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NFKB1 −94ins/delATTG polymorphism is a novel prognostic marker in first line-treated multiple myeloma

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Summary

Nuclear factor kappa B (NFKB) plays an important role in multiple myeloma (MM), and bortezomib affects this pathway. We retrospectively analysed the effect of the *NFKB1* −94ins/delATTG polymorphism on the survival of 295 MM patients treated at a single centre. The median progression-free survival (PFS) was 790 (659–921) d in patients with *NFKB1* homozygous insertion genotype (I/I, $n = 99$) and 624 (515–733) d in deletion-carriers (I/D&D/D, $n = 196$, $P = 0{\cdot}013$). In multivariate analysis, I/I carriers showed a favourable PFS compared to I/D&D/D with a hazard ratio of 0·622 (0·457–0·847), $P = 0{\cdot}003$, in addition to international staging system (ISS) score, fluorescence *in situ* hybridization (FISH) risk score, age and bortezomib treatment. I/I patients benefited more from bortezomib treatment [PFS 902 (703–1101) and 580 (343–817), $P = 0{\cdot}008$] than I/D&D/D patients [PFS 659 (487–831) and 488 (323–653), $P = 0{\cdot}531$]; in addition the beneficial effect of low ISS score was not observed in the I/D&D/D group [PFS 639 (454–824) and 650 (458–842), $P = 0{\cdot}226$], while it was clear in I/I patients [PFS 1140 (803–1477) and 580 (408–752), $P < 0{\cdot}001$]. We conclude that homozygous carriers of the insertion allele of the *NFKB1* −94ins/delATTG polymorphism have a better prognosis and probably benefit more from bortezomib treatment than MM patients carrying the deletion allele.

Keywords: multiple myeloma, nuclear factor kappa B, bortezomib, polymorphism, *NFKB1*.

Multiple myeloma (MM) is an incurable disease affecting mainly elderly people with an overall survival (OS) of about 5 years depending on various patient- and disease-related factors. International scoring system (ISS) score, fluorescence *in situ* hybridization (FISH) and gene expression profiling (GEP) have become effective prognostic tools, however predicting the response of an individual patient at diagnosis is still difficult. The current approach to treat symptomatic myeloma is chemotherapy with a combination of novel agents (thalidomide, bortezomib and lenalidomide), steroids and classical chemotherapeutic drugs, followed by autologous stem cell transplantation (ASCT) in younger and fit patients. Bortezomib, the first in class proteasome inhibitor (PI) received accelerated approval from the U.S. Food and Drug Administration in relapsed refractory myeloma in 2003, and entered into phase 3 trials in first line setting (Richardson *et al*, 2003; San Miguel *et al*, 2008; Harousseau *et al*, 2010). Currently it is standard upfront treatment in most European countries.

Nuclear factor kappa B (NFKB) is a key player in myeloma. It regulates the transcription of proteins that mediate cell cycle progression, apoptosis, drug resistance, cytokine and chemokine production. However, the precise mechanism of its activation and the exact role of NFKB in the pathogenesis of MM are yet to be fully characterized. Previous studies have shown that NFKB is a heterodimer composed of NFKB1 (p50) and RELA (p65), and is inactivated by its association with inhibitor kappa B (IkB) family inhibitors. Therefore, NFKBIA (also termed IkB-alpha) plays a crucial role in regulating NFKB activation. When the IkB kinase (IKK) complex phosphorylates the NFKBIA protein, it is ubiquitinated and degraded by the proteasomes, allowing translocation of NFKB1 and RELA into the nucleus where it binds to specific DNA sequences in the promoters of target genes

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(Hideshima *et al*, 2009). Bortezomib and other PIs block the proteasomal degradation of NFKBIA, thereby inhibiting the canonical activation of the NFKB pathway. Recent studies suggested that alternative ways of NFKB activation can also be important in MM. Hence, inhibition of the canonical pathway alone may be insufficient to block NFKB activity, and NFKB inhibition may not be the only way how PIs work in MM (Annunziata *et al*, 2007; Keats *et al*, 2007). For example, NFKB has an important role in inflammation, and cancers often exploit inflammatory components to improve their survival (Aggarwal & Sung, 2011). There are several polymorphisms within the NFKB pathway that were previously associated with the development and prognosis of various tumours including MM (Du *et al*, 2011). *NFKB1* −94ins/delATTG is particularly interesting as it affects the promoter activity of the *NFKB1* gene (Karban *et al*, 2004) and had not been tested before in MM in the context of these novel agents. There is one study showing that MM patients who are homozygous carriers of the wild type insertion allele of this gene polymorphism may benefit more from maintenance treatment with interferon-alpha (IFN-α, IFNA1) (Vangsted *et al*, 2009). Other studies described a potential connection between the presence of the deletion allele and various inflammatory conditions, such as ulcerative colitis (Borm *et al*, 2005) and psoriasis (Li *et al*, 2008). In addition, heterozygous and homozygous carriers of the deletion allele show increased susceptibility to colon (Lewander *et al*, 2007) and small cell lung cancer (Oltulu *et al*, 2014). The *NFKB1* −94ins/delATTG polymorphism is an insertion/deletion of four bases in the promoter region of the *NFKB1* gene encoding both of the NFKB1 isoforms, p50 and p105. The allele containing the deletion is less able to bind transcription factors and produces lower transcript levels in luciferase reporter systems. Consequently, carriers of the del-allele have lower cellular levels of NFKB1 (Karban *et al*, 2004; Vangsted *et al*, 2009).

