

Downregulation of *DCC* sensitizes multiple myeloma cells to bortezomib treatment

DORIVAL MENDES RODRIGUES-JUNIOR¹, THAÍS PRISCILA BIASI¹,
GABRIELA ESTRELA DE ALBUQUERQUE¹, VIVIANE CARLIN¹, MARCUS VINICIUS BURI²,
JOEL MACHADO-JUNIOR¹ and ANDRE LUIZ VETTORE¹

¹Department of Biological Sciences, Laboratório de Biologia Molecular do Câncer, UNIFESP, Universidade Federal de São Paulo, Campus Diadema, São Paulo 04039-032; ²Department of Biochemistry, Institute of Pharmacology, Universidade Federal de São Paulo, Campus São Paulo, São Paulo 04044-020, Brazil

Received July 30, 2018; Accepted December 12, 2018

DOI: 10.3892/mmr.2019.10142

Abstract. Multiple myeloma (MM) is an incurable disease; a better understanding of the molecular aspects of this hematological malignancy could contribute to the development of new treatment strategies and help to improve the survival rates of patients with MM. Previously, the methylation status of the *deleted in colorectal cancer (DCC)* gene was correlated with the survival rate of patients with MM, thus the main goal of this study was to understand *DCC* contribution to MM tumorigenesis, and to assess the impact of *DCC* inhibition in the MM response to treatment with bortezomib. Our results demonstrated that hypermethylation of the *DCC* promoter inhibits gene expression, and *DCC* silencing is significantly correlated with a reduction in cell viability and an increase in cell death induced by bortezomib. In conclusion, our results suggested that hypermethylation is an important mechanism of *DCC* expression regulation in MM and that the absence of *DCC* contributes to the enhanced sensitivity to treatment with bortezomib.

Introduction

Multiple myeloma (MM) is a neoplastic disorder characterized by expansion and accumulation of clonal plasma cells in the bone marrow (BM), secretion of monoclonal immunoglobulin and presence of osteolytic bone lesions (1,2). This plasma cell dyscrasia is the second most common hematological malignancy (after non-Hodgkin's lymphoma) accounting for ~1% of all cancer diagnoses (3). Over the last two decades, significant progress has been made in the management of MM because of

the introduction of new therapeutic agents, such as proteasome inhibitors (PIs) and immunomodulatory agents (IMiDs) (4).

PIs such as bortezomib and carfilzomib have assumed a central role for the management of MM. Bortezomib can be used at all phases of MM treatment, from frontline to combination therapy, or for the re-treatment of relapsed disease (5-8). Despite these newer agents, responses to therapy are transient and patients treated with PIs tend to develop resistance and to become refractory to their treatment regimens (9-11). Therefore, MM remains an incurable disease with a fatal outcome and new approaches to enhance the response to proteasome-targeted drugs are an unmet need.

In a previous study, we evaluated the methylation pattern in a group of genes in MM patients and observed an association between *Deleted in colorectal cancer (DCC)* hypermethylation and poor survival (12), suggesting that this gene could play a role in MM tumorigenesis. It is well known that *DCC* encodes a cell surface receptor whose ligand is Netrin 1. This receptor can act in cell-cell and cell-matrix interactions and it is involved in both epithelial and neuronal-cell differentiation (13,14). *DCC* has been proposed as a tumor suppressor gene because most of the advanced colorectal carcinomas show losses of the 18q region where this gene is located (15,16). The loss of *DCC* expression is not restricted to colorectal cancer, and it is also found in other tumors such as stomach, pancreas, esophagus, prostate, bladder, breast, neuroblastomas and gliomas (17,18). However, the rarity of point mutations in *DCC* coding sequences, associated to the lack of a tumor predisposition phenotype in *DCC* hemizygous mice and to a correlation with the relapse site in gastric cancer, has raised questions about its role as a tumor suppressor (19-21). Hence, this study was performed to gain an understanding of the *DCC* biological function and its role in MM tumorigenesis, as well as to evaluate this receptor contribution to bortezomib treatment response.

Materials and methods

Cell line culture. The cell lines RPMI8226 and SKO007 were a kind gift from Octavia L. Caballero (Ludwig Institute for Cancer Research, New York branch) and the cell line

Correspondence to: Professor Andre Luiz Vettore, Department of Biological Sciences, Laboratório de Biologia Molecular do Câncer, UNIFESP, Rua Pedro de Toledo, 669-11º andar, Universidade Federal de São Paulo, Campus Diadema, São Paulo 04039-032, Brazil
E-mail: andre.vettore@gmail.com

Key words: bortezomib, *deleted in colorectal cancer*, methylation, multiple myeloma

U266 was provided by Anamaria Camargo Aranha (Ludwig Institute for Cancer Research, São Paulo branch). All cell lines were maintained in suspension with RPMI 1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% non-essential amino acids (MEM NEAA) and 0.01 $\mu\text{g/ml}$ of penicillin-streptomycin (both Gibco; Thermo Fisher Scientific, Inc.). These cells were grown in an incubator at 37°C with 5% CO₂. Routinely, cells were treated with drug concentrations previously described in the literature. The bortezomib treatment was conducted by keeping the cells in the presence of 10 nM of the drug for 48 h. For demethylation study, RPMI8226 cells were seeded on day 0 and treated with 10 μM of 5'-aza-2'-deoxycytidine (decitabine, DAC; Sigma-Aldrich; Merck KGaA) for 3 days. DNA and RNA were extracted at days 0 and 3, and stored at -80°C.

