FULL PAPER

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Gene expression-based prediction of myeloma cell sensitivity to histone deacetylase inhibitors

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Background: Multiple myeloma (MM) is still a fatal plasma cell cancer. Novel compounds are currently clinically tested as a single agent in relapsing patients, but in best cases with partial response of a fraction of patients, emphasising the need to design tools predicting drug efficacy. Histone deacetylase inhibitors (HDACi) are anticancer agents targeting epigenetic regulation of gene expression and are in clinical development in MM.

Methods: To create a score predicting HDACi efficacy, five MM cell lines were treated with trichostatin A (TSA) and gene expression profiles were determined.

Results: The expression of 95 genes was found to be upregulated by TSA, using paired supervised analysis with Significance Analysis of Microarrays software. Thirty-seven of these 95 genes had prognostic value for overall survival in a cohort of 206 newly diagnosed MM patients and their prognostic information was summed up in a histone acetylation score (HA Score); patients with the highest HA Score had the shorter overall survival. It is worth noting that MM cell lines or patients' primary MM cells with a high HA Score had a significant higher sensitivity to TSA, valproic acid, panobinostat or vorinostat.

Conclusion: In conclusion, the HA Score allows identification of MM patients with poor survival, who could benefit from HDACi treatment.

The molecular events governing the onset and progression of malignant transformation involve oncogenic activation and inactivation of tumour suppressor genes, which help cancer cells over-riding the normal mechanisms controlling cellular survival and proliferation (Hahn and Weinberg, 2002; Vogelstein and Kinzler, 2004). These molecular events are triggered by DNA alterations (translocations, amplifications or deletions, mutations) and also by epigenetic modifications (Baylin, 2005). Epigenetic modifications include methylation of DNA cytosine residues and histone methylation or acetylation, and are critical in the initiation and progression of many cancers (Kondo, 2009). Acetylation of histone releases condensed chromatin into a more relaxed structure that is associated with greater levels of gene transcription. Histone acetylation and then gene transcription is controlled by histone acetyl transferases and histone deacetylases (HDACs) bringing and removing acetyl groups. Eighteen HDACs have been described and classified into four classes based on cellular localisation and function (Lane and Chabner, 2009). Class I HDACs are located in the nucleus and comprise HDACs 1, 2, 3 and 8. Class II HDACs comprise HDACs 4, 5, 7 and 9 (class IIa), which shuttle back and forth between the nucleus and the cytoplasm, and HDACs 6 and 10 (class IIb), which are expressed

