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Impact of global and gene-specific DNA methylation pattern in relapsed multiple myeloma patients treated with bortezomib☆

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ABSTRACT

We studied seventy-five patients with relapsed MM treated with bortezomib-based regimens. DNA was isolated from bone marrow samples at the time of relapse. Global methylation was determined by ELISA, and CpG island DNA methylation profile of 30 genes by a DNA methylation PCR system. Patients with more than 3.95% of total DNA methylated achieved better overall survival (OS) ($p = 0.004$). A relatively low methylation percentage (<1.07%) of *NFKB1* was also associated with longer OS after bortezomib treatment ($p = 0.015$). The combination of highly methylated global genome with low *NFKB1* methylation status defined a specific subset of patients with better prognosis.

1. Introduction

Bortezomib is one of the so-called novel drugs in the treatment of multiple myeloma (MM), together with thalidomide and lenalidomide [1]. The known activity of this agent is basically related to the 26S complex proteasome inhibition, with a wide number of intracellular pleiotropic effects [2]. Degradation of damaged or misfolded protein is crucial in secretory cells such as myeloma plasma cells, which can lead to a different regulation in the cell cycle, inflammatory response, tumor suppression and apoptosis [3], resulting in a global tumor load decrease. Clinical studies confirmed a significant beneficial effect in relapsed and/or refractory MM patients, leading to bortezomib approval in this setting [4]. In younger patients who are eligible for transplantation, bortezomib-based treatment has become a standard of care on the basis of the rate of high-quality responses before autologous stem-cell transplantation, with its consequent improvement in outcome after transplantation [5,6].

The incorporation of novel drugs, particularly thalidomide, bortezomib and lenalidomide has resulted in a significant improved survival of patients with MM [7,8]. However, MM is still an incurable disease with an important shortening in life expectancy [9]. Thus, once patients become refractory to current treatments the prognosis is ominous [10]. The response to the widely used bortezomib is particularly variable in relapsed patients. Cytogenetics and other biological variables have been studied in different settings as predictors of response, with heterogeneous results [11,12]. Other molecular mechanisms such as genomic DNA polymorphisms [13], RNA gene-expression [14], microRNA expression [15] and DNA methylation pattern [16], could account for this variability in response. The aim of the present study was to investigate the global DNA methylation pattern and specific methylation state in 30 genes in DNA from bone marrow cells and to correlate these findings with progression free survival (PFS) and overall-survival (OS) after bortezomib treatment in patients with relapsed myeloma.

2. Materials and methods

2.1. Patients

Seventy-five patients (37M/38F; median age 65 years, range 29–80) with relapsed MM were treated from December 2002 to March 2010 with bortezomib-based regimens at our institution. Median follow-up for patients alive was 31

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Table 1
Main patient characteristics.

Variable	*N* = 75 patients
Median age, years (range)	65 (29–80)
Gender (M/F)	37/38
M-protein type (%)	
IgG	48.7
IgA	23.7
Light chains	21.1
IgD	3.9
Oligosecretory	1.3
Light chain subtype (%)	
Kappa	61.8
Lambda	36.8
Median BM plasma cells (%)	46
Extramedullary involvement (%)	13.2
Median time from diagnosis, months (range)	34.8 (6–156)
Median no. of previous lines of therapy (range)	2 (1–6)
Previous bortezomib exposure (%)	8
Previous ASCT (%)	52

BM: bone marrow; ASCT: autologous stem cell transplantation.

months (range 6 to 45+). Main characteristics of the patients are shown in Table 1. The diagnosis of MM was established according to the criteria of the Chronic Leukemia-Myeloma Task Force [17]. Response to treatment, progression and relapse were defined according to European Blood and Marrow Transplantation (EBMT) criteria [18,19]. The Ethics Committee of Hospital Clínic of Barcelona provided institutional review board-approval for this study. No patient was lost of follow-up.

2.2. DNA isolation

Genomic DNA was isolated from slides from bone marrow aspirates with plasma cell infiltration at time of relapse. Bone marrow aspirates had an increased number of bone marrow plasma cells in all cases (median infiltration 46%, range 10–100%) and were obtained just before the administration of bortezomib. Briefly, bone marrow slides were treated with xylol for 7–14 days and subsequently in decreasing ethanol concentrations (100%, 96% and 70%, v/v) and DNase-free double distilled water, during 24 h each step. DNA extraction was performed, using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) incorporating pre-treatment with RNase according to manufacturer's protocol.

2.3. Global and specific DNA methylation analysis

Global methylation status was determined in all patients by a specific ELISA assay (MethylFlash™ Methylated DNA quantificacion Kit; Epigentek, Farmingdale, NY) in total DNA, which is based on recognition and quantification of the DNA methylated fraction by a 5-methylcytosine (5-mC) antibody; the assay was performed according to manufacturer's protocol.