RNA extraction, cDNA synthesis and RT-qPCR. RNA extraction was performed using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After extraction, 25 μg of total RNA were treated with *RQ1 RNase-Free DNase* (Promega Corp., Madison, WI, USA) to eliminate the presence of genomic DNA and 2 μg of total RNA were subjected to cDNA synthesis using the SuperScript III First-Strand Synthesis System (Invitrogen; Thermo Fisher Scientific, Inc.). The cDNA obtained was diluted 10-fold before use. The *DCC* mRNA expression was determined by RT-qPCR using an *ABI 7500 Sequence Detection System* (Applied Biosystems) and SYBR-Green reagent (both Thermo Fisher Scientific, Inc.). The primer sequences are available upon request. All reactions were performed in triplicate. The $2^{-\Delta\Delta C_t}$ method was employed to evaluate the expression of *DCC* in the MM cell lines (22). In a previous study, using geNorm algorithm to assess gene expression stability of different housekeeping genes in MM samples, we determined *GAPDH* and *ACTB* as the most stable combination of genes to be used to normalize the expression data in MM (23). Therefore, mean C_t values of these two genes were used for the normalization of RT-qPCR data and the results were illustrated in arbitrary units.

Methylation-specific PCR. Bisulfite treatment of DNA converts unmethylated cytosine to uracil while methylated ones remain as cytosine. Sodium bisulfite conversion of 2 μg of genomic DNA was performed as previously described (24). Bisulfite-modified DNA was used as a template for fluorescence-based real-time PCR (qMSP) as described previously (25). Primers and probes were the same used by de Carvalho *et al* (12). All reactions were performed in triplicate. Leukocyte DNA obtained from a healthy individual was methylated *in vitro* using SSSI methyltransferase enzyme (New England Biolabs Inc., Ipswich, MA, USA) to generate methylated DNA at all CpG sites (positive control). The calculation of the methylation level was performed using the $2^{-\Delta\Delta C_t}$ equation (22).

Western blotting. Cell lines were harvested, washed in ice-cold phosphate-buffered saline solution and resuspended in lyses buffer (50 mM Tris-HCl pH 7.4; 100 mM NaCl; 0.5% NP-40) containing a protease inhibitor cocktail

(Roche, Applied Science, Penzberg, Germany). The proteins were resolved in SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in Tris-buffered saline solution containing 0.05% of Tween-20 and 5% of nonfat dry milk for 1 h at room temperature. Further, the membranes were incubated with the appropriate primary antibodies for 2 h using the following dilutions: 1:100 DCC (cat. no. A-20; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and 1:1,000 α/β -Tubulin (cat. no. 2148; Cell Signaling Technologies, Inc., Danvers, MA, USA). As secondary antibodies, we used goat anti-mouse (cat. no. 14-13-06; Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) and anti-rabbit IgG HRP-linked (cat. no. 7074; Cell Signaling Technologies, Inc.), conjugated with peroxidase. Proteins were detected using the Chemiluminescent HRP Substrate (EMD Millipore) and visualized in an ImageQuantLass 4000 system (GE Healthcare Life Sciences).

DCC esiRNA transfections. The U266 cells were transfected with esiRNAs (cat. no. EHU014471; Mission esiRNA1; Sigma-Aldrich; Merck KGaA) targeting *DCC* or with a pmaxGFP™ control vector (Lonza Group, Ltd., Basel, Switzerland). Transfections were performed by electroporation using a Nucleofector apparatus following the manufacturer's recommendations (Cell Line Nucleofector kit C, program X-005; Lonza Group, Ltd.). Upon 24 h after transfection, the number of cells expressing GFP was counted using an inverted fluorescence microscope in order to estimate the transfection success (data not shown) (Zeiss Imager M1-AX10). After 48, 72 or 96-h post-transfection, cells were collected and subjected to downstream analysis.

Cell viability assay. Cell viability was quantified using PrestoBlue Cell Viability Reagent (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Briefly, MM cells (WT or freshly transfected with esiDCC) were seeded into 96-well plates at an initial density of 12×10^3 cells per well and incubated at 37°C. After 48 h, 10 nM of bortezomib was added. Two h before the end of the treatment (total of 96 h), 10 μl of PrestoBlue reagent was added to each well and, at completion of 96 h-incubation, fluorescence (540 nm excitation/590 nm emissions) was measured using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Experiments were performed in sextuplicate. Data was expressed as mean \pm SD of the sextuplicate assays.

