the patient or control cohorts (Table 2). The *TRAF3* haplotype 1 [GAACAG] was less frequent among patients (24.1% *versus* 36.8% in the controls; *P*<0.0001; *P*=0.043 after 1,000 permutation tests, OR 0.543, 95% CI, 0.410-0.718). *TRAF3* haplotypes 3 [AGACAG] and 4 [GGACAG] were significantly more frequent among patients (*P*=0.030, and *P*=0.001, respectively) and the frequency of the haplotype 4 was significantly different even after the permutation test (*P*=0.010); however, the difference became statistically insignificant for haplotype 3 after the permutation test (*P*=0.297). Otherwise, no other haplotype of the *IKBα* and *NFKB2* genes was associated with MM.

Patients' characteristics

The characteristics of all the MM patients are shown in *Online Supplementary Table S2*. To investigate the role of *IKBα*, *NFKB2*, and *TRAF3* gene polymorphisms on the outcome of bortezomib-based therapy, 83 relapsed/refractory MM patients who received bortezomib-based regimens were analyzed (*Online Supplementary Table S2*). Previous therapies included melphalan and prednisone; vincristine, adriamycin, and dexamethasone; melphalan, prednisone, cyclophosphamide, MeCCNU, and vincristine; thalidomide and dexamethasone; and stem cell transplantation (*Online Supplementary Table S2*). Patients in the study had received a median of two previous therapies. On the basis of EBMT criteria, the overall response rate (partial and complete responses) was not different in patients who had received previous therapies based on SNP state, no matter what kind of regimen they had received (*data not shown*).

The median age of the 83 patients treated with borte-

Table 1. Genotype frequencies within the *IKBα*, *NFKB2*, *TRAF3* genes in controls and patients.

