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

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Running title: DM Score predicts myeloma cells sensitivity to 5-azacitidine

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Introduction

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 hematologic malignancies (Smith, *et al* 2009). In 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 (Heuck, *et al* 2013, Walker, *et al* 2010). Hypermethylation of *GPX3*, *RBP1*, *SPARC* and *TGFBI* genes was demonstrated to be associated with significantly shorter overall survival, independent of age, 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 FDA (U.S. Food and Drug Administration)-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 both in DNA and RNA including rRNAs, tRNAs, mRNAs and miRNAs (Hollenbach, *et al* 2010). Once incorporated into DNA, 5-azacytidine and decitabine will lead to DNMTs depletion, DNA hypomethylation and DNA damage induction (Hollenbach, *et al* 2010). Via incorporation into newly synthesized RNA, 5-azacytidine will interfere with the processing of RNAs inhibiting protein synthesis (Hollenbach, *et al* 2010). We have recently reported the building of a DNA methylation score (DM Score) predicting

for the efficacy of decitabine to eliminate MM cells (MMCs). Given that 5-azacitidine can incorporate in both DNA and RNA and block DNA methylation and RNA translation, we have currently investigated whether DM Score could also predict for MMCs sensitivity to 5-azacitidine.

Materials and methods

Human Myeloma Cell Lines (HMCLs) and primary multiple myeloma cells of patients.

Human myeloma cell lines XG-1, XG-2, XG-5, XG-6, XG-12, XG-13, XG-16, XG-19, XG-20, RPMI8226, LP1 and SKMM2 (HMCLs, N=12) were obtained as previously described (Moreaux, *et al* 2011) or purchased from DSMZ and American Type Culture Collection. 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 multiple myeloma (n=14) at the university hospital of Montpellier were obtained after patients' written informed consent in accordance with the Declaration of Helsinki and agreement of the Montpellier University Hospital Center 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 analyzed using our bioinformatics platforms (<http://rage.montp.inserm.fr/> and <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>).

Sensitivity of myeloma cell lines and primary myeloma cells to 5-azacitidine.

HMCLs were cultured with graded 5-azacitidine (Sigma, St Louis, MO) concentrations. HMCLs cell growth was quantified with a Cell Titer Glo Luminescent Assay (Promega, Madison, WI) and half 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-PE mAb (Immunotech, Marseille, France) as described (Moreaux, *et al* 2012).

Gene set enrichment analysis (GSEA)

We compared the gene expression levels from high DM Score versus low DM Score patients and picked up the genes which had significant different expression for Gene set enrichment analysis (GSEA).

Results and Discussion

The efficacy of DM Score to predict MMCs sensitivity to 5-azacitidine was investigated on 12 HMCLs with high or low DM Score. The 6 HMCLs with high DM Score exhibited a 3 fold higher 5-azacitidine sensitivity ($P = 0.01$; median IC₅₀ = 2.43 μ M; range: 1.12 to 8.25 μ M) compared to the 6 ones with a low DM Score (median IC₅₀ = 7.45 μ M; range: 5.73 to 27.9) (Figure 1A). DM score could also predict for the ability of 5-azacytidine to eliminate patients' primary MMCs

cultured together with their BM 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) ($P < .05$, Figure 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 MM cell lines and primary MMCs to the two-clinical grade inhibitors of DNMT. These 47 genes include 22 genes associated with a bad prognosis and 25 with good one (Figure 2). Using GSEA analysis, MMCs of patients with a high DM Score show a significant enrichment in genes associated with proliferation (gene sets: REACTOME CELL CYCLE, CELL CYCLE G2 M and PLASMA CELLS VS PLASMABLAST DN, $P < 0.001$, supplementary Figure S1 and supplementary Tables S1, S2 and S3). On the other hand, MMCs of patients with a low DM Score show 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, $P < 0.001$, supplementary Figure S2 and supplementary Tables S4).

Thus, the higher sensitivity of MMCs of patients with a high DM Score to DNMT inhibitors could be explained by the fact these inhibitors are mainly active in cell cycling cells since 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 and Momparler 2013). Furthermore, DM Score is significantly higher in patients with a

high GEP-based proliferation index(Hose, *et al* 2011) (Supplementary Figure S3), emphasizing these data.