We hypothesized that *NFKB1* −94ins/delATTG could have an effect on myeloma susceptibility and treatment outcome, especially in patients treated with PI-based regiments.

Subjects, materials and methods

Patients, clinical data, treatment and response criteria

Between January 2004 and September 2013, 295 newly diagnosed MM patients were treated with first line chemotherapy at the St. Laszlo Hospital, Budapest, Hungary. This retrospective study evaluated the clinical parameters at diagnosis, the types of treatment protocols and outcome parameters of these patients. Within the bortezomib-treated group most transplant-eligible patients were treated with bortezomib-thalidomide-dexamethasone (VTD) (Cavo *et al*, 2012), while melphalan-prednisolon-bortezomib (MPV) (Palumbo *et al*, 2010) was the standard therapy for older patients. A few other patients had bortezomib-doxorubicin-dexamethasone (PAD) (Oakervee *et al*, 2005) or bortezomib-dexamethasone (VD) (Richardson *et al*, 2005) chemotherapy. The following other, non-bortezomib containing protocols were also applied: cyclophosphamide-thalidomide-dexamethasone (Morgan *et al*, 2011), vincristin-doxorubicin-dexamethasone (VAD) (Monconduit *et al*, 1986), lenalidomide-dexamethasone (Rajkumar *et al*, 2010) and melphalan-prednisolon (MP).

Treatment continued until best response and then the transplant-eligible patients received a high dose cyclophosphamide-primed stem cell mobilization, followed by high dose melphalan-conditioned ASCT. No maintenance treatment was given. All patients received antibacterial prophylaxis with amoxicillin and acyclovir as antiviral agent. The median follow up was 1184 d (1019 d for the bortezomib-treated patients and 1626 d for the non-bortezomib-treated group).

Response criteria [complete response (CR), very good partial response (VGPR), partial response (PR) no response (NR) and progressive disease (PD)] and survival measures [progression-free survival (PFS) and overall survival (OS)] were defined according to published guidelines (Durie *et al*, 2006). Response was assessed at the end of the treatment, in case of transplanted patients following the ASCT; 11 patients had no response assessment due to early death.

In the healthy control group, 149 healthy blood donors (male/female: 72/77, age: 37·9 ± 10·9 years) were genotyped for the presence of *NFKB1* −94ins/delATTG variant (Szamosi *et al*, 2009).

The study was approved by the Hungarian National Ethics Committee and participants signed informed consents.

Fluorescence in situ *hybridization*

Fluorescence *in situ* hybridization (FISH) was performed in each MM cases on bone marrow slides using probes for chromosome 13q and 17p deletion, translocation (11;14), (4;14), (14;16) and 1q amplification. FISH results were available in 275 out of 295 patients. For the purpose of this study, patients with t(4;14), t(14;16), 1q amplification and del(17p) were grouped together as a high risk cohort. Previous large studies have shown that del(13q) in patients lacking t(4;14) and del(17p) was no longer of prognostic significance (Avet-Loiseau *et al*, 2007; Palumbo *et al*, 2014).

Genotyping

Genomic DNA was isolated from bone marrow or peripheral blood according to the manufacturers' recommendations with Gentra Puragene Blood Kit (Qiagen, Crawley, UK). The −94ins/delATTG polymorphism in the *NFKB1* promoter (rs28362491) was genotyped by polymerase chain reaction (PCR)-Van91I restriction fragment length polymorphism (RFLP) as described (Karban *et al*, 2004; Szamosi *et al*, 2009).

Statistical methods

Comparisons of dichotomous variables were performed by Fisher's exact test; continuous variables were compared with Mann–Whitney test. Log-rank test was performed to compare PFS and OS. Following univariate analysis, variables with P values <0·05 in the entire cohort were included in a Cox proportional hazards model for PFS and OS. Adjusted hazard ratios (HR), 95% confidence intervals (CI) values, and tests for interaction were computed. The analyses were carried out using the SPSS (version 20.0) software package (SPSS, Chicago, IL, USA).

Results

We analysed the *NFKB1* −94ins/delATTG polymorphism in 295 newly diagnosed MM patients treated at a single centre, a large teaching hospital in Budapest. Ninety-nine patients (33·6%) had homozygous insertion/insertion (I/I), 161 patients (54·6%) heterozygous insertion/deletion (I/D) and 35 patients (11·9%) had the deletion/deletion (D/D) genotype. Allele frequencies (AF ± 95% CI) observed in the total MM patient group (39·2 ± 4·0%), and in different MM subgroups (set up by age, ISS score, FISH and PI treatment) were not different from healthy controls (38·6 ± 5·6%). Due the low case number in the D/D group the D/D and I/D subgroups were merged as deletion carriers.