SNP ID*	Location in gene	Major/minor alleles	Subjects	Total	AA (%)†	AB (%)†	BB (%)†	Genotypic *P*‡ (*P*§)	Allelic *P*‡ (*P*§)	Odds ratio†‖ (95% CI)	*P*	*P*¶
IKBα												
rs3138053	3' near gene	A/G	Control	272	222 (81.6)	46 (16.9)	4 (1.4)	0.560	0.286	0.808	0.281	0.964
			Patients	249	212 (85.1)	34 (13.65)	3 (1.2)	(0.822)	(0.810)	(0.548-1.192)		
rs2233406	3' near gene	C/T	Control	271	199 (73.4)	68 (25.1)	4 (1.5)	0.082	***0.036***	0.693	***0.024***	0.354
			Patients	250	204 (81.6)	43 (17.2)	3 (1.2)	(0.419)	(0.229)	(0.499-0.961)		
rs2233409	3' near gene	C/T	Control	267	212 (79.4)	52 (19.5)	3 (1.1)	0.071	***0.043***	0.702	0.067	0.383
			Patients	249	213 (85.5)	36 (14.5)	0 (0.0)	(0.405)	(0.244)	(0.479-1.029)		
rs1050851	exon_2	C/T	Control	247	234 (94.7)	12 (4.8)	1 (0.4)	0.478	0.269	0.667	0.353	0.954
			Patients	228	220 (96.5)	8 (3.5)	0 (0.0)	(0.960)	(0.791)	(0.282-1.579)		
rs3138054	intron_3	G/A	Control	270	261 (96.7)	9 (3.3)	0 (0.0)	0.536	0.539	0.726	0.535	1.000
			Patients	248	242 (97.6)	6 (2.4)	0 (0.0)	(0.906)	(0.789)	(0.262-2.010)		
rs2233419	intron_4	C/T	Control	268	255 (95.2)	13 (4.9)	0 (0.0)	0.255	0.262	1.490	0.255	0.951
			Patients	249	231 (92.7)	18 (7.2)	0 (0.0)	(0.817)	(0.783)	(0.246-2.978)		
rs8904	exon_6	C/T	Control	272	99 (36.4)	127 (46.7)	46(16.9)	0.348	0.270	1.027	0.679	0.799
			Patients	251	87 (34.7)	109 (43.4)	55(21.9)	(0.901)	(0.792)	(0.905-1.167)		
rs696	exon_6	G/A	Control	270	96 (35.6)	128 (47.4)	46(17.0)	0.481	0.351	1.024	0.710	0.909
			Patients	250	85 (34.0)	112 (44.8)	53(21.2)	(0.927)	(0.886)	(0.903-1.161)		
rs2273650	exon_6	C/T	Control	271	148 (54.6)	99 (36.5)	24 (8.9)	0.684	0.528	0.969	0.750	0.989
			Patients	250	140 (56.0)	93 (37.2)	17 (6.8)	(0.709)	(0.899)	(0.801-1.174)		
rs3138055	5' near gene	G/A	Control	267	83 (31.1)	134 (50.2)	50(18.7)	0.125	0.178	1.016	0.789	0.403
			Patients	250	75 (30.0)	110 (44.0)	65(26.0)	(0.543)	(0.692)	(0.906-1.139)		
NFKB2												
rs7076748		G/C	Control	259	113 (43.6)	117 (45.2)	29(11.2)	0.885	0.645	1.038	0.623	1.000
			Patients	246	102 (41.5)	115 (46.7)	29(11.8)	(0.954)	(0.929)	(0.894-1.207)		
rs127693165	5' near gene	G/A	Control	269	195 (72.5)	68 (25.3)	6 (2.9)	0.212	0.095	1.261	0.087	0.459
			Patients	248	162 (65.3)	79 (31.9)	7 (3.3)	(0.689)	(0.381)	(0.973-1.632)		
rs12772374	intron_6	A/G	Control	272	194 (71.3)	71 (26.1)	7 (2.6)	0.218	0.120	1.246	0.091	0.490
			Patients	249	160 (64.3)	82 (32.9)	7 (2.8)	(0.667)	(0.452)	(0.970-1.602)		
rs7897947	intron_8	T/G	Control	270	111 (41.1)	129 (47.8)	30(11.1)	0. 988	0.989	0.994	0.935	1.000
			Patients	246	102 (41.5)	116 (472)	28(11.4)	(1.000)	(1.000)	(0.860-1.149)		
rs11574851	exon_19	C/T	Control	270	245 (90.7)	24 (8.9)	1 (0.4)	0.650	0.372	1.268	0.357	0.974
			Patients	247	218 (88.3)	28 (11.3)	1 (0.4)	(0.931)	(0.852)	(0.764-2.104)		
rs7077329	intron_22	C/T	Control	266	172 (64.7)	78 (29.3)	16 (6.0)	0.603	0.477	0.955	0.704	0.942
			Patients	246	163 (66.3)	73 (29.7)	10 (4.1)	(0.964)	(0.919)	(0.752-1.212)		
rs1056890	3' near gene	C/T	Control	270	163 (66.1)	87 (32.2)	20 (7.4)	0.336	0.121	0.855	0.175	0.602
			Patients	248	164 (66.1)	71 (28.6)	13 (5.2)	(0.840)	(0.457)	(0.681-1.073)		
TRAF3												
rs7143468		G/A	Control	268	70 (26.1)	131 (48.9)	67(25.0)	0.098	***0.034***	1.512	0.066	0.397
			Patients	248	47 (19.0)	124 (50.0)	77(31.0)	(0.290)	(0.105)	(0.995-2.298)		
rs12147254	intron_1	G/A	Control	268	66 (24.6)	127 (47.4)	75(28.0)	***<0.0001***	***<0.0001***	0.709	***<0.001***	***<0.001***
			Patients	249	116 (46.6)	101 (40.6)	32(12.9)	***(<0.001)***	***(<0.001)***	(0.619-0.817)		
rs11846158	intron_1	A/G	Control	271	127 (46.9)	112 (41.3)	32(11.8)	0.181	0.058	0.820	0.952	1.000
			Patients	249	99 (39.5)	111 (44.8)	39(15.7)	(0.440)	(0.211)	(0.556-1.209)		
rs3783384	intron_5	C/T	Control	272	114 (41.9)	121 (44.5)	37(13.6)	0.293	0.115	1.089	0.229	0.653
			Patients	245	90 (36.7)	111 (45.3)	44(18.0)	(0.600)	(0.313)	(0.948-1.251)		
rs12588538	intron_8	A/G	Control	262	152 (58.0)	89 (34.0)	21 (8.0)	0.499	0.241	1.123	0.241	0.903
			Patients	246	130 (52.9)	93 (37.8)	23 (9.4)	(0.764)	(0.525)	(0.925-1.364)		
rs11160707		G/A	Control	264	210 (79.6)	49 (18.6)	5 (1.8)	0.984	0.855	1.029	0.868	1.000
			Patients	247	195 (79.0)	47 (19.0)	5 (2.0)	(0.982)	(0.861)	(0.933-1.445)		