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

In conclusion, the DM Score allows 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 with technical assistance.

KB designed and supervised the research and wrote the paper.

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Figure legends

Figure 1: DM Score predicts for sensitivity of human myeloma cell lines and primary myeloma cells of patients to 5-azacitidine.

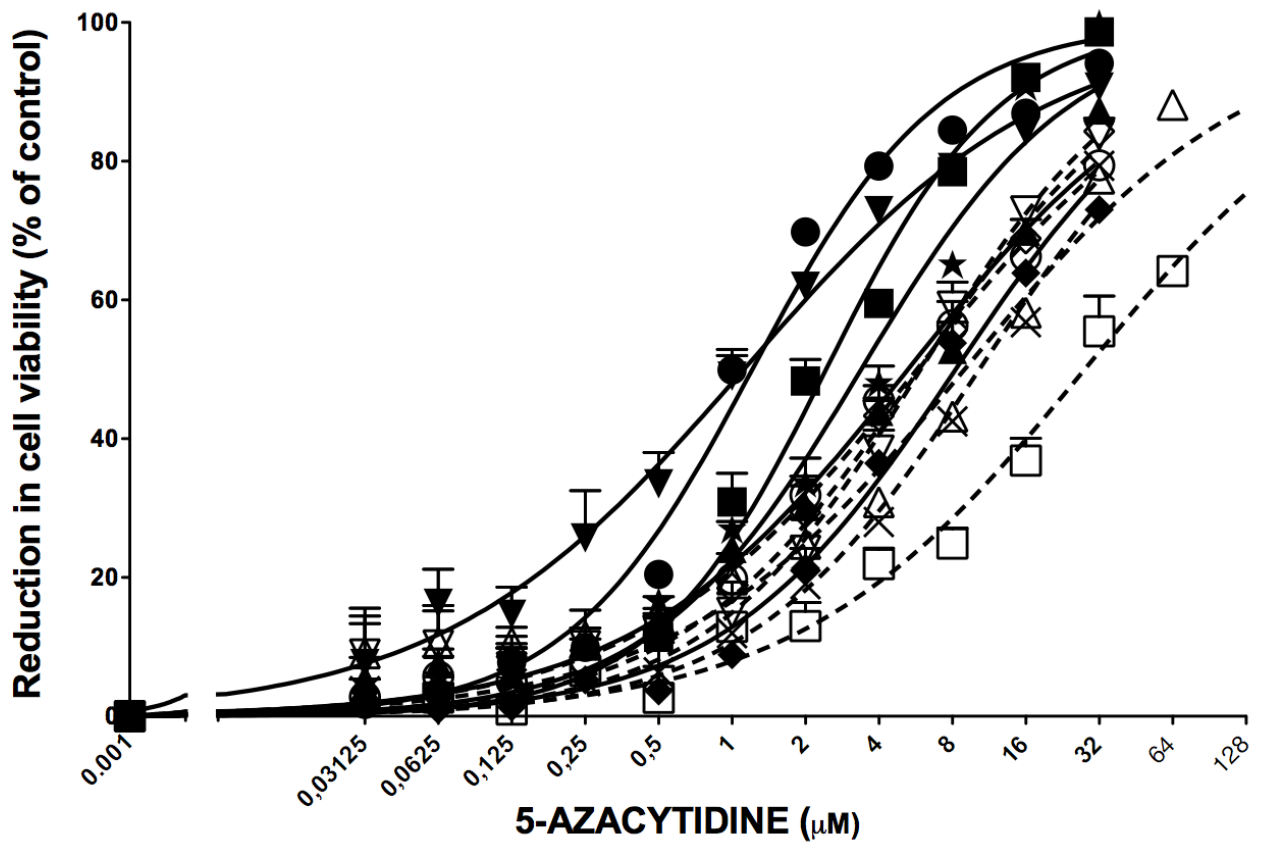
(A) HMCLs with a high DM Score (n = 6) exhibit significant higher 5-azacitidine sensitivity compared to HMCLs with a low DM Score (n = 6). HMCLs were cultured for 4 days in 96-well flat-bottom microtiter plates in RPMI 1640 medium, 10% FCS, 2 ng/ml IL-6 culture medium (control), and graded 5-azacitidine concentrations. Data are mean values plus or minus standard deviation (SD) of 5 experiments determined on sextuplet culture wells.