Study population

Patient characteristics are shown in Table I. Among the 295 newly diagnosed MM patients, 148 were males and 147 females, with a median age of 60 (27–84) years. The majority of patients had either IgG (58%) or IgA (20%) myeloma and 17% had light chain (LC) disease. Among the remaining 14 (5%) patients there were 3 IgD, 2 IgM, 1 IgE and 8 true non-secretory diseases. ISS was calculated in each case at diagnosis, with 32, 26 and 42% of the patients having an ISS score of 1, 2 and 3 respectively. According to our pre-defined FISH risk stratification, 74 patients (27%) were in the high-risk and 201 (73%) in the low-risk groups.

One hundred and seventy-four patients [90 males and 84 females; median age: 60 (28–84) years] had bortezomib-based treatment and 121 [58 males and 63 females; median age: 60 (27–84) years] had other, non-bortezomib containing protocols. The treatment decision was at the discretion of the treating physician. Within the bortezomib-treated cohort, 88 patients received VTD, 33 received PAD, 45 MPV and eight VD treatment. The majority (83%) of the VTD- and PAD-treated patients also received ASCT. In the non-bortezomib treated group, the majority of patients had either thalidomide-based therapy (54 patients) or VAD protocol (43 patients) and almost half of them (48% thalidomide-treated and 46% VAD-treated patients) received ASCT consolidation. Only four patients had lenalidomide-dexamethasone and 20 received MP. Treatment continued until best response or intolerable toxicities. Sixty-three per cent of the bortezomib-treated patients received ASCT whereas ASCT frequency was lower (39%) in the non-bortezomib group, reflecting the fact that, from 2008, VTD became the preferred induction in transplant candidates, while oral protocols were more feasible for frailer patients in our hospital. The median age of the transplanted and non-transplanted patients was 57 and 67, respectively ($P < 0·001$).

Patients in the I/I group were significantly older than in the I/D&D/D group [median age 63 (range: 32–84) years vs. 60 (range: 27–84) years, $P = 0·049$] but apart from this, there was no significant difference in the distribution of disease

Table I. Patients' characteristics according to their *NFKB1* −94ins/delATTG genotypes.

	All	I/I	I/D&D/D	*P*
n (%)	295	99	196	
Sex				
Male	148 (50·2)	52 (52·5)	96 (49·0)	0·622
Female	147 (49·8)	47 (47·5)	100 (51·0)	
Median age, years (range)	60 (27–84)	63 (32–84)	60 (27–84)	**0·049**
ISS score				
1 + 2	158 (58·5)	51 (56·7)	107 (59·4)	0·695
3	112 (41·5)	39 (43·3)	73 (40·6)	
FISH				
Low risk	201 (73·1)	67 (73·6)	134 (72·8)	1·000
High risk*	74 (26·9)	24 (26·4)	50 (27·2)	
Bortezomib				
Yes	174 (59)	64 (64·6)	110 (56·1)	0·170
No	121 (41)	35 (35·4)	86 (43·9)	
Chemotherapy				
VTD	88 (29·8)	33 (33·3)	55 (28·1)	0·350
PAD	33 (11·2)	11 (11·1)	22 (11·2)	1·000
MPV	45 (15·3)	19 (19·2)	26 (13·3)	0·230
VD	8 (2·7)	1 (1·0)	7 (3·6)	0·275
Thal	54 (18·3)	16 (16·2)	38 (19·4)	0·528
Len	4 (1·4)	1 (1·0)	3 (1·5)	1·0
VAD	43 (14·6)	11 (11·1)	32 (16·3)	0·295
MP	20 (6·8)	7 (7·1)	13 (6·6)	1·0
ASCT				
Yes	157 (53·2)	52 (52·5)	105 (53·6)	0·902
No	138 (46·8)	47 (47·5)	91 (46·4)	

ASCT, autologous stem cell transplantation; D/D, homozygous deletion; FISH, fluorescence *in situ* hybridization; I/D heterozygous insertion plus deletion; I/I, homozygous insertion; ISS, International scoring system; Len, lenalidomide-based; MP, melphalan, prednisolone; MPV, melphalan, prednisolone, bortezomib; PAD, bortezomib, doxorubicin, dexamethasone; Thal, thalidomide-based without bortezomib; VAD, vincristine, doxorubicin, dexamethasone; VD, bortezomib, dexamethasone; VTD, bortezomib, thalidomide, dexamethasone.

Significant *P* values are in bold.

*FISH high risk: t(4;14), t(14;16), del 17p and 1q amplification; low risk: all others.

characteristics and prognostic markers across the different *NFKB1* −94ins/delATTG genotypes (Table I).

The effect of conventional prognostic factors on response and survival

In order to analyse the impact of variations in *NFKB1* genotype on therapy, we first looked at the effect of conventional risk factors (age, ISS score and FISH) on the response, PFS and OS (Table SI).

The median PFS and OS for the entire cohort ($n = 295$) was 670 ± 43·6 and 1966 ± 108·9 d. Age at diagnosis had a significant effect on PFS and OS. The median PFS in patients aged below and above 60 years was 768 and 582 d ($P = 0·009$), respectively, and the median OS was 2304 and 1888 d ($P = 0·016$) respectively. Using 70 years of age as a cut-off point, PFS was 739 and 458 d ($P < 0·001$), and the OS 2120 and 1029 d, respectively ($P < 0·001$). ISS score was also confirmed as a significant factor in survival. PFS in patients with an ISS score of 1, 2 and 3 were 761, 783 and 600 d ($P = 0·003$), and OS in the same groups were 3136, 2304 and 1255 d, respectively ($P < 0·001$) (Table SI, in further analyses ISS scores 1 and 2 were merged).

