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

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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

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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 μg/ml of penicillin-streptomycin (both Gibco; Thermo Fisher Scientific, Inc.). These cells were grown in an incubator at 37˚C with 5% CO_2. 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 $2^{-\Delta\Delta Ct}$ 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 Ct 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 $2^{-\Delta\Delta Ct}$ 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 $12x10^3$ 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 ± SD of the sextuplicate assays.

Cell death assay. Briefly, $1x10^5$ 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 $CaCl_2$). 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

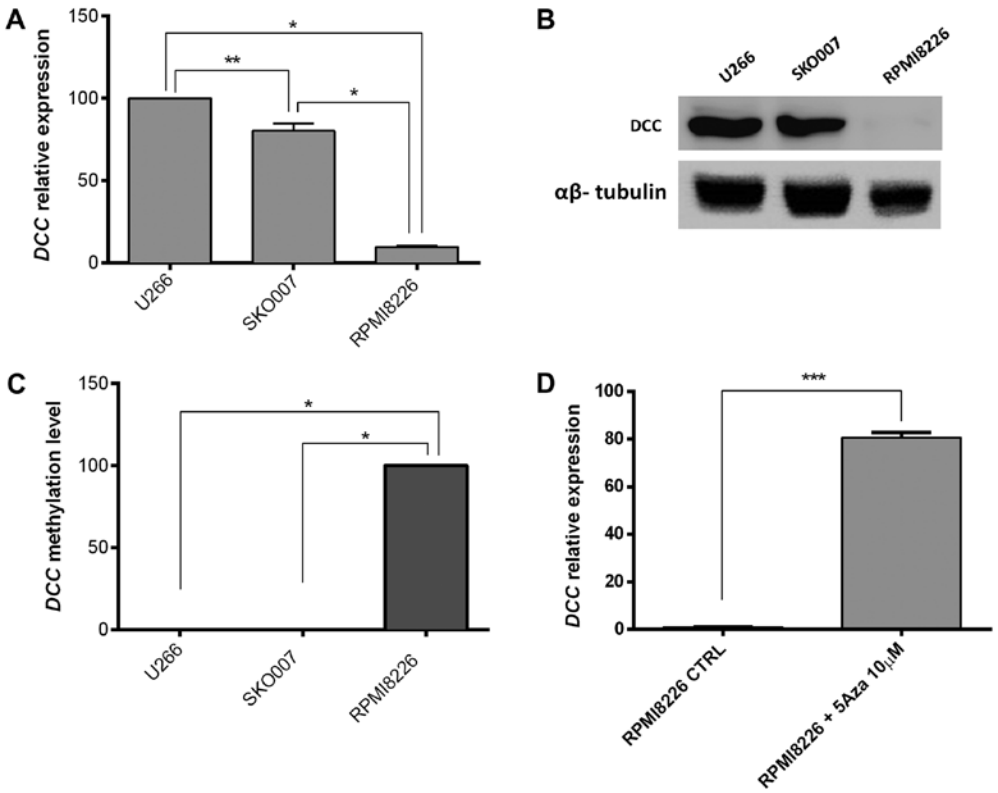

Figure 1. DCC expression in MM cells is regulated by methylation. (A) Expression pattern of *DCC* mRNA in RPMI8226, SKO007 and U266 MM cell lines was evaluated by reverse transcription-quantitative polymerase chain reaction. (B) DCC expression in MM cell lines was evaluated by western blotting. The cell crude extracts were transferred to a membrane and probed with anti-DCC antibody or with anti-α/β-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 μM of DAC for 3 days. The treatment with the demethylating agent induced a 82-fold increment in the *DCC* expression level. CRTL: RPMI8226 cells collected at day 0 of the DAC treatment. All the relative expression level was calculated using the $2^{-\Delta\Delta Cq}$ method. Data are presented as the mean ± 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, SKOO07 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 e 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.