Cell death assay. Briefly, 1×10^5 MM cells were seeded in each well of a 12-well dish with 1 ml of RPMI-1640 medium. A total of 24 h after seeding, bortezomib was added. After 48 h of incubation with bortezomib, cells were harvested by centrifugation (250 x g; 5 min), washed with PBS and resuspended in 100 μl of binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl and 2.5 mM CaCl₂). Next, 0.5 μl of Annexin V-conjugated with FITC (cat. no. 556419; BD Biosciences, Franklin Lakes, NJ, USA) and 0.5 μl of Propidium Iodide (PI; cat. no. P4170; Sigma-Aldrich; Merck KGaA) were added to the cells and incubated for 60 min at room temperature in the dark. Flow cytometer was performed using standard procedures on Accuri C6 (BD Biosciences). MM cells marked only with 0.5 μl of Annexin V or with 0.5 μl of PI were

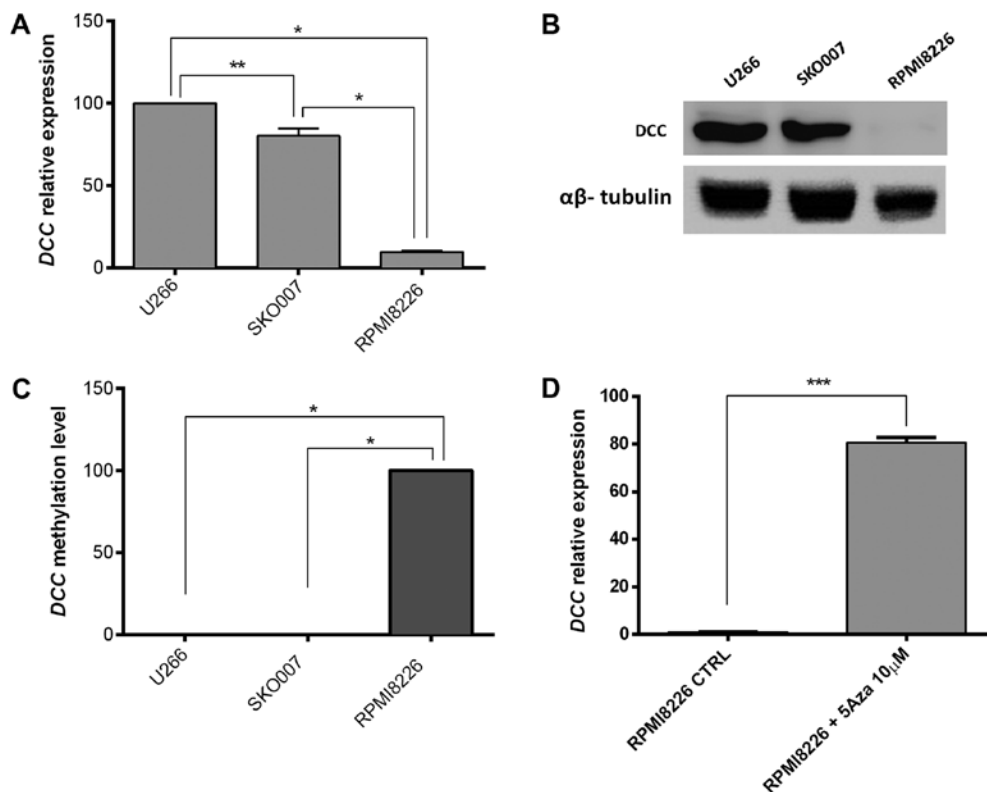


Figure 1. DCC expression in MM cells is regulated by methylation. (A) Expression pattern of *DCC* mRNA in RPMI8226, SKO007 and U266 MM cell lines was evaluated by reverse transcription-quantitative polymerase chain reaction. (B) DCC expression in MM cell lines was evaluated by western blotting. The cell crude extracts were transferred to a membrane and probed with anti-DCC antibody or with anti- $\alpha\beta$ -TUBULIN antibody. (C) Methylation status of the *DCC* promoter region in RPMI8226, SKO007 and U266 cell lines was evaluated by qMSP. (D) RPMI8226 cells were treated with 10 μ M of DAC for 3 days. The treatment with the demethylating agent induced a 82-fold increment in the *DCC* expression level. CTRL: RPMI8226 cells collected at day 0 of the DAC treatment. All the relative expression level was calculated using the $2^{-\Delta\Delta C_t}$ method. Data are presented as the mean \pm standard deviation of three independent experiments. * $P < 0.0001$, ** $P = 0.0045$ by one-way analysis of variance and Bonferroni's post-test method; *** $P = 0.0002$ by Welch's t-test. *DCC*, deleted in colorectal cancer; MM, multiple myeloma; DAC, 5'-aza-2'-deoxycytidine; CTRL, control.

used to calibrate the flow cytometer according to Annexin V and PI labeling. This assay was performed in triplicate. For this analysis, the percentage of cell death represents the sum of MM cells staining Annexin V+/PI- and Annexin V+/PI+.

Statistical analysis. The statistical significance of *DCC* transcripts or methylation levels was calculated using the Welch t test or one-way ANOVA, as appropriated. Comparisons of the values obtained in cell viability and cell death assays were performed using one-way ANOVA. All multiple paired comparisons were conducted by means of the Bonferroni's post-test method to maintain the 5% significance level.

Results

Methylation status of *DCC* in MM cells. First of all, to better understand the regulation of *DCC* expression in MM cells, we evaluated the presence of *DCC* transcripts and protein in three different MM lines (RPMI8226, SKO007 and U266). This analysis showed high *DCC* mRNA expression in SKO007 and U266, while RPMI8226 presented a lower transcript level of this gene (100, 80.19 \pm 9.66%, respectively; Fig. 1A) and, as expected, the DCC polypeptides were observed only in U266 and SKO007 cells (Fig. 1B). In order to correlate the status of *DCC* methylation with the transcript and protein expression observed, the methylation pattern of the promoter region of

this gene was evaluated in MM cell lines. According to this analysis, the *DCC* promoter region was highly methylated in the RPMI8226 cell line, which presents *DCC* low expression, whereas no methylation could be detected in the *DCC* high expression cell lines SKO007 and U266 (Fig. 1C; $P < 0.0001$).