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in the cytoplasm only. Class III HDACs include the sirtuin family, which does not act primarily on histones, and class IV includes HDAC11 (Lane and Chabner, 2009; Neri *et al*, 2012). Histone deacetylase inhibitors (HDACi) are now being used in the treatment of some hematologic malignancies (Kelly *et al*, 2010). Histone deacetylase inhibitors are classified into four classes according to their chemical structure: aliphatic acids (valproic acid (VPA) and sodium phenylbutyrate), hydroxamates (panobinostat, trichostatin A (TSA), vorinostat, belinostat (PXD101), NVP-LAQ824 and givinostat (ITF2357)), cyclic peptide (romidepsin (depsipeptide)) and benzamides (MS-275, MGCD0103) (Neri *et al*, 2012). Histone deacetylase inhibitors include inhibitors specific to class I HDACs (MGCD0103, romidepsin and MS-275) and pan-HDACi (TSA, panobinostat, vorinostat and belinostat) (Neri *et al*, 2012). Multiple myeloma (MM) is a plasma cell (PC) neoplasm characterised by the accumulation of malignant PCs, termed MM cells (MMCs), primarily within the bone marrow (BM). Despite the recent introduction of novel agents such as bortezomib or lenalidomide, MM remains an almost incurable disease (Moreau, 2012). Multiple myeloma arises through progressive accumulation of multiple genetic abnormalities that include primarily overexpression of a D-type cyclin gene, *CCND1* (cyclin D1) in the case of t(11;14)(q13;q32.3) translocation, *CCND3* (cyclin D3) in the case of the rare t(6p23;14q32) translocation or *CCND2* (cyclin D2) on the background of a t(14q32;16q23) translocation involving *CMAF* or t(4;14)(p16.3;q32.3) involving WHSC1/*FGFR3*. *CCND* genes are also overexpressed in hyperdiploid MM patients because of gene amplification or downregulation of miRNAs that target *CCND* genes (Bergsagel and Kuehl, 2005; Rio-Machin *et al*, 2013). Secondary genetic abnormalities include *NRAS* or *KRAS* mutations, *TP53* monoallelic deletion and mutations, *MYC* alterations, mutations of genes coding for NF-κB pathway (Hideshima *et al*, 2004; Bergsagel and Kuehl, 2005; Morgan *et al*, 2012). Histone deacetylase inhibitors have already been evaluated in MM including TSA (Lavelle *et al*, 2001), vorinostat (Mitsiades *et al*, 2003, 2004), depsipeptide (Khan *et al*, 2004), KD5170 (Feng *et al*, 2008), NVP-LAQ824 (Catley *et al*, 2003), VPA (Kaiser *et al*, 2006; Neri *et al*, 2008) and panobinostat (Neri *et al*, 2012). Histone deacetylase inhibitors induce G1 cell cycle arrest in MMCs through dephosphorylation of retinoblastoma protein and increase expression of p53 and p21 (Lavelle *et al*, 2001; Mitsiades *et al*, 2003; Neri *et al*, 2008). Histone deacetylase inhibitors induce apoptosis by dowregulation of Bcl-2 family members (Mitsiades *et al*, 2003; Khan *et al*, 2004) and overcome drug resistance mediated by the BM environment (Mitsiades *et al*, 2003). Furthermore, glucose-regulated protein 78 (GRP78) was recently identified as a novel non-histone target of HDACi (Rao *et al*, 2010; Kahali *et al*, 2011). Glucose-regulated protein 78 has a central role in the unfolded protein response (UPR). Glucose-regulated protein 78 acetylation following HDACi treatment was described to activate UPR and contributes to the antitumour activity of HDACi. Class 1 HDACs binding to GRP78, within the endoplasmic reticulum (ER), represent a novel mode of UPR regulation and an interesting mechanism of HDACi action (Kahali *et al*, 2012). The ER of normal PCs and MMCs is well developed to accommodate the production and secretion of large amounts of immunoglobulins. That is why association of HDACi with proteasome inhibitors could be promising in MM treatment (Hideshima and Anderson, 2013). When used as a single agent in patients with relapsing/refractory MM, HDACi have shown modest antitumour activity (Richardson *et al*, 2008; Niesvizky *et al*, 2011). In combination with other anti-MM treatments, HDACi can induce durable antitumour responses (Badros *et al*, 2009; Harrison *et al*, 2011).

To improve the clinical testing of the efficacy of novel agents, a major stake is identify patients who could benefit from treatment by finding biomarkers predictive for sensitivity of MMCs to HDACi. We recently reported the development of a gene expression-based risk score predicting the sensitivity of MMCs to DNA methylation inhibitors (Moreaux *et al*, 2012). In this study, we used the same strategy to build a histone acetylation (HA) score, based on genes whose expression is deregulated by HDACi in MMCs. Histone acetylation score score makes it possible to identify a subgroup of 42% of patients with short overall survival (OS), whose MMCs are highly sensitive to HDAC inhibition.

MATERIALS AND METHODS

Human myeloma cell lines. Human myeloma cell lines (HMCLs, $N=40$) were obtained as described previously (Zhang *et al*, 1994; Rebouissou *et al*, 1998; Tarte *et al*, 1999; Gu *et al*, 2000; Moreaux *et al*, 2011). Human myeloma cell lines' phenotypic and molecular characteristics have been described previously (Moreaux *et al*, 2011). Human myeloma cell lines' microarray data have been deposited in the ArrayExpress public database (accession numbers: E-TABM-937 and E-TABM-1088).