*SNP: single-nucleotide polymorphism; CI: confidence interval. * According to National Center for Biotechnology Information SNP database rs number. †Number of individuals (%): AA, wild-type homozygote; AB, heterozygote; BB, polymorphic homozygote. ‡Two-sided χ^2 test. §After 1000 permutation tests by SHEsis and PLINK software. ‖Comparison of polymorphic allele carriers (BB + AB) with non-carriers (AA). ¶After 1000 permutation tests by PLINK software. Further adjustment of age (continuous) and sex yielded similar results. Statistically significant values are marked in bold and italics.*

Table 2. Haplotype frequencies of *IKBα*, *NFKB2*, and *TRAF3* genes in controls and patients.

Haplotype*		Control†	Patients†	Odds ratio (95% CI)	P‡	P§
IKBα						
1	ACCCGCTACA	0.379	0.396	1.076 (0.821-1.412)	0.595	1.000
2	ACCCGCCGTG	0.232	0.249	1.097 (0.808-1.489)	0.553	1.000
3	ACCCGCCGCG	0.205	0.187	0.888 (0.640-1.233)	0.478	1.000
4	GTTCGCCGCG	0.046	0.042	0.900 (0.480-1.689)	0.743	1.000
5	ATTCGCCGCG	0.024	0.011	0.470 (0.162-1.364)	0.155	0.980
6	GTCCGCCGCA	0.021	0.011	0.518 (0.176-1.529)	0.226	0.995
NFKB2						
1	CGAGCCC	0.333	0.321	0.963 (0.735-1.261)	0.784	1.000
2	GGATCCC	0.264	0.252	0.955 (0.715-1.275)	0.755	1.000
3	GGATCTT	0.205	0.174	0.825 (0.597-1.140)	0.244	0.992
4	GAGTCCC	0.110	0.128	1.201 (0.813-1.773)	0.357	0.999
5	GAGTTCC	0.046	0.053	1.174 (0.657-2.098)	0.588	1.000
6	GGAGCCC	0.010	0.008	0.808 (0.212-3.088)	0.755	1.000
TRAF3						
1	GAACAG	0.368	0.241	0.543 (0.410-0.718)	***<0.0001***	***0.043***
2	AGGTGG	0.168	0.202	1.260 (0.910-1.744)	0.163	0.739
3	AGACAG	0.119	0.166	1.492 (1.038-2.146)	***0.030***	0.297
4	GGACAG	0.069	0.132	2.099 (1.355-3.254)	***0.001***	***0.010***
5	AAACAG	0.078	0.051	0.636 (0.377-1.073)	0.114	0.927
6	AGGTAA	0.053	0.061	1.157 (0.673-1.989)	0.591	0.993
7	GAATAG	0.039	0.025	0.638 (0.307-1.324)	0.224	0.763
8	AGGTGA	0.032	0.042	1.314 (0.675-2.561)	0.420	0.999
9	GGGTGG	0.021	0.021	1.015 (0.428-2.406)	0.974	1.000

*Order of SNP according to location in gene (same as Table 1). †The sum of the percentages is not 100% because of rare haplotypes that are not presented in this table. ‡Two-sided χ^2 test. §After 1,000 permutation tests. Statistically significant values are marked in bold and italics.