To confirm that *DCC* low expression in RPMI8226 was associated with the hypermethylation of its promoter region, this MM cell line was treated with DAC (a recognized DNA demethylating agent). Remarkably, an 82-times increment could be observed in the *DCC* expression in RPMI8226 cells treated with this demethylating agent (Fig. 1D; $P = 0.0002$).

MM cells response to bortezomib treatment. To evaluate the association between bortezomib resistance in MM cells and the *DCC* expression, it was determined the bortezomib effect in SKO007 and U266 (high expression of *DCC*), as well as in RPMI8226 (low expression of *DCC*). This assay showed a significant lower number of viable cells in RPMI8226 submitted to bortezomib treatment in comparison to the other two MM cell lines ($P < 0.0001$; Fig. 2A). In accordance with this, the total number of dead cells after bortezomib treatment is higher in the RPMI8226 in comparison to SKO007 or U266 ($P < 0.0001$; Fig. 2B). These results demonstrated that the effect of the bortezomib on RPMI8226 (the cell line with the lowest expression level of *DCC*) is stronger in comparison to the other two MM cell lines, which presented higher *DCC* expression.

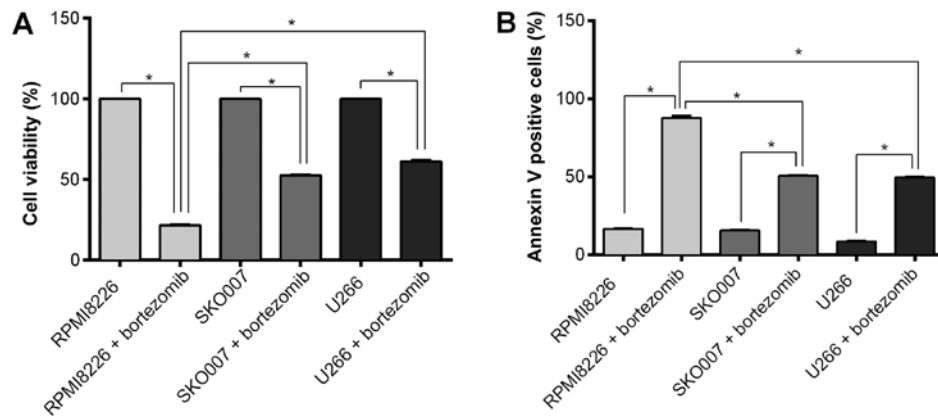


Figure 2. Effect of bortezomib treatment in multiple myeloma cells. RPMI8226, SKO007 and U266 cells were treated with 10 nM of bortezomib for 48 h. (A) Proportion of viable cells was estimated using PrestoBlue Cell Viability Reagent. Data are presented as the mean \pm standard deviation of six independent experiments. (B) Cell death rate was evaluated by flow cytometer using Annexin V/Propidium Iodide staining. All the cells stained positive for Annexin V were considered dead cells. * $P < 0.0001$ by one-way analysis of variance and Bonferroni's post-test method. MM, multiple myeloma.

DCC silencing decreases cell viability and promotes cell death. To put some light in the functional role of DCC in MM tumorigenesis, we conducted a transient *DCC* knockdown in the U266 line. The *DCC* silencing was confirmed by quantitative PCR and western blot analysis. As shown in Fig. 3, when compared to control cells, *DCC*-silenced cells exhibited a dramatic reduction in *DCC* transcript and polypeptide levels.

No significant difference in cell viability of U266-WT and U266-esiDCC cells was observed in the absence of bortezomib. However, *DCC*-silenced cells exhibited a significant decrease in the cell viability in the presence of this PI (36.9 and 49.9%, respectively; Fig. 3C). As shown in Fig. 3D, the *DCC*-silenced U266 cells exhibited a significant increase in the number of dead cells upon bortezomib treatment in comparison to U266 WT cells (75.3 vs. 54.1%, respectively). Interestingly, these results suggest that the blockage of DCC activity increases bortezomib-induced cell death in MM cells, suggesting that DCC absence could be able to sensitize MM cells to bortezomib treatment.

Discussion

Over the last decade, the introduction of novel agents such as immunomodulatory drugs (thalidomide and lenalidomide) and PIs has changed the treatment landscape of MM and prolonged overall survival of patients with this plasma cell disease (26). Nevertheless, MM is still regarded as an incurable disease for most patients. Therefore, a major challenge is to enlarge the molecular aspects knowledge of this hematological malignancy and the consequent development of rational combination therapies that could efficiently destroy MM cells.