Among the tested FISH abnormalities, del(17p) and t(4;14) had significant impact on PFS. In case of del(17p) ($n = 6$), the median PFS was only 136 d compared to 670 d of patients without the deletion ($P < 0·001$) and the OS was 603 d and 1962 d, respectively ($P = 0·054$). For t(4;14), the median PFS was only 488 d compared to 701 d for patients without the deletion ($P = 0·026$), however, there was no difference in OS (1683 d and 1966 d, respectively, $P = 0·643$). In addition, there was a trend for worse PFS and OS in patients with 1q amplification at diagnosis ($P = 0·068$ and 0·089). Using the pre-defined FISH risk stratification, we observed a significant difference in PFS but not in OS [PFS 545 d in high and 727 d in low risk FISH ($P = 0·002$); OS 1611 d in high and 2099 d in low risk FISH patients ($P = 0·115$)]. Other factors, such as immunoglobulin subtype and sex, had no effect on either PFS or OS and therefore were not included in the multivariate and subgroup analyses.

As expected, patients receiving bortezomib-based therapy had a superior outcome compared to other patients, with a significant difference for PFS (739 vs. 578 d, $P = 0·028$), but not OS ($P = 0·217$); patients that underwent ASCT fared significantly better (PFS 893 vs. 365 d, $P < 0·001$; OS 2386 vs. 1530 d, $P < 0·002$).

The effect of the NFKB1 −94ins/delATTG polymorphism on the quality of response to treatment

In the whole cohort, there was no difference between the *NFKB1* subgroups in terms of quality of response. The proportion of CRs, VGPRs, PRs and NR/PDs were 37·5%, 16·7%, 36·5%, 9·4% in the I/I group and 39·9%, 18·1%, 28·2%, 13·8% in the I/D&D/D group, respectively, with no significant difference. Bortezomib-treated patients showed significantly deeper responses than the other groups: CRs, VGPRs, PRs and NR/PDs were 53·5 vs. 17·5% ($P < 0·001$), 19·4 vs. 14·9% ($P = 0·346$), 24·1 vs. 41·2% ($P = 0·003$) and 2·9 vs. 26·3% ($P < 0·001$) respectively.

In the bortezomib-treated group the *NFKB1* genotypes were distributed evenly across the four response categories (Table II), nevertheless when we compared the number of treatment cycles required by *NFKB1* I/I and I/D&D/D patients to reach their best responses, I/D&D/D patients required significantly more cycles of chemotherapy to reach their best responses. While patients with homozygous insertion (I/I genotype) needed 3·78 cycles on average, deletion carriers (I/D&D/D genotypes) required 4·32 ($P = 0·008$). This difference was also significant between I/I and I/D&D/D patients who achieved CR due to the higher number of quick responders ($P = 0·012$, Table II).

The effect of the NFKB1 −94ins/delATTG polymorphism on survival of the whole cohort

When we analysed the effect of the *NFKB1* −94ins/delATTG polymorphism on survival, we demonstrated that the presence of at least one D allele had a significant effect on PFS. The median PFS was 670 d in the entire cohort, 790 in the

Table II. Best responses and average number of bortezomib cycles needed to reach the best response in upfront bortezomib-treated MM patients according to *NFKB1* genotype. Four bortezomib-treated patients were not assessed for response due to early death.

	All			I/I			I/D & D/D			
Best response	*n*	%	Cycles	*n*	%	Cycles	*n*	%	Cycles	*P* (cycle number)
CR	91	53·5	4·08	35	55·6	3·71	56	52·3	4·30	**0·012**
VGPR	33	19·4	3·94	10	15·9	3·80	23	21·5	4·00	0·305
PR	41	24·1	4·27	17	27·0	3·76	24	22·4	4·63	0·243
NR, PD	5	2·9	4·80	1	1·6	6·0	4	3·7	4·50	0·400
Total	170	100·0	4·12	63	100·0	3·78	107	100·0	4·32	**0·008**

CR, complete response; D/D, homozygous deletion; I/D heterozygous insertion plus deletion; I/I, homozygous insertion; NR, no response; PD, progressive disease; PR, partial response; VGPR, very good partial response.

Significant *P* values are in bold.

Patients in the I/D&D/D group required significantly more cycles of chemotherapy to reach their best responses.

I/I group and 624 in the I/D&D/D group ($P = 0{\cdot}013$; Fig 1A). In terms of OS there was no significant difference: the OS was 1931 and 1966 d in the I/I and I/D&D/D groups ($P = 0{\cdot}535$), and 1966 in the whole cohort (Table SI).