Table 3. Survival of patients with combined *NFKB2* rs12769316, rs1056890, and *TRAF3* rs11160707 genotype.

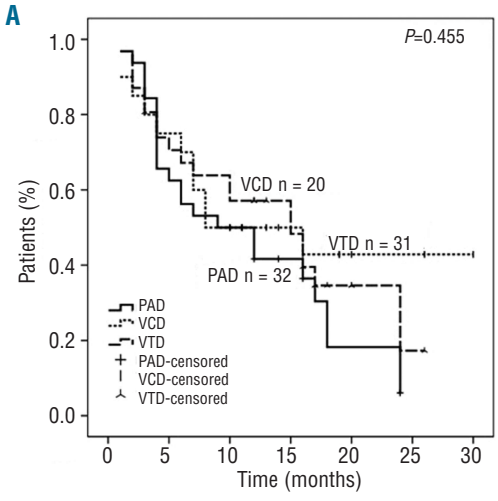
NFKB2 rs12769316	NFKB2 rs1056890	TRAF3 rs11160707	N. patients n=83	Progression-free survival*,† Median PFS (mo)	P	Hazard ratio (95% CI)	P	Overall survival*,† Median OS (mo)	P	Hazard ratio (95% CI)	P
GG	CC	GG	22	7		1		28		1	
GA+AA	CC	GG	19	15	0.672	0.896 (0.422-1.903)	0.775	-	***0.007***	0.094 (0.012-0.767)	***0.027***
GG	CT+TT	GG	21	7	0.758	1.055 (0.737-1.511)	0.769	24	0.645	1.114 (0.699-1.775)	0.651
GA+AA	CT+TT	GG	3	12	0.826	0.948 (0.578-1.555)	0.833	-	0.892	1.049 (0.521-2.114)	0.893
GG	CC	GA+AA	8	17	0.336	0.886 (0.687-1.144)	0.354	-	***0.044***	0.243 (0.084-1.876)	0.396
GA+AA	CC	GA+AA	4	24	0.423	0.893 (0.665-1.201)	0.455	-	0.544	0.881 (0.579-1.340)	0.553
GG	CT+TT	GA+AA	4	-	0.066	0.574 (0.225-1.468)	0.247	-	0.655	0.942 (0.651-1.312)	0.659
GA+AA	CT+TT	GA+AA	2	16	0.508	0.911 (0.681-1.218)	0.530	-	0.266	0.623 (0.167-2.330)	0.482

*Wild-type homozygote as reference. † "-" indicates that median overall survival (OS) was not reached. Statistically significant values are marked in bold and italics. PFS: progression-free survival; mo.: months.

zomib-based regimens was 58 years (range, 40-78 years); all patients were in Durie-Salmon stage III. Overall, 63 patients (75.9%) had a partial response or better to bortezomib-based therapy; 11 patients (13.3%) had a complete response, 21 patients (25.3%) had a very good partial response, and 31 (37.3%) had a partial response. β2-microglobulin (with a cut-off level of 3.5 mg/L) was a prognostic marker for poor progression-free survival (HR 2.1, 95% CI 1.318-3.921, log-rank test *P*=0.009) and overall survival (HR 3.584, 95% CI 1.062-10.870, log-rank test *P*=0.011). Other parameters, including age, sex, serum albumin, hemoglobin, C-reactive protein, and lactate dehydrogenase, were not associated with survival. No significant difference was observed in response rate, progression-free survival and overall survival between any two treatment arms and in progression-free survival and overall survival among the groups receiving the PAD, VCD, and VTD regimens (progression-free survival, *P*=0.455; overall survival, *P*=0.387) (Figure 1).