Previous data showing frequent hypermethylation of *DCC* in MM patients and its association with poor prognosis (12) raised the question of what would be the DCC contribution to MM tumorigenesis. It is well accepted that hypermethylation leads to the promoter obstruction, which hinders gene transcription, and subsequently causes gene silencing (27). Thus, first and foremost, we verified if the aberrant methylation of the *DCC* promoter translates into gene expression inhibition in MM cells. Our results fully support this correlation, since the absence of

aberrant methylation in promoter region was associated to the presence of mRNA and DCC protein, as observed in SKO007 and U266 cell lines, while, on the other hand, the *DCC* hypermethylation (as observed in RPMI8226) correlates with the lack of *DCC* transcription and the scarcity of DCC polypeptides.

DCC was firstly described in colorectal carcinomas as a potential tumor suppressor gene located on chromosome 18q (15). This chromosome region is often subjected to the loss of heterozygosity, and the absence of *DCC* in colorectal cancer has been correlated with poor prognosis, tumor progression and increased risk of metastasis (16). It was also reported that DCC might induce apoptosis in colorectal tumors in the absence of netrin 1, while the presence of this ligand is able to block DCC-induced cell death (28). Moreover, the *DCC* overexpression in ovarian cancer cells suggests an important role in suppressing cell viability and inducing apoptosis through regulation of the levels of β -catenin (29,30).

On the other hand, the *DCC* role as a tumor suppressor remains controversial. The chromosome 18q region, where *DCC* is located, also harbors other genes known as tumor suppressors, such as *SMAD2* and *SMAD4*. Furthermore, the lack of predisposition to intestinal tumors in mice carrying a *Dcc* mutation was interpreted as an evidence that *DCC* gene may not has a significant role in suppressing colorectal tumors (19,31). Beside this, Bamias *et al* (21) showed an association between the loss of DCC and a better prognosis in resected gastric cancer. These studies indicate that *DCC* does not act as a tumor suppressor gene in all situations and could play different roles in different cancer tissues. Therefore, in this context, it seems that a major question is to understand when the presence/loss of DCC offers selective advantages for cancer cells. To the best of our knowledge, this is the first study to demonstrate that the *DCC* absence increases MM cells sensitivity to bortezomib treatment. The lack of *DCC* induces a reduction in the cell viability rate and increases the number of dead cells in response to bortezomib treatment, which suggest that this cell surface receptor may not have a tumor suppressor activity in MM.

Bortezomib is a reversible inhibitor that primarily targets the $\beta 5$ -subunit (PSMB5) subunit/chymotrypsin-like activity of the 26S proteasome and also, to a somewhat lesser extent, the caspase-like activity harbored by the $\beta 1$ (PSMB6)