Primary MMCs and gene expression profiling. Patients presenting with previously untreated MM ($N=206$) or monoclonal gammopathy of undetermined significance ($N=5$) at the university hospitals of Heidelberg and Montpellier as well as seven healthy donors have been included in the study with the approval of the ethics committee of Montpellier and Heidelberg after obtaining written informed consent in accordance with the Declaration of Helsinki. Clinical parameters and treatment regimens of the MM patients included in the Heidelberg–Montpellier cohort were described previously (Moreaux *et al*, 2012).

Normal BM PCs and myeloma cells were purified as published previously (Moreaux *et al*, 2012) and whole genome gene expression profiling assayed with Affymetrix U133 2.0 plus microarrays (Affymetrix, Santa Clara, CA, USA) (ArrayExpress accession number E-MTAB-372). Affymetrix data of an independent cohort of 345 MM patients from the University of Arkansas for Medical Sciences (UAMS, Little Rock, AR, USA) were also used (Gene Expression Omnibus accession number GSE2658. http://www.ncbi.nlm.nih.gov/geo/). T(4;14) translocation was evaluated using *MMSET* spike expression (Kassambara *et al*, 2012b) and del17p13 surrogated by *TP53* probe set signal (Xiong *et al*, 2008) for UAMS-TT2 patients.

Change in gene expression profile of myeloma cell lines by HDACi. Five HMCLs (XG-5, XG-6, XG-7, XG-20 and LP1) were treated without or with 0.33 μmol l^{-1} TSA (Sigma, St Louis, MO, USA) for 24 h in RPMI 1640, 10% fetal bovine serum supplemented with IL-6 for IL-6-dependent HMCLs (Moreaux *et al*, 2011, 2012). Whole genome gene expression profiling was assayed with Affymetrix U133 2.0 plus microarrays (Affymetrix).

Sensitivity of myeloma cell lines and primary myeloma cells to HDACi. Human myeloma cell lines were cultured with graded TSA, VPA (Sigma), vorinostat (SAHA) (Sigma) or panobinostat (LBH-589) (Sigma) concentrations. Human myeloma cell lines' cell growth was quantified with a Cell Titer Glo Luminescent Assay (Promega, Madison, WI, USA) as described(Moreaux *et al*, 2012). The half inhibitory concentration (IC_{50}) was determined using GraphPad Prism (http://www.graphpad.com/scientific-software/prism/).

Primary myeloma cells of 13 patients were cultured with or without graded concentrations of TSA and MMC cytotoxicity was evaluated using anti-CD138-PE mAb (Immunotech, Marseille, France) as described (Mahtouk *et al*, 2004; Moreaux *et al*, 2012).

Bioinformatics and statistics. Gene expression data were analysed using SAM (Significance Analysis of Microarrays)

software (Cui and Churchill, 2003) and our bioinformatics platforms (http://rage.montp.inserm.fr/ and http://amazonia.montp.inserm.fr/) (Reme *et al*, 2008; Tanguy Le Carrour, 2010) as published (Moreaux *et al*, 2012). All computations were performed using R 2.15.1 (http://www.r-project.org/) and bioconductor 2.0. Survival analyses were investigated using the Kaplan–Meier method and Cox's proportional hazards model as published (Moreaux *et al*, 2012).

The HA Score was built using our previously published methodologies to develop prognostic scores using a set of prognostic genes coding for related proteins (Kassambara *et al*, 2012a, b; Moreaux *et al*, 2012). Histone acetylation score is the sum of the Cox β-coefficients of each of the 37 TSA-deregulated genes with a prognostic value, weighted by ± 1 if the patient MMC signal for a given gene is above or below the probe set Maxstat value of this gene (Moreaux *et al*, 2012).

