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DNA methylation score is predictive of myeloma cell sensitivity to 5-azacitidine

Epigenetics is characterized by a wide range of changes that are reversible and orchestrate gene expression. Recent studies of the epigenome have shown that epigenetic modifications play a role in cancer physiopathology including haematological malignancies (Smith *et al*, 2009). In multiple myeloma (MM), DNA hypomethylation was reported as the predominant early change during myelomagenesis that is gradually transformed to DNA hypermethylation in relapsed cases and during the progression of the disease (Walker *et al*, 2010; Heuck *et al*, 2013). Hypermethylation of *GPX3*, *RBPI*, *SPARC* and *TGFBI* genes was demonstrated to be associated with significantly shorter overall survival, independent of age, International Staging System (ISS) score and adverse cytogenetics (Kaiser *et al*, 2013). DNA methylation is regulated by DNA methyltransferases (DNMT; Hollenbach *et al*, 2010). Decitabine (5-aza-2'-deoxycytidine) or 5-azacytidine are both U.S. Food and Drug Administration (FDA)-approved DNMT inhibitors for the treatment of myelodysplastic syndrome (MDS). 5-azacytidine is a ribonucleoside and decitabine is a deoxyribonucleoside. Decitabine is incorporated only in DNA whereas 5-azacytidine incorporates in both DNA and RNA including rRNAs, tRNAs, mRNAs and miRNAs (Hollenbach *et al*, 2010). Once incorporated into DNA, 5-azacytidine and decitabine will lead to DNMT depletion, DNA hypomethylation and DNA damage induction (Hollenbach *et al*, 2010). Via incorporation into newly synthesized RNA, 5-azacytidine will interfere with RNA processing, inhibiting protein synthesis (Hollenbach *et al*, 2010). We have recently reported the building of a DNA methylation score (DM Score) that predicts for the efficacy of decitabine to eliminate MM cells (MMCs). Given that 5-azacytidine is

incorporated in both DNA and RNA and can block DNA methylation and RNA translation, we have currently investigated whether the DM Score could also predict for MMCs sensitivity to 5-azacytidine.

Human myeloma cell lines (HMCLs, $n = 12$) XG-1, XG-2, XG-5, XG-6, XG-12, XG-13, XG-16, XG-19, XG-20, RPMI8226, LP1 and SKMM2 were obtained as previously described (Moreaux *et al*, 2011) or purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and American Type Culture Collection (Rockville, MD, USA). Microarray data are deposited in the ArrayExpress public database (accession numbers E-TABM-937 and E-TABM-1088). Bone marrow of patients presenting with previously untreated MM ($n = 14$) at the university hospital of Montpellier was obtained after patients' written informed consent in accordance with the Declaration of Helsinki and agreement of the Montpellier University Hospital Centre for Biological Resources. MMCs were purified as previously published (Hose *et al*, 2011; Moreaux *et al*, 2012) and whole genome gene expression profiling assayed with Affymetrix U133 2.0 plus microarrays (Affymetrix, Santa Clara, CA, USA). Gene expression data were analysed using our bioinformatics platforms (<http://rage.montp.inserm.fr/>)(<http://amazonia.montp.inserm.fr/>) and computations performed using R 2.15.1 (<http://www.r-project.org/>) and bioconductor 2.0 (<http://www.bioconductor.org/>).

Human myeloma cell lines were cultured with graded 5-azacytidine (Sigma, St Louis, MO, USA) concentrations. HMCL cell growth was quantified with a Cell Titer Glo Luminescent Assay (Promega, Madison, WI, USA) and the

50% inhibitory concentration (IC₅₀) was determined using GraphPad Prism software (<http://www.graphpad.com/scientific-software/prism/>).

Primary myeloma cells of 14 patients were cultured with or without graded concentrations of 5-azacitidine and MMC cytotoxicity evaluated using anti-CD138-phycoerythrin

monoclonal antibody (Immunotech, Marseille, France) as described (Moreaux *et al*, 2012).

We compared the gene expression levels from patients with high DM Score *versus* low DM Score and selected the genes that had significantly different expression for gene set enrichment analysis (GSEA).