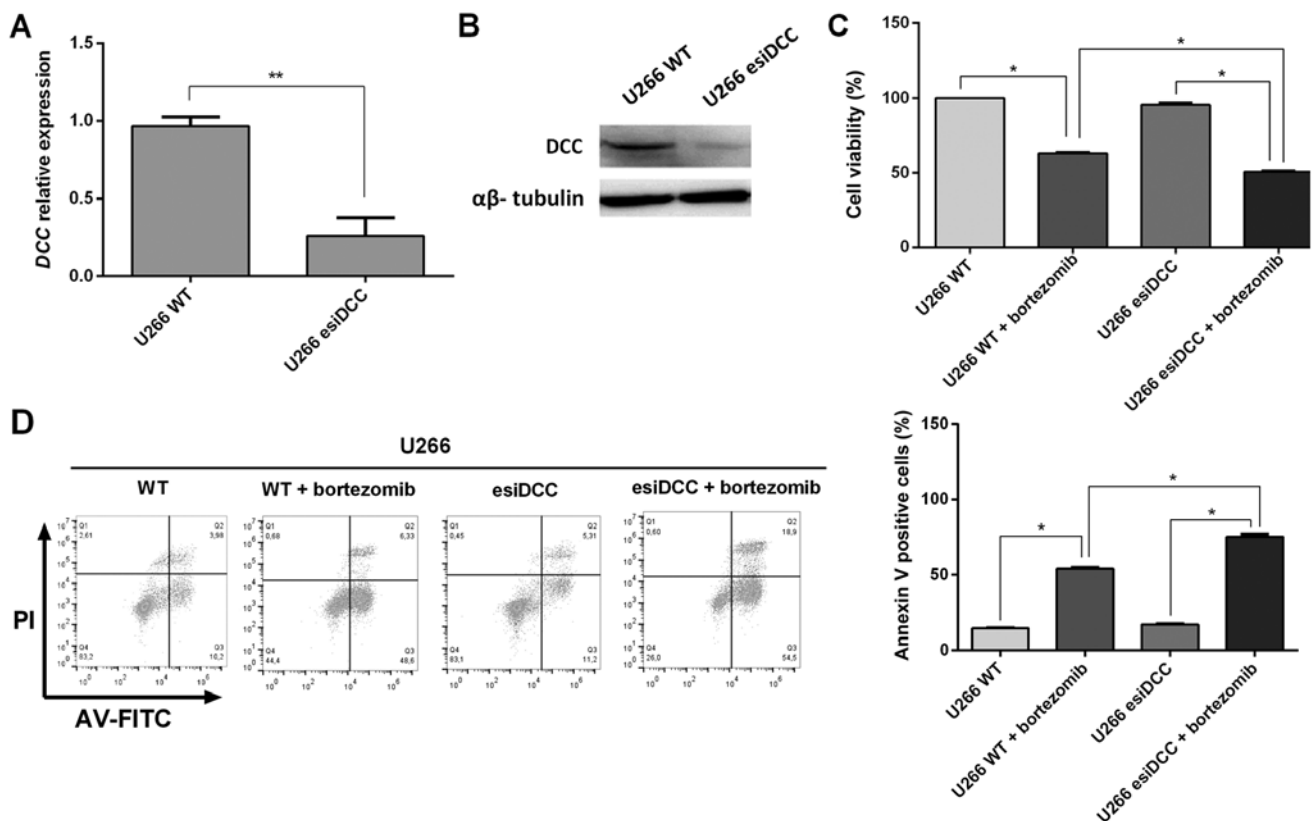


Figure 3. In the absence of DCC, MM cells are more sensitive to the bortezomib treatment. The expression level of DCC transcripts and protein were evaluated in U266-WT and U266-esiDCC cells by (A) Reverse transcription-quantitative chain reaction (** $P=0.0032$ by Welch t-test) and (B) western blotting. (C) Number of U266 WT and U266 esiDCC viable cells was estimated using PrestoBlue reagent before and after treatment with 10 nM bortezomib. No significant difference was observed in the cell viability of U266 WT and U266 esiDCC cells not treated with bortezomib ($P=0.1396$). However, a significant reduction in the cell viability was observed in the knocked-down cells treated with bortezomib ($P<0.0001$). (D) Dot plot diagrams obtained by flow-cytometry analysis of bortezomib treated U266-WT and U266-esiDCC cells after dual staining with AV and PI. The upper-left quadrant represents unviable cells (PI positive and Annexin negative), the upper-right quadrant represents cell that are in late apoptosis or necrosis (both Annexin and PI positive). The lower-left quadrant represents viable cells (both Annexin and PI negative). The lower-right quadrant represents cells in early apoptosis/cell apoptosis (Annexin positive and PI negative). Representative dot plots of three independent experiments are given. The transient silencing of *DCC* expression in U266 cell line leads to a significant increment in the proportion of AV positive cells induced by bortezomib treatment ($P<0.0001$). No significant difference was observed in the proportion of dead cells in the U266 WT and U266 esiDCC without exposition to bortezomib ($P=0.1000$). One-way analysis of variance and Bonferroni's post-test method was used. *DCC*, *deleted in colorectal cancer*; MM, multiple myeloma; PI, propidium iodide; AV, Annexin V; FITC, fluorescein isothiocyanate; WT, wild-type.

proteasome subunit. At higher concentrations, bortezomib inhibits trypsin-like proteolytic activity facilitated by $\beta 2$ (PSMB7) proteasome subunits (32,33). Due to this proteasome inhibition, there is an accumulation of misfolded proteins, resulting in endoplasmic reticulum stress to cause unfolded protein response and cell apoptosis (34). Other effects of this drug in MM include inhibition of angiogenesis and DNA repair system, as well as decrease of osteoclast activity (35). In 2003, the United States Food and Drug Administration (FDA) approved bortezomib for use in relapsed/refractory MM, and, quickly, this boronic acid-based reversible PI became recognized as the most effective anticancer drug for MM treatment (36). However, patients treated with bortezomib will ultimately develop resistance and experience clinical progression, thus, characterizing the mechanisms of PI resistance has become of great interest.

To better understand the bortezomib resistance, Chauhan *et al* (37) provided the first evidence that heat shock protein 27 confers bortezomib resistance in lymphoma cells. Besides this, cancer testis antigens (CTA) appears to contribute to apoptosis suppression, avoiding the effect of caspase inhibitors in MM cells (38). MAGE C1/CT7 and MAGE A3 might

play an important role protecting MM cells against spontaneous apoptosis and their absence contributes to increased bortezomib cytotoxic effects (39,40). Moreover, Hu *et al* (41) showed that CD9 expression increases MM cells sensitivity to bortezomib treatment by inducing apoptosis. Additionally, Moschetta *et al* (42) suggested that cMet is a potential therapeutic target for MM because the presence of this protein conferred a protective effect when MM cells were treated with bortezomib.

Thus, our results are the first to suggest that MM cells could become more susceptible to bortezomib cytotoxic effects when *DCC* is absent. Along the same line, the miRNA hsa-miR-631, which was able to re-sensitize bortezomib-resistant MM cell line (43), was recently described as targeting *DCC* transcript and inhibiting its transduction (44). Further, our results showed that the *DCC* biological function in MM should be related to cell death regulation, exerting an anti-apoptotic role in these cells in response to bortezomib treatment. In some way, these findings seem to be contrary to our previous observations, which the presence of *DCC* (no hypermethylation) was associated with better prognosis (12). However, it is worth mentioning that none of the patients included in the former

study were treated with bortezomib (they were all submitted to VAD, melphalan/prednisone or thalidomide/prednisone therapy schemes). Thus, the difference in the chemotherapy regimen adopted avoids a direct comparison of the results observed in both studies.

In conclusion, our results indicate that the absence of DCC increase the response to bortezomib in MM. Based on these findings, we can speculate that drugs blocking DCC activity should increase the efficacy of bortezomib-based therapy approaches, although further studies are required to confirm this hypothesis and to better discriminate the role of DCC in the MM cell response to bortezomib.

Acknowledgements

Not applicable.