RESULTS

Identification of prognostic genes whose expression is upregulated by TSA treatment of MMCs. Genes upregulated by TSA treatment of MMCs were identified by treating five HMCLs with 0.33 μM TSA for 24 h, a concentration-inducing histone acetylation in mammalian cells, in particular in myeloma cells *in vitro* (Yoshida *et al*, 1990; Heller *et al*, 2008). In addition, this TSA treatment did not affect myeloma cell viability (Supplementary Table S1) (Heller *et al*, 2008). Using SAM supervised paired analysis, expression of 95 genes was found significantly upregulated by TSA treatment (FDR $<5\%$; Supplementary Table S2). Trichostatin A-regulated genes are significantly enriched in genes related to 'Immunological disease and Inflammatory disease' pathway ($P<0.05$; Ingenuity pathway analysis, data not shown). It is worth noting that TSA-deregulated genes were also found to be upregulated by Panobinostat treatment in MMC (Supplementary Figure S1). We next investigated associations of TSA-deregulated genes with OS using Maxstat R function. The aim was to identify genes regulated by HDAC that have potentially important disease-modulating functions. The results of our analysis were corrected for multiple testing using the Benjamini–Hochberg algorithm. Investigating the expression of these 95 TSA-regulated genes in primary MMCs of a cohort of 206 newly diagnosed patients (HM cohort), 16 genes had a bad prognostic value and 21 a good one (Table 1). The prognostic information of HDACi-regulated genes was gathered within an HA Score as indicated in Materials and Methods. The value of HA Score in normal, premalignant or malignant PCs is displayed in Figure 1. Cells from MGUS patients had a significantly higher HA Score than normal BMPCs ($P<0.001$), MMCs of patients had a significantly higher HA Score than normal BMPCs or PCs from MGUS patients ($P<0.001$), and HMCLs had the highest score ($P<0.001$) (Figure 1). Figure 2B shows the contribution of the 16 bad prognostic and the 21 good prognostic genes for HA score. Bad prognostic genes are highly expressed in patients with high HA score and the reverse for good prognostic genes. Investigating the HA Score in the eight groups of the molecular classification of MM (Zhan *et al*, 2006), HA Score was significantly higher in the proliferation subgroup ($P<0.001$) associated with a poor prognosis and significantly lower in the CD2 subgroup ($P<0.001$) (Zhan *et al*, 2006) (Figure 3). Among the 37 genes of the HA Score, 7 code for proteins that have been described as lysine acetylation target proteins and 18 have been identified as HDACi targets (Choudhary *et al*, 2009; Niesen and Blanck, 2009; Bantscheff *et al*, 2011; Iwahashi *et al*, 2011) (Supplementary Tables S5 and S6). Gene expression profiles of HA Score genes in purified MMC and normal BM subpopulations are listed in Supplementary Figure S2. Supplementary Figure S2 shows a highly variable expression of each of the 37 genes in primary MMCs of the patients, indicating they all contribute to unravel disease heterogeneity.

Table 1. Prognostic value of TSA-deregulated genes in primary MMC of newly diagnosed patients

Probe set	Name	Adjusted *P*-value (Benjamini–Hochberg multiple testing correction)	Hazard ratio
Bad prognostic genes			
204563_at	*SELL*	0.04	1.94
203567_s_at	*TRIM38*	0.04	1.96
201012_at	*ANXA1*	0.02	2.01
205352_at	*SERPINI1*	0.04	2.03
204944_at	*PTPRG*	0.01	2.12
222651_s_at	*TRPS1*	0.03	2.17
214875_x_at	*APLP2*	0.01	2.19
203854_at	*IF*	0.03	2.34
209958_s_at	*PTHB1*	0.01	2.35
209969_s_at	*STAT1*	0.009	2.37
205552_s_at	*OAS1*	0.01	2.50
226269_at	*GDAP1*	0.008	2.69
210432_s_at	*SCN3A*	0.007	2.71
224701_at	*PARP14*	0.01	2.94
214079_at	*DHRS2*	4.76e − 05	3.11
226158_at	*KLHL24*	0.01	3.44
Good prognostic genes			
34408_at	*RTN2*	2.42e − 05	0.28
225842_at	—	9.96e − 05	0.32
208894_at	*HLA-DRA*	0.01	0.36
212464_s_at	*FN1*	0.01	0.37
202391_at	*BASP1*	7.01e − 05	0.37
228726_at	*SERPINB1*	0.009	0.38
235301_at	*KIAA1324L*	0.01	0.39
206385_s_at	*ANK3*	0.007	0.40
230233_at	*RASGEF1B*	0.04	0.42
215193_x_at	*HLA-DRB1*	0.01	0.43
212636_at	*QKI*	0.02	0.44
212998_x_at	*HLA-DQB1*	0.01	0.47
223218_s_at	*NFKBIZ*	0.03	0.47
209198_s_at	*SYT11*	0.03	0.48
211990_at	*HLA-DPA1*	0.02	0.49
218918_at	*MAN1C1*	0.04	0.49
215388_s_at	*CFH*	0.04	0.52
228152_s_at	*FLJ31033*	0.03	0.53
216834_at	*RGS1*	0.04	0.54
203695_s_at	*DFNA5*	0.04	0.54
219833_s_at	*EFHC1*	0.04	0.55