(B) Mononuclear cells from tumour samples of 14 patients with MM were cultured for 4 days in the presence of IL-6 (2 ng/ml) with or without graded 5-azacitidine concentrations. At day 4 of culture, the count of viable CD138⁺ MMCs was determined using flow cytometry. The grey columns represent the mean \pm SD of primary myeloma cell counts (expressed as the percentage of the count without adding 5-azacytidine) of the 7 patients with a low DM Score and the white columns that of the 7 patients with a high DM Score.

Figure 2: Clustergram of the signals of the 47 genes used to build DM Score in myeloma cells of 206 previously untreated patients.

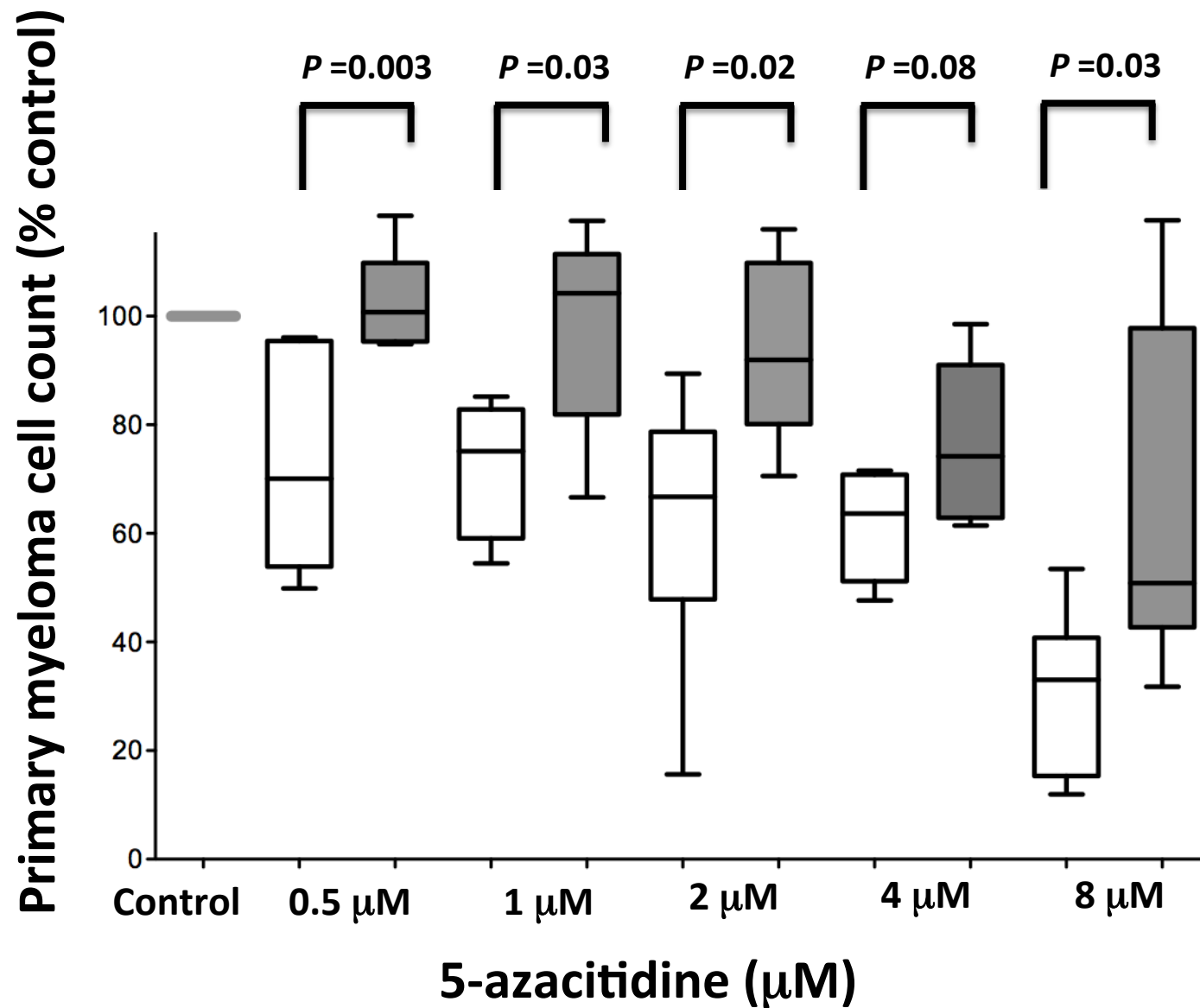
The signals of the 47 probe sets in MMCs of 206 patients, ordered by increasing DM Score, are displayed from low (deep blue) to high (deep red) expression.