Multivariate analysis (Cox proportional hazard model) showed that, in addition to age, ISS score, FISH and bortezomib treatment, the presence of *NFKB1* genotypes I/D&D/D was an independent risk factor for PFS in the whole cohort,

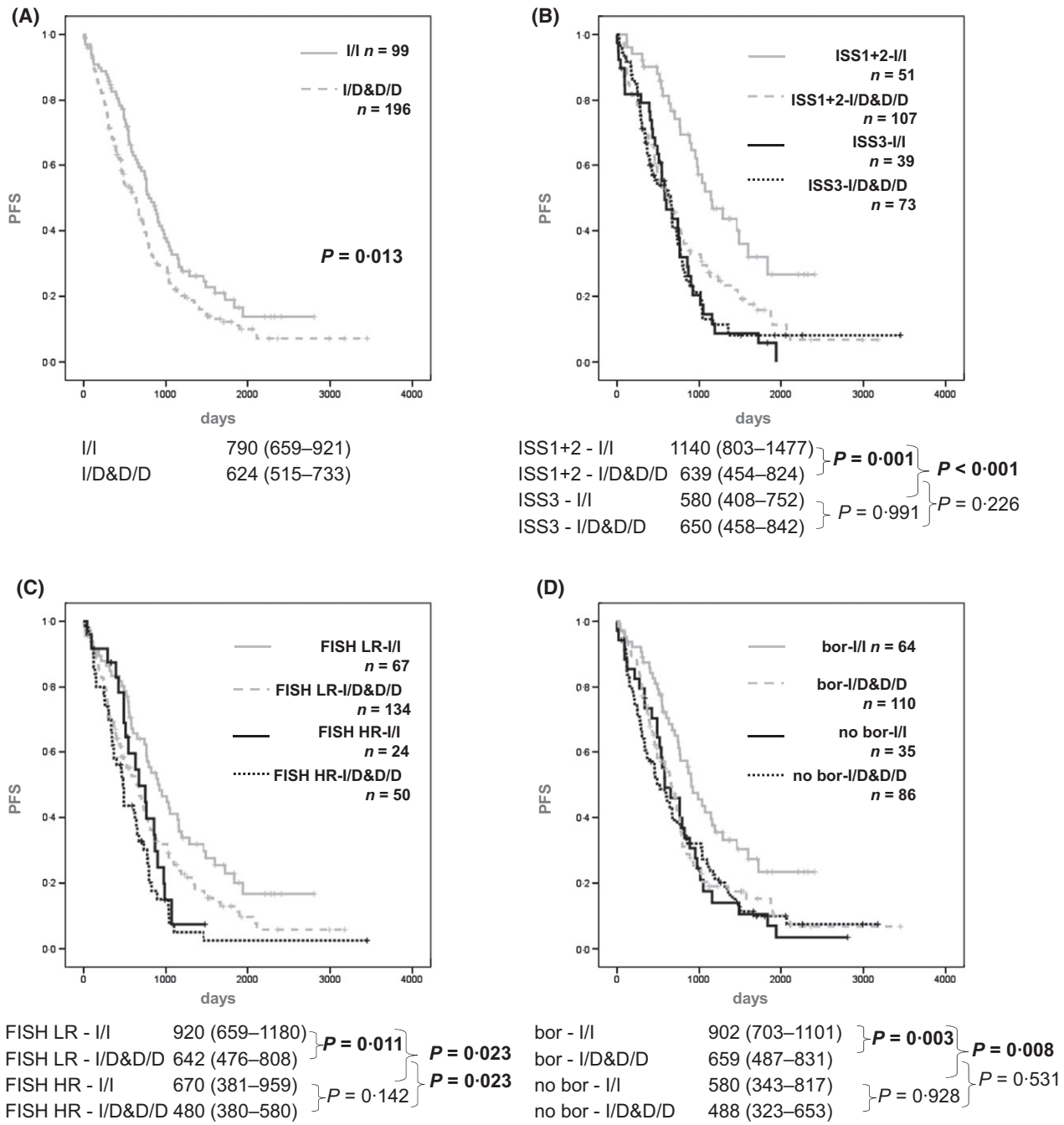

Fig 1. Comparisons of progression-free survival between *NFKB1* −94ins/delATTG I/I genotype vs. deletion carriers (I/D and D/D combined). PFS for all patients (A); in subgroups according to ISS score (1 + 2 and 3) (B); in subgroups with low risk (LR) and high risk (HR) FISH abnormalities (C); and in subgroups according to bortezomib treatment (D). Below the graphs, median PFS (95% CI) and *P*-values calculated by paired Kaplan–Meier analysis for each subgroup are shown. There was a significant survival advantage for *NFKB1* I/I compared to I/D&D/D patients in the whole cohort, in each subgroup with good prognosis and in the bortezomib-treated subgroup. Also, the benefit generally conferred by low ISS score and bortezomib treatment was significantly more pronounced in the *NFKB1* I/I cohort and non-significant in I/D&D/D patients. bor, bortezomib; CI, confidence interval; D/D, homozygous deletion; FISH, fluorescence *in situ* hybridization; HR, high risk; I/D heterozygous insertion plus deletion; I/I, homozygous insertion; ISS, International scoring system; LR, low risk; PFS, progression-free survival.

and also, in some of the subgroups (Fig 2). Similarly, the use of bortezomib was found to be an independent protective factor in the Cox model (Table SII). In terms of OS, only age and ISS score were significant independent risk factors; FISH and *NFKB1* were not (Table SII).