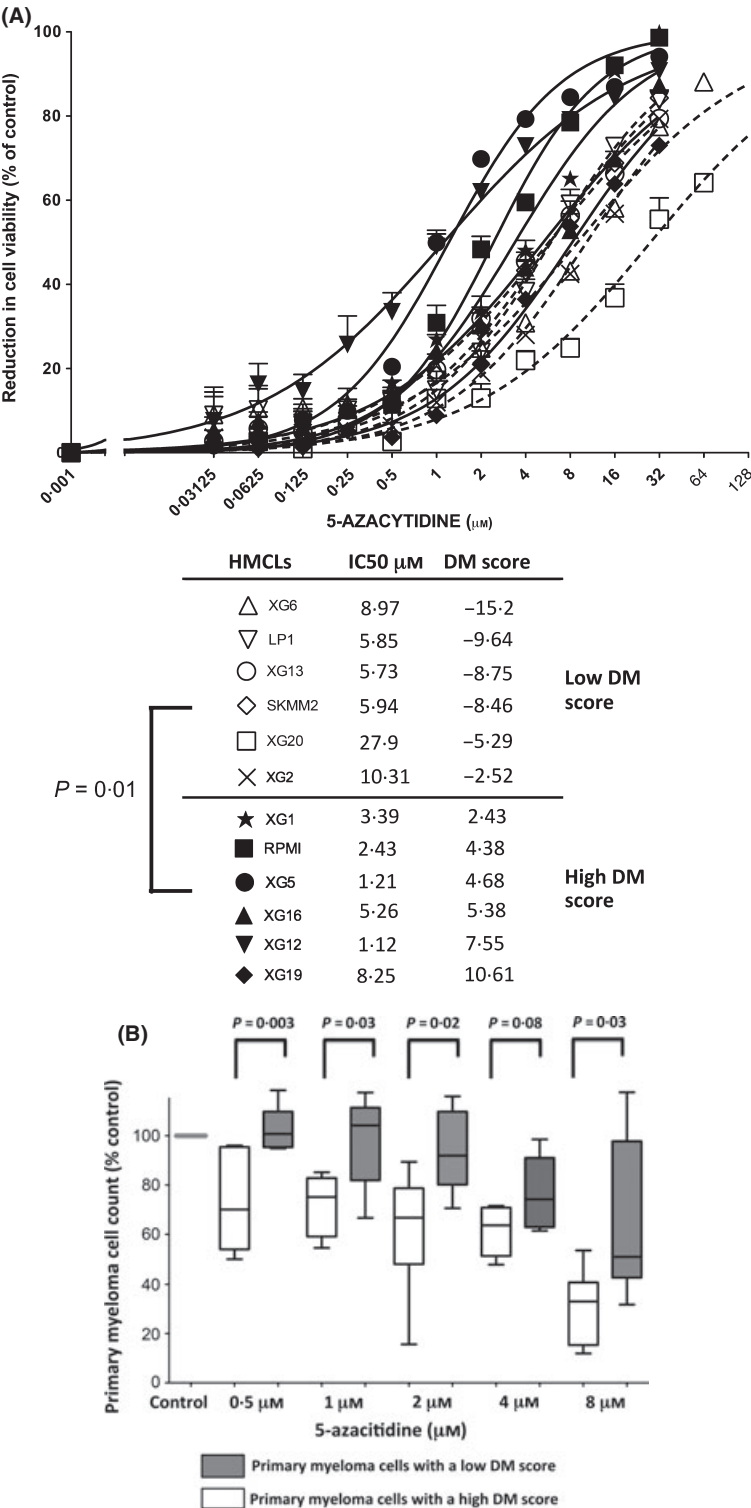


Fig 1. DNA methylation Score predicts for sensitivity of human myeloma cell lines and primary myeloma cells of patients to 5-azacitidine. (A) Human myeloma cell lines (HMCLs) with a high DM Score ($n = 6$) exhibit significantly higher 5-azacitidine sensitivity compared to HMCLs with a low DM Score ($n = 6$). HMCLs were cultured for 4 d in 96-well flat-bottom microtitre plates in RPMI 1640 medium, 10% fetal calf serum, 2 ng/ml interleukin six culture medium (control), and graded 5-azacitidine concentrations. Data are mean values $\pm$ standard deviation (SD) of five experiments determined on sextuplet culture wells. (B) Mononuclear cells from tumour samples of 14 patients with multiple myeloma were cultured for 4 d in the presence of IL6 (2 ng/ml) with or without graded 5-azacitidine concentrations. At day 4 of culture, viable CD138⁺ MMCs were counted using flow cytometry. The grey boxes represent the mean $\pm$ SD of primary myeloma cell counts (expressed as the percentage of the count without adding 5-azacytidine) of the seven patients with a low DM Score and the white boxes indicate the seven patients with a high DM Score.

The efficacy of the DM Score to predict MMC sensitivity to 5-azacitidine was investigated on 12 HMCLs with high or low DM Scores. The 6 HMCLs with high DM Score exhibited a 3-fold higher 5-azacitidine sensitivity ($P = 0.01$; median $IC_{50} = 2.43 \mu\text{mol/l}$; range: 1.12–8.25 $\mu\text{mol/l}$) compared to the 6 HMCLs with a low DM Score (median $IC_{50} = 7.45 \mu\text{mol/l}$; range: 5.73–27.9; Fig 1A). DM Score could also predict for the ability of 5-azacytidine to eliminate patients' primary MMCs cultured together with their bone marrow environment. The MMCs of patients with a high DM Score ($n = 7$) exhibited a significant 1.6-fold higher 5-azacytidine sensitivity compared to low DM Score patients ($n = 7$; $P < 0.05$, Fig 1B).