Funding

The present study was funded by São Paulo Research Foundation (FAPESP; grant no. 2012/14837-7; 2010/20218-2). DMR-Jr was recipient of scholarship from São Paulo Research Foundation (FAPESP; grant no. 2012/01597-8).

Availability of data and materials

The dataset used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DMR-J conducted the studies, analyzed the data and drafted the manuscript. TPB, GEDA, VC, MVB and JM-J contributed to the data analysis. ALV contributed to the design and coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Morgan GJ: Advances in the biology and treatment of myeloma. *Br J Haematol* 105 (Suppl 1): S4-S6, 1999.
- Smith ML and Newland AC: Treatment of myeloma. *QJM* 92: 11-14, 1999.
- Röllig C, Knop S and Bornhäuser M: Multiple myeloma. *Lancet* 385: 2197-2208, 2015.
- Mateos MV, Ocio EM and San Miguel JF: Novel generation of agents with proven clinical activity in multiple myeloma. *Semin Oncol* 40: 618-633, 2013.
- Cvek B: Proteasome inhibitors. *Prog Mol Biol Transl Sci* 109: 161-226, 2012.
- Moreau P, Richardson PG, Cavo M, Orłowski RZ, San Miguel JF, Palumbo A and Harousseau JL: Proteasome inhibitors in multiple myeloma: 10 years later. *Blood* 120: 947-959, 2012.
- McBride A and Ryan PY: Proteasome inhibitors in the treatment of multiple myeloma. *Expert Rev Anticancer Ther* 13: 339-358, 2013.
- Grosicki S, Barchnicka A, Jurczyszyn A and Grosicka A: Bortezomib for the treatment of multiple myeloma. *Expert Rev Hematol* 7: 173-185, 2014.
- Brenner H, Gondos A and Pulte D: Recent major improvement in long-term survival of younger patients with multiple myeloma. *Blood* 111: 2521-2526, 2008.
- Kumar SK, Rajkumar SV, Dispenzieri A, Lacy MQ, Hayman SR, Buadi FK, Zeldenrust SR, Dingli D, Russell SJ, Lust JA, *et al*: Improved survival in multiple myeloma and the impact of novel therapies. *Blood* 111: 2516-2520, 2008.
- Pulte D, Redaniel M, Brenner H, Jansen L and Jeffreys M: Recent improvement in survival of patients with multiple myeloma: Variation by ethnicity. *Leuk Lymphoma* 55: 1083-1089, 2014.
- de Carvalho F, Colleoni GW, Almeida MS, Carvalho AL and Vettore AL: TGFbetaR2 aberrant methylation is a potential prognostic marker and therapeutic target in multiple myeloma. *Int J Cancer* 125: 1985-1991, 2009.
- Hedrick L, Cho KR, Fearon ER, Wu TC, Kinzler KW and Vogelstein B: The DCC gene product in cellular differentiation and colorectal tumorigenesis. *Genes Dev* 8: 1174-1183, 1994.
- Keino-Masu K, Masu M, Hinck L, Leonardo ED, Chan SS, Culotti JG and Tessier-Lavigne M: Deleted in colorectal cancer (DCC) encodes a netrin receptor. *Cell* 87: 175-185, 1996.
- Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM and Bos JL: Genetic alterations during colorectal-tumor development. *N Engl J Med* 319: 525-532, 1998.
- Fearon ER, Cho KR, Nigro JM, Kern SE, Simons JW, Ruppert JM, Hamilton SR, Preisinger AC, Thomas G, Kinzler KW, *et al*: Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science* 247: 49-56, 1990.
- Cho KR and Fearon ER: DCC: Linking tumour suppressor genes and altered cell surface interactions in cancer? *Eur J Cancer* 31A: 1055-1060, 1995.
- Fearon ER: DCC: Is there a connection between tumorigenesis and cell guidance molecules? *Biochim Biophys Acta* 1288: M17-M23, 1996.
- Fazeli A, Dickinson SL, Hermiston ML, Tighe RV, Steen RG, Small CG, Stoeckli ET, Keino-Masu K, Masu M, Rayburn H, *et al*: Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. *Nature* 386: 796-804, 1997.
- Forcet C, Ye X, Granger L, Corset V, Shin H, Bredesen DE and Mehlen P: The dependence receptor DCC (deleted in colorectal cancer) defines an alternative mechanism for caspase activation. *Proc Natl Acad Sci USA* 98: 3416-3421, 2001.
- Bamias AT, Bai MC, Agnantis NJ, Michael MC, Alamanos YP, Stefanaki SV, Razi ED, Skarlos DV, Kappas AM and Pavlidis NA: Prognostic significance of the deleted in colorectal cancer gene protein expression in high-risk resected gastric carcinoma. *Cancer Invest* 21: 333-340, 2003.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Andrade VC, Vettore AL, Panepucci RA, Almeida MS, Yamamoto M, De Carvalho F, Caballero OL, Zago MA and Colleoni GW: Number of expressed cancer/testis antigens identifies focal adhesion pathway genes as possible targets for multiple myeloma therapy. *Leuk Lymphoma* 51: 1543-1549, 2010.
- Vidal DO, Paixão VA, Brait M, Souto EX, Caballero OL, Lopes LF and Vettore AL: Aberrant methylation in pediatric myelodysplastic syndrome. *Leuk Res* 31: 175-181, 2007.
- Rettori MM, de Carvalho AC, Bomfim Longo AL, de Oliveira CZ, Kowalski LP, Carvalho AL and Vettore AL: Prognostic significance of TIMP3 hypermethylation in post-treatment salivary rinse from head and neck squamous cell carcinoma patients. *Carcinogenesis* 34: 20-27, 2013.
- Palumbo A and Anderson K: Multiple myeloma. *N Engl J Med* 364: 1046-1060, 2011.
- Garinis GA, Patrinos GP, Spanakis NE and Menounos PG: DNA hypermethylation: When tumour suppressor genes go silent. *Hum Genet* 111: 115-127, 2002.
- Mehlen P and Fearon ER: Role of the dependence receptor DCC in colorectal cancer pathogenesis. *J Clin Oncol* 22: 3420-3428, 2004.