Before further subgroup analyses, we performed statistical tests for interaction between *NFKB1* genotype and the other factors involved in the Cox analysis for PFS. This showed a statistically significant interaction for the *NFKB1* genotype with ISS score but not with FISH and bortezomib therapy (Fig 2).

The impact of the NFKB1 −94ins/delATTG polymorphism on survival in different subgroups

We observed a significant PFS benefit for *NFKB1* I/I patients in all subgroups with favourable risk features (Fig 1B,C). Within the subgroup with ISS score 1 + 2, the median PFS of the I/I vs. I/D&D/D patients was 1140 vs. 639 d ($P = 0{\cdot}001$) and it was 580 vs. 650 d ($P = 0{\cdot}991$) in the subgroup with ISS score 3 (Fig 1B). There was no significant difference between the OS of the same groups [2565 vs. 2465 ($P = 0{\cdot}646$) and 1075 vs. 1269 ($P = 0{\cdot}395$) respectively] (Table SI).

Within the low risk FISH group, we observed a significant difference between the PFS of the I/I and I/D&D/D patients (920 vs. 642 d, $P = 0{\cdot}011$), which was not significant among the high risk patients (670 vs. 480 d, $P = 0{\cdot}142$) (Fig 1C). There was no significant difference between the OS of the I/I and I/D&D/D carriers in either FISH subgroup (Table SI).

Within the bortezomib-treated cohort, the difference between the two genetic subgroups was highly significant. In this group ($n = 174$), the PFS was 902 in the I/I group and 659 in the I/D&D/D group ($P = 0{\cdot}003$). Importantly, there was no similar difference between the PFS of the two *NFKB1* groups within the non-bortezomib treated cohort ($n = 121$) (Fig 1D).

Although we attempted to stratify patients according to the eight different treatment protocols applied in this study, we concluded that most of the groups were not large enough to draw any meaningful conclusions regarding the survival of the involved I/I and I/D&D/D patients separately. The largest group was the VTD-treated patients ($n = 88$, followed by ASCT $n = 78$), for whom the PFS was 1154 (I/I) and 727 d (I/D&D/D) in the two genetic groups ($P = 0{\cdot}002$), with the median OS not reached in the I/I group and 1962 d in the I/D&D/D group ($P = 0{\cdot}014$). We could not demonstrate a significant difference in PFS in any of the other treatment groups (data not shown).

We analysed the patient groups separately according to *NFKB1* I/I and I/D&D/D genotypes. Interestingly, while in the subgroup of *NFKB1* I/I carriers, low ISS score, low risk FISH and the presence of bortezomib treatment showed significant effects on PFS (protective factors with decreased hazard ratio), in the I/D&D/D carrier subgroup, neither ISS score nor bortezomib treatment had a significant impact on survival (Fig 1B–D).

Discussion

Myeloma susceptibility

There was no significant difference in the allele frequencies of the *NFKB1* −94ins/delATTG polymorphism between our

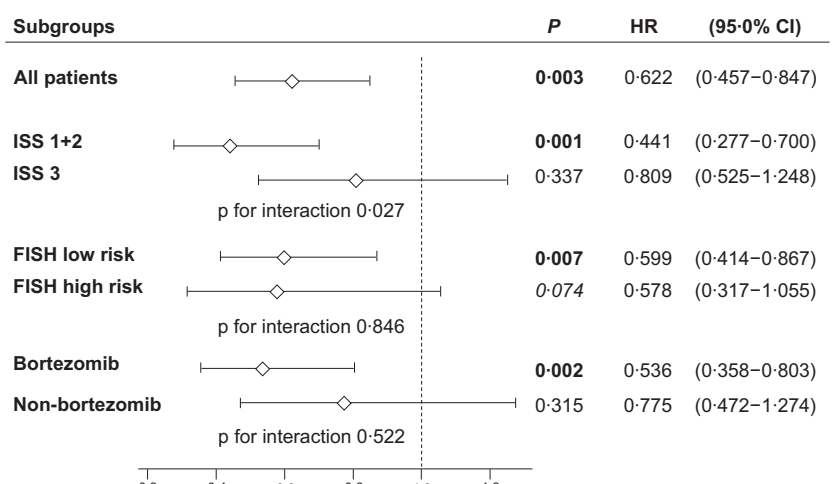

Fig 2. Risk factors in subgroups of patients. Hazard ratio (HR, diamonds) with 95% confidence intervals (horizontal lines) calculated by multivariate analysis (Cox-model) for PFS and *NFKB1* −94ins/delATTG genotype in various subgroups adjusted to age, ISS score, FISH and bortezomib treatment. *P* values of interaction testing between *NFKB* genotype and the respective covariates are inserted below the symbols for ISS score, FISH results and the use of bortezomib treatment respectively. Multivariate analysis identified the *NFKB1* polymorphism as an independent prognostic factor for PFS in ISS low risk, FISH low risk and bortezomib-treated patients. Statistical test for interaction was significant between NFKB1 and ISS score but was not significant between NFKB1 and either FISH score or bortezomib treatment. FISH high risk: t(4;14), t(14;16), del 17p and 1q amplification; low risk: all others. CI, confidence interval; D/D, homozygous deletion; FISH, fluorescence *in situ* hybridization; HR, hazard ratio; I/D heterozygous insertion plus deletion; I/I, homozygous insertion; ISS, International scoring system; PFS, progression-free survival.