Thus, the DM Score, which was built using 47 genes whose expression is deregulated by decitabine in HMCLs and which have prognostic value for patients' overall survival, can predict for the sensitivity of HMCLs and primary MMCs to the two-clinical grade inhibitors of DNMT. These 47 genes include 22 genes associated with a bad prognosis and 25 associated with a good prognosis (Fig 2). Using GSEA analysis, MMCs of patients with a high DM Score showed a significant enrichment in genes associated with proliferation (gene sets: REACTOME CELL CYCLE, CELL CYCLE G2 M and PLASMA CELLS VS PLASMABLAST DN, $P < 0.001$, Figure S1 and Tables SI, SII and SIII). On the other hand, MMCs of patients with a low DM Score showed a significant enrichment in genes coding for solute carrier group of membrane transport proteins (gene sets: REACTOME TRANSPORT OF INORGANIC CATIONS ANIONS AND AMINO ACIDS OLIGOPEPTIDES, $P < 0.001$, Figure S2 and Table SIV).

Thus, the higher sensitivity of MMCs of patients with a high DM Score to DNMT inhibitors could be explained by the fact that these inhibitors are mainly active in cell cycling cells, as incorporation into DNA is restricted to the S-phase (Hollenbach *et al*, 2010), and also by a reduced drug export

in MMCs of these patients, resulting in higher intracellular drug accumulation (Karahoca & Momparler, 2013). Furthermore, the DM Score is significantly higher in patients with a high GEP-based proliferation index (Hose *et al*, 2011; Figure S3), emphasizing these data.

Clinical trials are ongoing to evaluate the safety of DNMT inhibitors as monotherapy or in combination with lenalidomide or dexamethasone in MM (Maes *et al*, 2013) and investigation of the link between HM Score and the response of MM patients to DNMT inhibitors could be promising.

In conclusion, the DM Score enables the identification of MM patients who could benefit from treatment with two clinical grade DNMT inhibitors and the development of personalized treatment.

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Author contributions

MJ designed the research and wrote the paper. HD and GH collected bone marrow samples and clinical data and participated in the writing of the paper. VJL participated in the bioinformatics studies. BA provided technical assistance. KB designed and supervised the research and wrote the paper.

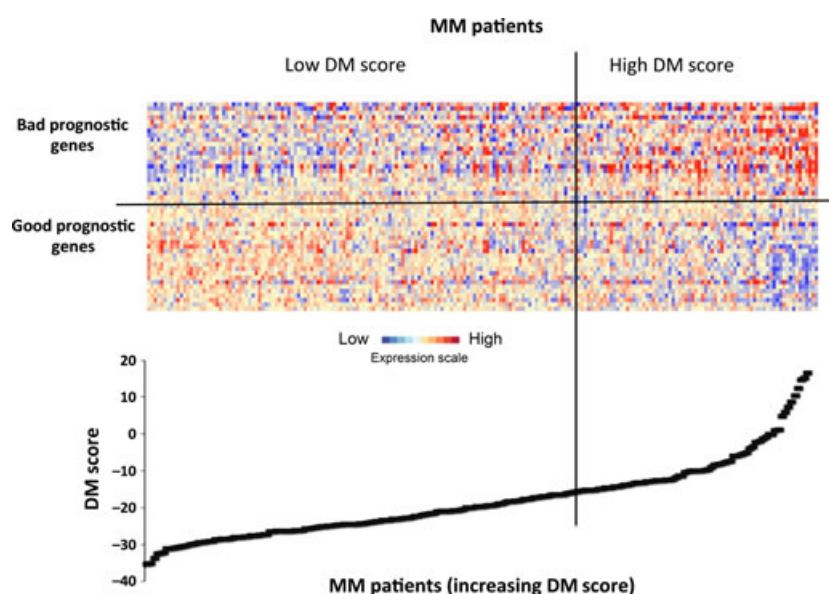


Fig 2. Clustergram of the signals of the 47 genes used to build DNA methylation Score in myeloma cells of 206 previously untreated patients. The signals of the 47 probe sets in MMCs of 206 patients, ordered by increasing DM Score, are displayed from low (deep blue) to high (deep red) expression.

Conflict of interest

The authors have no conflict of interest to declare.

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Supporting Information

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

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Fig S1. Top gene sets significantly associated with high DM score.

Fig S2. Top gene sets significantly associated with low DM score.

Fig S3. DNA methylation score in the 3 groups of the GEP based growth proliferation index (GPI).

Table SI. Genes set enrichment analysis revealed a significant overrepresentation of the REACTOME CELL CYCLE set in high DM Score patients compared to low DM Score patients ($P < 0.001$).

Table SII. Genes set enrichment analysis revealed a significant overrepresentation of the CELL CYCLE G2/M set in high DM Score patients compared to low DM Score patients ($P < 0.001$).

Table SIII. Genes set enrichment analysis revealed a significant overrepresentation of the PLASMA CELLS VS PLASMABLAST set in high DM Score patients compared to low DM Score patients ($P < 0.001$).

Table SIV. Genes set enrichment analysis revealed a significant overrepresentation of the REACTOME TRANSPORT OF INORGANIC CATIONS ANIONS AND AMINO ACID OLIGOPEPTIDES set in low DM Score patients compared to high DM Score patients ($P < 0.001$).