Abbreviations: TSA = trichostatin A; MMC = multiple myeloma cell.

Prognostic value of HA score in two independent cohorts of patients. When used as a continuous variable, HA Score had prognostic value ($P\leqslant 10^{-4}$, results not shown). Using Maxstat R function, a maximum difference in OS was obtained with an HA Score $= -11.3$, splitting patients into a high-risk group of 42.7% patients (HA Score > -11.3) with a 43.5 months median OS and a low-risk group of 57.3% patients (HA Score $\leqslant -11.3$) with not reached median survival (Figure 2A). High-risk patients are characterised by a higher expression of the 16 bad prognostic genes and a lower expression of the 21 good ones used for HA Score building (Figure 2B). Using univariate Cox analysis, HA Score, UAMS-HRS, IFM-score and GPI had prognostic value as

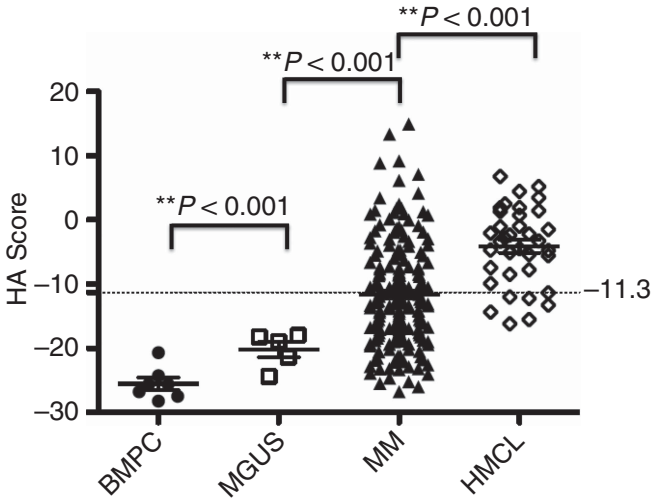

Figure 1. Histone acetylation score in normal and malignant PCs. Histone acetylation score in normal BMPCs ($N=7$), in premalignant PCs of patients with monoclonal gammopathy of undetermined significance (MGUS, $N=5$), in MM cells of patients with intramedullary MM ($N=206$) and in HMCLs ($N=40$). ** Indicate that the score value is significantly different with a P-value <0.01.

well as t(4;14), del17p, β2m, albumin and ISS using the HM patient cohort (Supplementary Table S3). When these parameters were compared two by two, HA Score tested with β2m and t(4;14) remained significant. When tested together, HA Score, β2m, t(4;14) and GPI kept prognostic value. The HA Score, computed using HM cohort parameters, is also prognostic in an independent cohort of 345 patients from UAMS (UAMS-TT2 cohort). The median OS of patients with high HA Score was 71.4 months and not reached for patients with low HA Score ($P<0.0001$) (Figure 2A). Using Cox univariate analysis, UAMS-HRS, IFM and GPI scores as well as t(4;14) and del17p had prognostic value. Serum concentrations of β2m or albumin are not publicly available for this cohort. When analysed two by two, HA Score remained significant compared with UAMS-HRS, IFM, GPI, t(4;14) and del17p in the UAMS-TT2 cohort (Supplementary Table S3). When these parameters were tested together, HA Score, UAMS-HRS, t(4;14) and del17p kept prognostic value in UAMS-TT2 cohort.