patients and healthy Hungarian individuals, thus, in our cohort there was no association between this polymorphism and the risk of myeloma. Other groups described an increased frequency of various malignancies in patients with *NFKB1* −94ins/delATTG ID and DD genotypes, including colon cancer (Lewander *et al*, 2007), small cell lung cancer (Oltulu *et al*, 2014), bladder (Li *et al*, 2013) and prostate cancer (Zhang *et al*, 2009). On the other hand, in nasopharyngeal cancer Zhou *et al* (2009) found increased risk in the I/I group. To our knowledge, this is the first publication that compares *NFKB1* −94ins/delATTG allele frequencies between MM patients and normal individuals.

NFKB1 encodes the genes for the p50 and p105 NFKB1 isoforms, ubiquitous transcription regulators important for multiple diseases associated with inflammation and immunity. Karban *et al* (2004) found that carriers of *NFKB1* −94ins/delATTG D allele showed significantly reduced promoter activity *in vitro*, which was particularly pronounced following 24 h of exposure to lipopolysaccharide extract. The exact connection between these phenomena and the increased susceptibility to colitis in patients with the *NFKB1* D allele was not clear, but they hypothesized that poor activation of the NFKB pathway may weaken the normal cellular defences against intestinal bacteria that may allow bacteria crossing the intestinal mucosa not to be properly cleared by the immune system, and hence contribute to on-going intestinal inflammation (Karban *et al*, 2004).

Regarding cancer susceptibility, it can be concluded that previous studies showed conflicting results. NFKB can promote carcinogenesis by enhancing proliferation and angiogenesis, suppressing apoptosis and immune response (Karin, 2006). Recently Zou *et al* (2011) suggested that the deletion allele is protective for cancer susceptibility among Asians and is a risk allele in Caucasians.

Response to chemotherapy and survival

We observed a longer PFS among patients carrying *NFKB1* −94ins/delATTG I/I in comparison to those with I/D&D/D. This difference in PFS was highly significant, and even more pronounced in subgroups with better prognosis, such as ISS score 1 and 2 and low risk FISH group. It is important to mention that I/I patients fared better even if they were significantly older than I/D&D/D carriers.

The differences increased further when we focused on the bortezomib-treated subgroup, especially the VTD-treated patients and those having had ASCT consolidation. However, we have to keep in mind that treatment choice in this retrospective study was not independent of other factors. From 2008, VTD was the preferred induction in transplant eligible patients and the majority of the VTD-treated patients therefore had ASCT consolidation as well. Also, early death is a well-known complication in myeloma, with a higher prevalence in high risk patients, who therefore have a lower chance of surviving long enough to receive ASCT. Based on our results, *NFKB1* −94ins/delATTG I/I seems to have a stronger effect on the survival of myeloma patients with better prognosis according to ISS score and FISH and those who had bortezomib treatment.

It is interesting and may help to explain this phenomenon that, among the 174 bortezomib-treated patients there was a significant correlation between the *NFKB1* subgroup and the number of bortezomib cycles these patients required before reaching their best responses. Although the number of patients reaching CR and VGPR were similar, I/I patients typically required <4 cycles of chemotherapy whereas the I/D&D/D patients needed more.

It is also important to note that while the *NFKB1* I/I genotype cohort showed a clear survival advantage in ISS score 1 + 2 and low risk FISH patients and those treated with bortezomib, the I/D&D/D patients seemed to have a poor prognosis regardless of ISS score and did not seem to benefit significantly from bortezomib treatment.

Multivariate analysis identified *NFKB1* I/I genotype and bortezomib treatment as significant independent protective factors. However we were not able to demonstrate a statistical interaction between bortezomib treatment and *NFKB1* genotype using the Cox model. At the same time, with univariate analysis showed a significant, 8-month PFS advantage for *NFKB1* I/I patients compared to I/D&D/D carriers within the bortezomib-treated group with no similar difference among the non-bortezomib treated patients. We believe this is clinically substantial. We speculate that the lack of statistical interaction between bortezomib treatment and *NFKB1* genotype is due to the following reasons. (i) The relatively low case numbers in some of the subgroups, (ii) the different follow-up times in non-bortezomib and bortezomib-treated patients, and, most importantly, (iii) the fact that this is a retrospective non-randomized study where the decision between bortezomib and non-bortezomib protocols may have been biased in many cases, resulting in an increased number of low risk ISS and FISH patients in the non-bortezomib subgroup.

The lack of significant difference in OS is probably partly due to the fact that the follow-up period was relatively short. During the disease course a typical myeloma patient is exposed to various combination chemotherapies, bortezomib being only one of them. If we accept that the beneficial effect of *NFKB1* polymorphism is due to interaction with bortezomib and not with other treatments, then this may explain why we failed to demonstrate a clear OS benefit in our patients.