Genotypes and treatment response

The genotype distribution of SNP and the correspon-



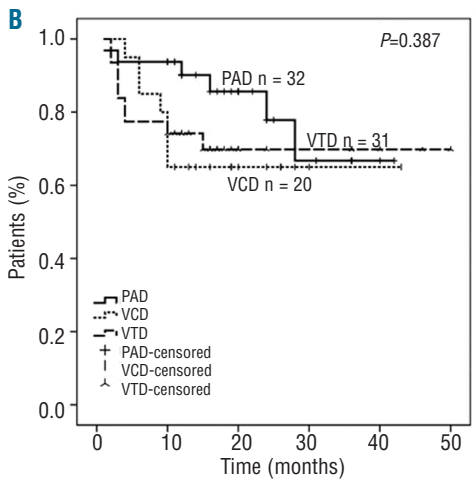
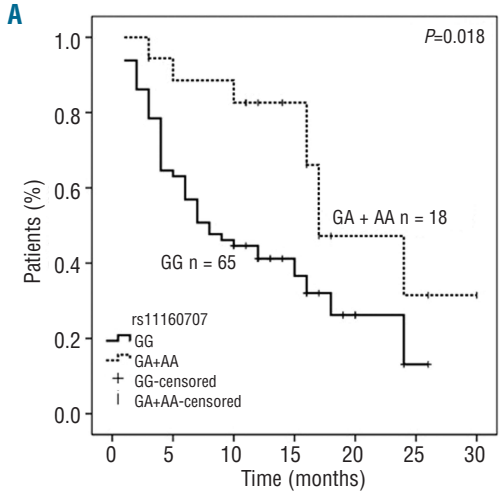
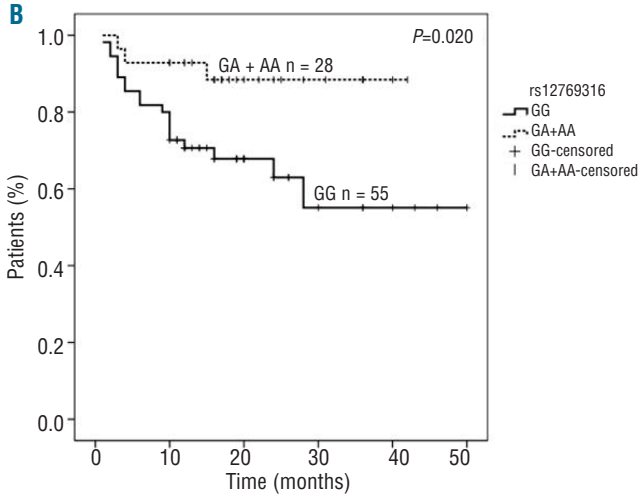


Figure 1. Survival of patients treated with bortezomib-based regimens. Kaplan-Meier curves for (A) progression-free survival of MM patients. (HR 0.869; 95% CI 0.637-1.186, log rank test *P*=0.455); (B) overall survival of MM patients. (HR 1.310; 95% CI 0.806-2.129, log rank test *P*=0.387).