Histone acetylation score allows identification of HMCLs or patients' primary MMCs sensitive to TSA *in vitro*. We investigated whether HA Score could predict for the sensitivity of HMCLs to HDAC inhibitors. Ten out of 40 HMCLs (Moreaux *et al*, 2011) with the highest or lowest HA Score were selected to assay for TSA sensitivity. The five HMCLs with the highest HA Score displayed

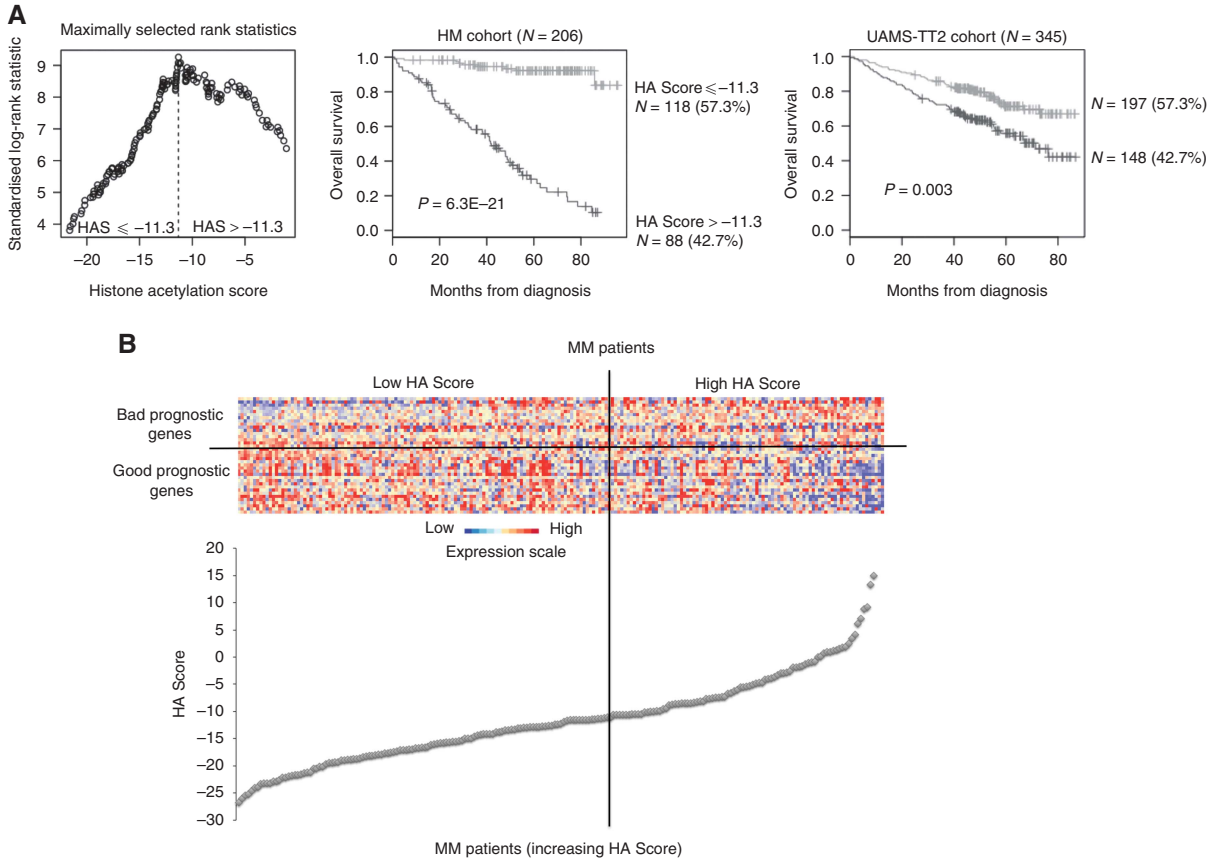

Figure 2. Prognostic value of HA Score in MM. (**A**) Patients of the HM cohort were ranked according to increased HA Score and a maximum difference in OS was obtained with HA Score $=-11.3$ splitting patients into high-risk (42.7%) and low-risk (57.3%) groups. Histone acetylation score also had a prognostic value of an independent cohort of 345 patients from University of Arkansas for Medical Science (UAMS) treated with TT2 therapy (UAMS-TT2 cohort). The parameters to compute the HA Score of patients of UAMS-TT2 cohort and the proportions delineating the two prognostic groups were those defined with HM cohort. (**B**) Clustergram of HA Score genes ordered from best to worst prognosis. The level of the probe set signal is displayed from low (deep blue) to high (deep red) expression. MM patients ($N=206$) were ordered by increasing GE-based risk score.

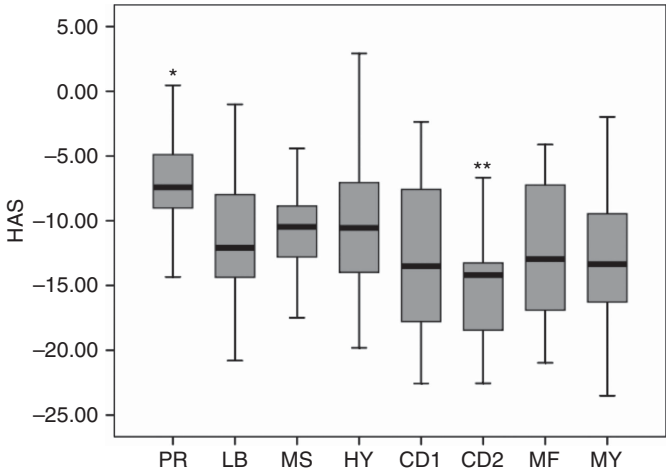

Figure 3. Histone acetylation score in MM molecular subgroups. The HA Score was computed for MMCs of patients belonging to the eight groups of the University of Arkansas for Medical Science (UAMS) molecular classification of multiple myeloma, using UAMS-TT2 cohort. *Score value is significantly higher in the group compared with all the patients of the cohort ($P<0.05$). **Score value is significantly lower in the group compared with all the patients of the cohort ($P<0.05$). Abbreviations: CD1, cyclin D1; CD2, cyclin D2; HY, hyperdiploid; LB, low bone disease; MF, MAF; MS, MMSET; MY, myeloid; PR, proliferation.