29. Meimei L, Peiling L, Baoxin L, Changmin L, Rujin Z and Chunjie H: Lost expression of DCC gene in ovarian cancer and its inhibition in ovarian cancer cells. *Med Oncol* 28: 282-289, 2011.
30. Cai Y, Hu CJ, Wang J and Wang ZH: Influence of deleted in colorectal carcinoma gene on proliferation of ovarian cancer cell line SKOV-3 in vivo and in vitro. *Chin Med Sci J* 26: 175-181, 2011.
31. Roush W: Putative cancer gene shows up in development instead. *Science* 276: 534-535, 1997.
32. Crawford LJ, Walker B, Ovaa H, Chauhan D, Anderson KC, Morris TC and Irvine AE: Comparative selectivity and specificity of the proteasome inhibitors BzLLLCOCHO, PS-341, and MG-132. *Cancer Res* 66: 6379-6386, 2006.
33. Kisselev AF, van der Linden WA and Overkleeft HS: Proteasome inhibitors: An expanding army attacking a unique target. *Chem Biol* 19: 99-115, 2012.
34. Nawrocki ST, Carew JS, Dunner K Jr, Boise LH, Chiao PJ, Huang P, Abbruzzese JL and McConkey DJ: Bortezomib inhibits PKR-like endoplasmic reticulum (ER) kinase and induces apoptosis via ER stress in human pancreatic cancer cells. *Cancer Res* 65: 11510-11519, 2005.
35. Rajkumar SV and Kyle RA: Multiple myeloma: Diagnosis and treatment. *Mayo Clin Proc* 80: 1371-1382, 2005.
36. San Miguel J, Bladé J, Boccadoro M, Cavenagh J, Glasmacher A, Jagannath S, Lonial S, Orłowski RZ, Sonneveld P and Ludwig H: A practical update on the use of bortezomib in the management of multiple myeloma. *Oncologist* 11: 51-61, 2006.
37. Chauhan D, Li G, Podar K, Hideshima T, Shringarpure R, Catley L, Mitsiades C, Munshi N, Tai YT, Suh N, *et al*: The bortezomib/proteasome inhibitor PS-341 and triterpenoid CDDO-Im induce synergistic anti-multiple myeloma (MM) activity and overcome bortezomib resistance. *Blood* 103: 3158-3166, 2004.
38. Yang B, O'Herrin SM, Wu J, Reagan-Shaw S, Ma Y, Bhat KM, Gravekamp C, Setaluri V, Peters N, Hoffmann FM, *et al*: MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines. *Cancer Res* 67: 9954-9962, 2007.
39. Atanackovic D, Hildebrandt Y, Jadcak A, Cao Y, Luetkens T, Meyer S, Kobold S, Bartels K, Pabst C, Lajmi N, *et al*: Cancer-testis antigens MAGE-C1/CT7 and MAGE-A3 promote the survival of multiple myeloma cells. *Haematologica* 95: 785-793, 2010.
40. de Carvalho F, Costa ET, Camargo AA, Gregorio JC, Masotti C, Andrade VC, Strauss BE, Caballero OL, Atanackovic D and Colleoni GW: Targeting MAGE-C1/CT7 expression increases cell sensitivity to the proteasome inhibitor bortezomib in multiple myeloma cell lines. *PLoS One* 6: e27707, 2011.
41. Hu X, Xuan H, Du H, Jiang H and Huang J: Down-regulation of CD9 by methylation decreased bortezomib sensitivity in multiple myeloma. *PLoS One* 9: e95765, 2014.
42. Moschetta M, Basile A, Ferrucci A, Frassanito MA, Rao L, Ria R, Solimando AG, Giuliani N, Boccarelli A, Fumarola F, *et al*: Novel targeting of phospho-cMET overcomes drug resistance and induces antitumor activity in multiple myeloma. *Clin Cancer Res* 19: 4371-4382, 2013.
43. Xi H, Li L, Du J, An R, Fan R, Lu J, Wu YX, Wu SX, Hou J and Zhao LM: hsa-miR-631 resensitizes bortezomib-resistant multiple myeloma cell lines by inhibiting UbcH10. *Oncol Rep* 37: 961-968, 2017.
44. Agarwal V, Bell GW, Nam JW and Bartel DP: Predicting effective microRNA target sites in mammalian mRNAs. *Elife*: 4, 12 Aug, 2015 doi: 10.7554/eLife.05005.