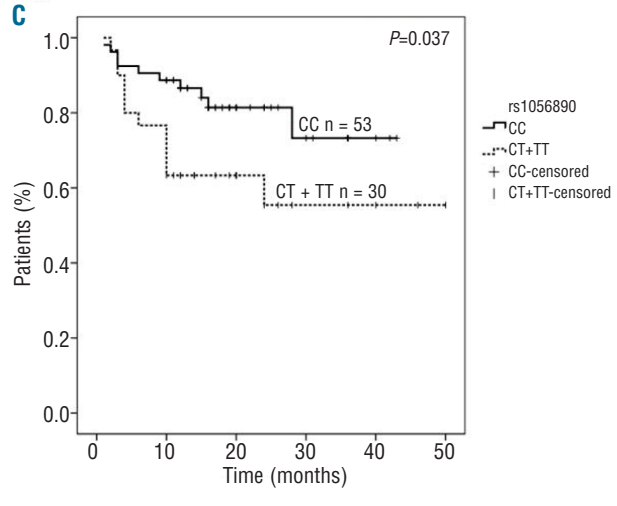


Figure 2. Genotypes and survival. Kaplan-Meier curves for (A) progression-free survival of MM patients according to *TRAF3* rs11160707 polymorphism status (HR 0.428; 95% CI 0.201-0.912, log rank test, *P*=0.018); (B) overall survival of MM patients according to *NFKB2* rs12769316 polymorphism status (HR 0.264; 95% CI 0.078-0.893, log rank test *P*=0.020), and (C) overall survival of MM patients according to *NFKB2* rs1056890 polymorphism status (HR 2.355; 95% CI 1.016-5.460, log-rank test *P*=0.037).

ding response rates are presented in *Online Supplementary Table S3*. On the basis of EBMT and IMWG criteria, the overall response rate among patients with the *NFKB2* gene rs12769316 GA+AA genotype was higher than that among patients with the GG genotype (89.3% *versus* 69.1%, *P*=0.042). For the *NFKB2* rs1056890, the proportion of patients in the CT+TT subgroup who achieved an overall response was lower than that in the CC subgroup (63.3% *versus* 83.0%, *P*=0.047). The overall response rate was not different between patients with other SNP (*Online Supplementary Table S3*). A multivariate logistic regression analysis was performed, combining *NFKB2* rs12769316, rs1056890, and *TRAF3* rs11160707 genotypes together; these SNP were not, however, shown to be independent factors for achieving overall response (*Online Supplementary Table S4*).

Genotypes and survival

At a median follow-up of 20 months (95% CI 15.97-25.14), the median overall survival, considering patients with all SNP, was not reached (*Online Supplementary Table S3*). The *TRAF3* rs11160707 GA+AA genotype was associated with a significantly superior progression-free survival (*P*=0.018) (Figure 2A), but had no effect on overall survival. With regards to the *NFKB2* gene, the rs12769316

GA+AA genotype was associated with a significantly superior overall survival in comparison with the GG genotype (*P*=0.020; Figure 2B). In contrast, patients with the rs1056890 CT+TT genotype had an inferior overall survival compared with patients with the CC genotype (*P*=0.037; Figure 2C). No statistically significant effect of the *IKBα* polymorphism was observed (*Online Supplementary Table S3*).

We further analyzed the distribution of patients' characteristics according to their genotypes: *NFKB2* rs12769316 GG *versus* GA+AA, *NFKB2* rs1056890 CC *versus* CT+TT, and *TRAF3* rs11160707 GG *versus* GA+AA (*Online Supplementary Table S5*). With the exception of patients with the *NFKB2* rs12769316 GA+AA genotypes, who tended to have a lower level of C-reactive protein compared to patients with the GG genotype (*P*=0.081), no significant differences in clinical features were observed (*Online Supplementary Table S5*).

Because *NFKB2* rs12769316, rs1056890, and *TRAF3* rs11160707 affected outcome, we performed an exploratory survival analysis (Table 3). Patients with the GA+AA/CC/GG genotypes at the rs12769316, rs1056890, and rs11160707 sites, respectively, had a significantly superior overall survival compared to that of patients with the wild-type genotype (*P*=0.007; Figure 3); however, this result could not be verified by the Cox proportional hazards model.

Subsequently, a multivariate analysis was performed to determine the effects of the *NFKB2* rs12769316, rs1056890, and *TRAF3* rs11160707 genotypes on overall response and survival. The results indicate that *TRAF3* rs11160707 polymorphic status is an independent factor that favors progression-free status (HR 0.428, 95% CI 0.201-0.911, *P*=0.028; *Online Supplementary Table S4*).