Figure 1A



HMCLs	IC50 μ M	DM Score	
△ XG6	8.97	-15.2	Low DM Score
▽ LP1	5.85	-9.64	
○ XG13	5.73	-8.75	
◇ SKMM2	5.94	-8.46	
□ XG20	27.9	-5.29	
× XG2	10.31	-2.52	
★ XG1	3.39	2.43	High DM Score
■ RPMI	2.43	4.38	
● XG5	1.21	4.68	
▲ XG16	5.26	5.38	
▼ XG12	1.12	7.55	
◆ XG19	8.25	10.61	

$P = .01$



■ Primary myeloma cells with a low DM Score
□ Primary myeloma cells with a high DM Score

Figure 2

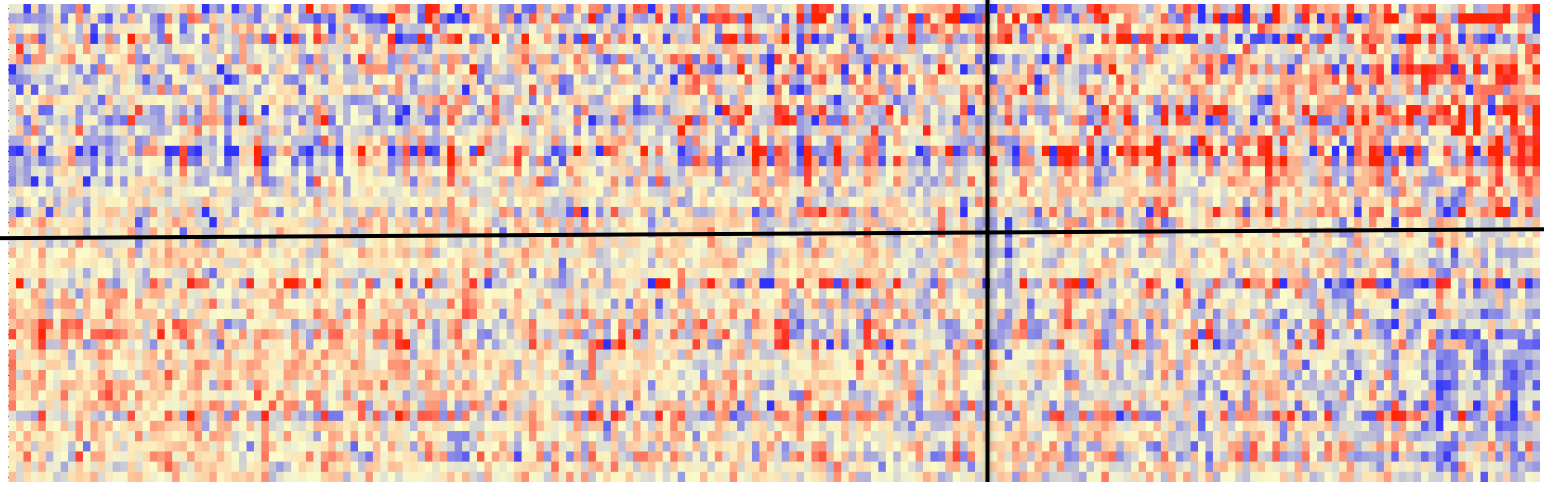
MM patients

Low DM Score

High DM Score

Bad prognostic genes

Good prognostic genes



Low  High
Expression scale

DM Score
20
10
0
-10
-20
-30
-40

MM patients (increasing DM Score)