The situation within the non-bortezomib treated cohort is more complex. As a group, these patients failed to show survival benefit in either *NFKB1* genotype subgroup. In a retrospective study, Vangsted *et al* (2009) analysed the effect of *NFKB1* −94ins/delATTG polymorphism on the survival of myeloma patients treated with VAD induction and ASCT with or without IFN-α maintenance. The main finding was that, within the IFN-α treated group, the presence of the I/I

genotype provided the patients with a significant survival benefit [time to treatment failure (TTF) 49·9 (I/I) vs. 34·4 (I/D&D/D) months, OS not reached in the I/I group after 120 months vs. 74·4 months in the I/D&D/D group, $P = 0{\cdot}09$ and 0·002] (Lenhoff *et al*, 2000; Vangsted *et al*, 2009). This difference was only observable in the IFN-α-treated cohort, while in the no maintenance group, I/I patients actually seemed to have a slightly (although not significantly) worse outcome. The authors speculated that this selective effect of IFN-α maintenance on homozygous I/I allele carriers indicates that the effect of IFN-α depends on the availability of NFKB, which is essential for both the innate and adaptive immune systems (Vangsted *et al*, 2009).

NFKB1 −94ins/delATTG polymorphism is an insertion/deletion polymorphism of four bases in the promoter region of the *NFKB1* affecting its capability to bind transcription factors. As a result, carriers of the D allele have lower levels of NFKB1 (Karban *et al*, 2004) and may have a different response to various transcription factors. Although bortezomib has many potential ways in which it can interact with the proliferation of MM cells, the main mechanism of action is probably through the NFKBIA-NFKB pathway. Our hypothesis is that, due to the higher expression of *NFKB1* in homozygous carriers of the I/I allele, myeloma cells could be more dependent on this pathway, and therefore its blocking could have a stronger effect on them compared to patients with I/D&D/D genotypes with lower NFKB1 levels.

The observed positive effect of the I/I allele was the strongest and most unequivocal among the VTD-treated patients, who also benefited from the immunomodulatory effect of thalidomide and the majority of whom received additional ASCT. It is possible that this difference is at least partially related to the effect of either the thalidomide or the ASCT or both. However, there was no significant difference between the two *NFKB1* genotypes in either patients having thalidomide treatment without bortezomib or in the group of ASCT-treated patients without bortezomib in the induction. Still, we cannot exclude the possibility that the observed survival benefit in the I/I patients is somehow related to the combined effect of bortezomib, thalidomide and ASCT, and therefore mainly affects VTD-treated patients having had ASCT. Of note, when analysing the MPV-treated group, the outcome of I/I patients compared to I/D&D/D carriers was not significantly different. On the other hand, the median age of the VTD and MPV patients was 58 and 73 years, respectively and, within the MPV-treated cohort, I/I patients were older than I/D&D/D carriers. As age is a very strong predictor of short survival, we think that the main factor behind the fact that the *NFKB1* polymorphism had virtually no effect on the survival of the MPV-treated patients was that old age suppressed the beneficial effect of the I/I genotype among these patients.

The effect of proteasome inhibition on the NFKB pathway is far from clear. Recently, investigators found that, similarly to other chemotherapeutic agents and radiotherapy, paradoxically bortezomib can also induce canonical NFKB activation via IkB downregulation. This phenomenon was recently described in endometrial cancer (Dolcet *et al*, 2006), gastrointestinal stromal tumour (Bauer *et al*, 2010) and also in myeloma (Hideshima *et al*, 2009), suggesting that bortezomib-induced cytotoxicity cannot be fully attributed to inhibition of the canonical NFKB activity in MM cells. Moreover, a previous study has demonstrated a highly activated noncanonical pathway in primary MM cells, and it is possible that bortezomib blocks noncanonical NFKB activity, due to inhibition of proteasome-dependent NFKB2/p100 conversion into the active p52 isoform (Keats *et al*, 2007). Clearly this effect could be different in patients with or without the D allele and therefore to understand exactly how *NFKB1* −94ins/delATTG genetic variants affect the survival of MM cells, it would be essential to investigate its effect on both canonical and noncanonical pathways of NFKB activation.

In conclusion, our data indicate that, patients who are homozygous carriers of the wild type insertion allele of the *NFKB1* −94ins/delATTG polymorphism have better outcome compared to patients carrying the variant deletion allele. This survival advantage is more pronounced in patients with low risk MM. Based on our data, homozygous insertion type patients may benefit more from treatment with VTD and ASCT. Therefore this polymorphism may be a prognostic marker in MM patients having first line treatment. Further studies are needed to confirm this association in independent cohorts and to clarify the molecular background of this effect on bortezomib therapy in MM.

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Authors' contribution

Varga, G., Andrikovics, H., Koszarska, M. and Balassa, K. performed the research; Varga, G., Mikala, G. and Masszi T. designed the research study; Adam, E. and Kozma, A. performed the cytogenetic analysis; Varga, G., Mikala, G. and Andrikovics, H. analysed the data; Varga, G., Mikala, G., Andrikovics, H., Tordai, A. and Masszi, T. wrote the paper. All authors read and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no competing financial interests in relation to the work described.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table SI. Univariate analysis of PFS and OS in all patients and different subgroups according to their *NFKB1* −94ins/delATTG genotype.

Table SII. Multivariate analysis of PFS and OS in all patients (top), bortezomib treated patients (middle) and non-bortezomib treated patients (bottom).

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