Specific-gene DNA methylation profile was determined in CpG islands of 30 genes in 42 patients with enough DNA by a DNA methylation PCR system based on DNA digestion with methylation sensitive and/or dependent restriction enzymes (Methyl-Profiler™ DNA Methylation PCR Array System; Qiagen, Hilden, Germany). According to manufacturer's protocol, DNA was treated with sensitive and/or dependent restriction enzymes, active against methylated and unmethylated DNA, respectively, and quantitative real time PCR with CpG island specific primers and SybrGreen® was performed to obtain the percentage of high and intermediate methylation status of CpG islands for each specific gene.

These 30 genes were selected based on two criteria: (1) their well-known relationship with hematological malignancies and their treatment, and (2) the availability of a commercial assay for specific related CpG islands in each gene previously selected. Thus, genes involved in pathways such as cytokine network, apoptosis, tumor suppression, transcription factors and cellular cycle were studied. The list of genes and CpG islands involved are detailed in Table 2.

2.4. Data analysis

Descriptive statistical analysis of the main characteristics of the patients was performed. Differences in response rate were assessed by Fisher exact test. Clustering analysis was performed according to both, treatment response and the global and specific methylation status in the genes with a non-parametric Wilcoxon test by using Multiexperiment Viewer Software vs 10.2 [20]. Association of global and specific DNA methylation status with PFS and OS, was determined using the Kaplan–Meier method and log-rank test. The cut-off value to dichotomize the continuous variables associated with PFS or OS was calculated by MaxStat with R 2.9.2 software (Vienna, Austria) [21]. Cox proportional hazards model was used to estimate the risk ratio of events (ORR) with the respective confidence interval (CI) after controlling for prognostic variables in multivariate analysis. Variables included at multivariate analysis were the number of previous treatment lines, autologous stem-cell transplantation, previous exposure to bortezomib, and global and specific DNA methylation status. Further, an additional analysis was performed including both, the combination of the global methylation status, and the specific methylation status for the genes which were associated with patient prognosis; with these analyses we defined the values of these parameters which define different subsets of patients in terms of OS. Statistical tests were performed with PASW software 18.0 for Windows® (Chicago, IL, USA). A p value < 0.05 was required for statistical significance.

Table 2
Genes and CpG islands studied.

Pathway	Gene	CpG island
Immune response	*CD40*	CpG island 25929
	MIF	CpG island 26718
Transcriptional coactivators	*EP300*	CpG island 28275
	CBP	CpG island 20205
Cytokine network	*CXCR4*	CpG island 03697
	CXCL12	CpG island 28488
	IL6R	CpG island 01705
	IL17RA	CpG island 26557
	TGFB1	CpG island 24670
	TGFBR2	CpG island 04667
Apoptosis	*TNFRSF13C*	CpG island 27048
	TNFRSF21	CpG island 09077
	TNFRSF25	CpG island 28446
	BCL2L11	CpG island 03511
Tumor suppression	*TP53*	CpG island 21488
	BRCA1	CpG island 28472
	DAPK1	CpG island 28491
	CDH1	CpG island 28479
	RASD1	CpG island 21627
Transcription factors	*NFKB1*	CpG island 06611
	NFKBIB	CpG island 24567
	IRF4	CpG island 08395
Cellular cycle	*CCNB1*	CpG island 07358
	CCND1	CpG island 15579
	CCNA2	CpG island 06719
	CCNE1	CpG island 24350
	CDKN2A	CpG island 28560
	CDKN1A	CpG island 28245
Cytokine stimulus response	*SOCS3*	CpG island 22727
Efflux transporter	*ABCG2*	CpG island 06547

3. Results

All 75 patients were evaluable for response. Overall response (OR) was achieved in 62% of the patients (complete remission 6.7%, partial response 44% and minimal response 10.7%), while 9 (12%) and 20 (26.7%) showed no response (NR) or progressive disease (PD), respectively. The median PFS and OS after bortezomib therapy were 6 and 19.6 months, respectively.

3.1. Global methylation status

A low global methylation status was observed in the whole group (median 4.68% of 5-mC, range 0.02–13.6). The value obtained by MaxStat to dichotomize the patient group was 3.95%. The relatively low-methylated group had a median of 2.43%, in comparison to the 6.35% in the higher one. Patients with more than 3.95% of total DNA methylated achieved better OS than patients with more unmethylated DNA (median 30 versus 15 months) (p = 0.004; Fig. 1).

3.2. Specific-gene methylation status

Concerning methylation on specific-genes, a methylation status lower than 3.97% in *CXCR4* was correlated with a longer PFS after

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