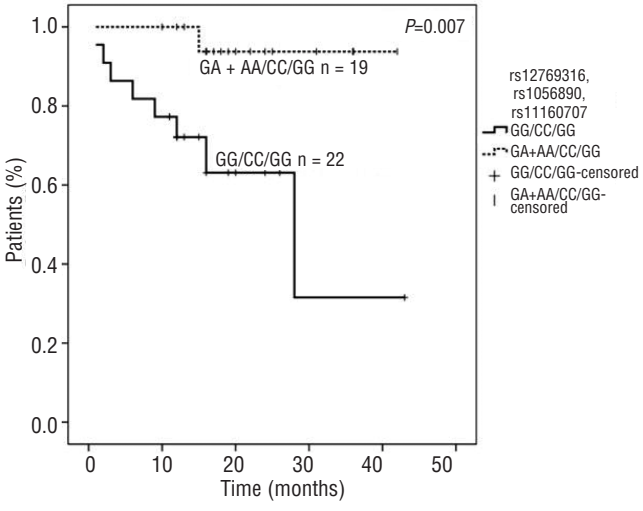


Figure 3. Combined genotypes and survival. Kaplan-Meier curves of overall survival for MM patients according to *NFKB2* rs12769316, rs1056890, and *TRAF3* rs11160707 polymorphism status (HR 0.094; 95% CI 0.012-0.767, log-rank test *P*=0.007).

Discussion

To examine our hypothesis that the NF-κB pathway may be associated with MM and patients' response to bortezomib, we analyzed the polymorphisms of NF-κB family members in 252 MM patients and compared them with those in 275 age- and sex-matched healthy controls. Interestingly, we observed that the development of MM in the Chinese Han population was associated with polymorphisms. Our results showed that SNP in the *IKBα* gene rs2233406 T allele are associated with a reduced risk of MM, although the *P* value was above the statistically significant level of 0.05 after the permutation test. With regards to the *TRAF3* gene, the rs12147254 A allele and a specific haplotype 1 of *TRAF3* [GAACAG] were associated with a decreased risk, while *TRAF3* haplotype 4 [GGACAG] was associated with an increased risk of MM. These findings suggest that *NFκB* gene polymorphisms play a role in myeloma disease.

Two previous studies also showed that certain *IKBα* polymorphisms are associated with an increased risk of MM.[24,25] Parker *et al.* found a higher frequency of the +418, +2787, and +2921 polymorphisms in 18 MM patients than in 24 controls.[24] Spink *et al.* examined 157 MM patients and 196 controls and found an association of +1678 (rs3138054) and +2025 (rs2233419) with MM.[25] In our study, we found an association of polymorphisms in rs2233406 and rs2233409 with MM in the Chinese Han population. These could reflect differences in ethnic background, limitations inherent to the studies, and the SNP selected in this study. The SNP rs2233406 T allele and rs2233409 T allele in *IKBα*, which were associated with a decreased risk of MM, are clustered in the upstream region, suggesting a possible effect on promoter function. Although polymorphisms located in the promoter region have been reported to be associated with sarcoidosis, trachoma, and invasive pneumococcal disease, the functional effects of polymorphisms of *IKBα* have not been investigated thus far.[27-29] As regards the *TRAF3* gene, two research groups recently reported homozygous inactivation of *TRAF3* in 13% of MM patients,[11,12] suggesting an important role of NF-κB in myeloma. Our study demonstrated an association of MM with rs7143468 and rs12147254, particularly the rs12147254 A allele, even after analysis by the permutation test. Moreover, the *TRAF3* haplotype 1 was associated with a decreased risk of MM and haplotype 4 with an increased risk in MM. Collectively, these observations suggest that the *TRAF3* gene polymorphism could be responsible for NF-κB activation. Although the rs12147254 SNP is located in an intronic region, intronic polymorphisms could also affect function through the promoter region,[38] splicing sites,[39] or intronic microRNA.[40] Further research is needed to identify the functional effects of the associated polymorphisms in each gene.