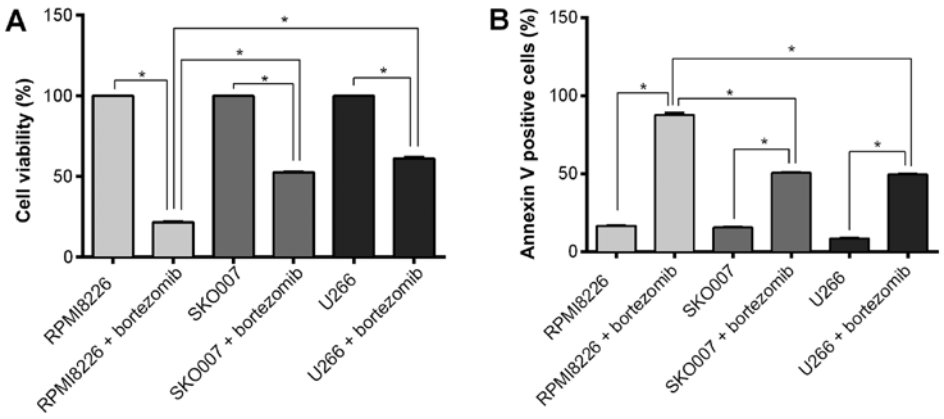

Figure 2. Effect of bortezomib treatment in multiple myeloma cells. RPMI8226, SKO007 and U266 cells were treated with 10 nM of bortezomib for 48 h. (A) Proportion of viable cells was estimated using PrestoBlue Cell Viability Reagent. Data are presented as the mean ± standard deviation of six independent 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 β5-subunit (PSMB5) subunit/chymotrypsin-like activity of the 26S proteasome and also, to a somewhat lesser extent, the caspase-like activity harbored by the β1 (PSMB6)

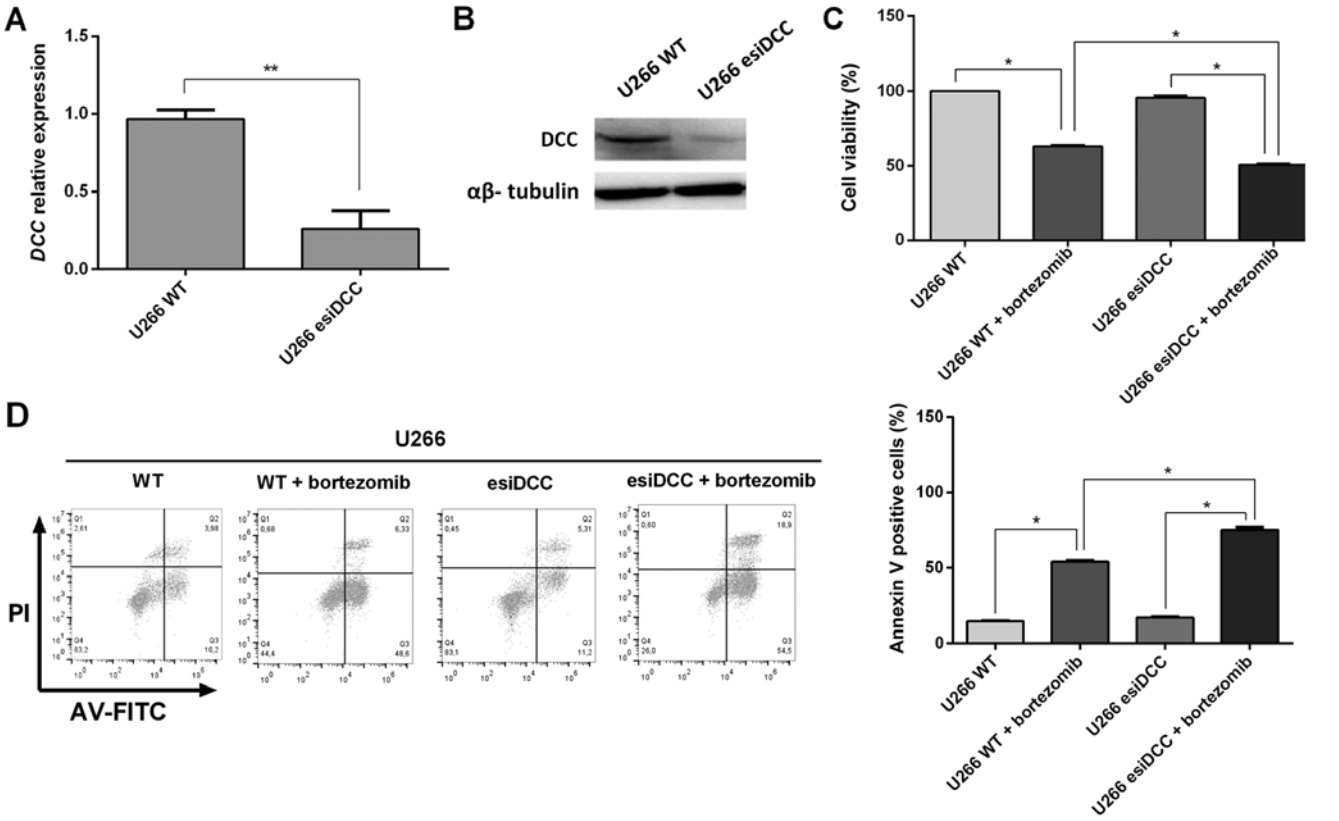

Figure 3. In the absence of DCC, MM cells are more sensitive to the bortezomib treatment. The expression level of DCC transcripts and protein were evaluated in U266-WT and U266-esiDCC cells by (A) Reverse transcription-quantitative chain reaction (**P=0.0032 by Welch t-test) and (B) western blotting. (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 β2 (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.

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