Bortezomib, a proteasome inhibitor currently used in the treatment of MM, proceeded from initial experiments in 2000 to USA FDA approval for its use in initial treatment of MM in 2008.[9] Although bortezomib was expected to have diverse effects on MM cell biology, the most common mechanism of its anti-myeloma activity is inhibition of the transcription factor, NF-κB.[41] Various clinical studies of bortezomib, particularly in combination with a variety of chemotherapeutic agents, have demonstrated its superiority to previous standards of care.[20-22] However, bortezomib also has several dose-limiting side effects and drug resistance can occur; some patients' responses to treatment are short and these patients have a poorer prognosis. The factors predicting relapsed and refractory diseases in

high-risk patients have been studied, and include certain cytogenetic abnormalities, high β2-microglobulin, and low serum albumin.[42] However, other prognostic factors require further characterization. Genetic variations in genes regulating the NF-κB pathway could partly explain why bortezomib-based regimens fail to achieve the expected therapeutic effects. To date, no study has been conducted to analyze the association between SNP status of the NF-κB pathway genes and clinical outcome. In this study, we evaluated the influence of polymorphisms in NF-κB family genes on the clinical outcome of 83 patients with relapsed or refractory MM who received bortezomib-based regimens. Recently, some multicenter and randomized trials have reported data comparing the safety and efficacy of bortezomib-based regimens for MM.[43,44] However, up to now there were no comparative data from large series of relapsed/refractory patients treated with PAD, VCD, and VTD regimens. Although the number of patients in our study is still limited, we did not find a differences with regard to response rate, progression-free survival and overall survival between patients treated with PAD, VCD, or VTD. Differences in treatment regimen should not, therefore, have influenced any association found between genotype and treatment outcome in this study, allowing us to investigate the cohort as a single group. Moreover, among the drugs used to treat patients, only bortezomib is known to affect the NF-κB pathway. Thus, it is reasonable to study the association of NF-κB family gene polymorphisms with the outcome of patients treated with bortezomib-based regimens.

We found that carriers of the rs12769316 GA+AA genotype of the *NFKB2* gene had a superior overall survival, whereas a significantly inferior overall survival was observed for those with the CT+TT genotype at rs1056890. It is notable that rs12769316 is located in the promoter region and rs1056890 in the 3'UTR, suggesting that they may regulate NF-κB signaling via over-expression of NF-κB2.[12] In the *TRAF3* gene, the rs11160707 is an independent and favorable factor for progression-free survival. Kears *et al.*[12] reported a better response to bortezomib and prolonged progression-free survival in patients with low levels of *TRAF3*, suggesting that constitutive activation of the non-canonical NF-κB pathway by inactivation of *TRAF3* may be associated with high sensitivity to the proteasome inhibitor. We, therefore, speculate that the influence of rs11160707 on progression-free survival may contribute to inactivation of *TRAF3*. *NFKB2* is a critical mediator of the non-canonical NF-κB pathway, and *TRAF3* is a recognized negative regulator of both the canonical and non-canonical NF-κB signaling pathways. Based on our finding that polymorphisms in *NFKB2* and *TRAF3* influenced the outcome of MM patients treated with bortezomib-containing regimens, it can be hypothesized that the effect of bortezomib treatment may depend on the availability of critical molecules in the NF-κB pathway. This may further support the recent observation that a subset of patients with primary MM who had activating mutations within the non-canonical NF-κB pathway was particularly sensitive to bortezomib.[12] Currently, various risk-stratification models have been proposed, most of which rely on a combination of cytogenetic abnormalities, with fluorescence *in situ* hybridization (FISH) analysis, and an estimate of tumor burden and/or proliferation.[23,45] Comprehensive risk-stratification analysis, which includes the determination of both cytogenetic abnormalities and the status of the studied SNP, would be very valuable. Unfortunately, some patients included in this study were treated in 2005 and 2006, when FISH analysis was not routinely performed on samples from patients with myeloma at our Institution. In conclusion, we have identified the *IKBα* and *TRAF3* gene polymorphisms associated with myeloma and have shown that *NFKB2* and *TRAF3* gene polymorphisms could have a substantial impact on the outcome of patients treated with bortezomib-based regimens. We believe that NF-κB family genes may represent a potentially useful indicator for preclinical assessments of myeloma risk and treatment response to bortezomib-based therapy.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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