a significant ($P=0.0004$) fivefold higher sensitivity to TSA (median $IC_{50}=10.97$ nM; range: 6.32–17.4 nM) than the five HMCLs with low HA Score (median $IC_{50}=52.33$ nM; range: 29.49–57.74 nM) (Figure 4). No difference in recurrent genetic abnormalities was found between HMCLs with the highest or lowest HA Score (Table 2). Histone acetylation score could also predict for sensitivity of patients' primary MMCs, cocultured with BM environment, to TSA. The TSA concentrations used to treat primary MM samples were chosen to cover the range of TSA concentrations yielding 50% inhibition of the growth of the 10 HMCLs displaying high and low HA Scores (Figure 4).

Primary MMCs of eight patients with an HA Score above the Maxstat cutoff (>-11.3; Figures 1 and 2) exhibited significant ($P<0.05$) 2.4-fold higher TSA sensitivity than MMCs of five patients with HA Score $\leqslant -11.3$ (Figure 5).

Histone acetylation score is predictive for sensitivity of human myeloma cells to other clinical grade HDACi *in vitro*. We sought to determine whether HA Score could predict for the sensitivity of myeloma cells to other clinical grade HDAC inhibitors (Neri *et al*, 2012). The five HMCLs with the highest HA Score exhibited a significant higher sensitivity to panobinostat, VPA or vorinostat (median $IC_{50}=1.16$ nM, 0.28 μM and 528 nM, respectively) than the five HMCLs with lowest HA Score ($P=0.007$, $P=0.009$ and $P=0.02$; median $IC_{50}=3.16$ nM, 0.43 μM and 897 nM, respectively) (Figure 6A–C).

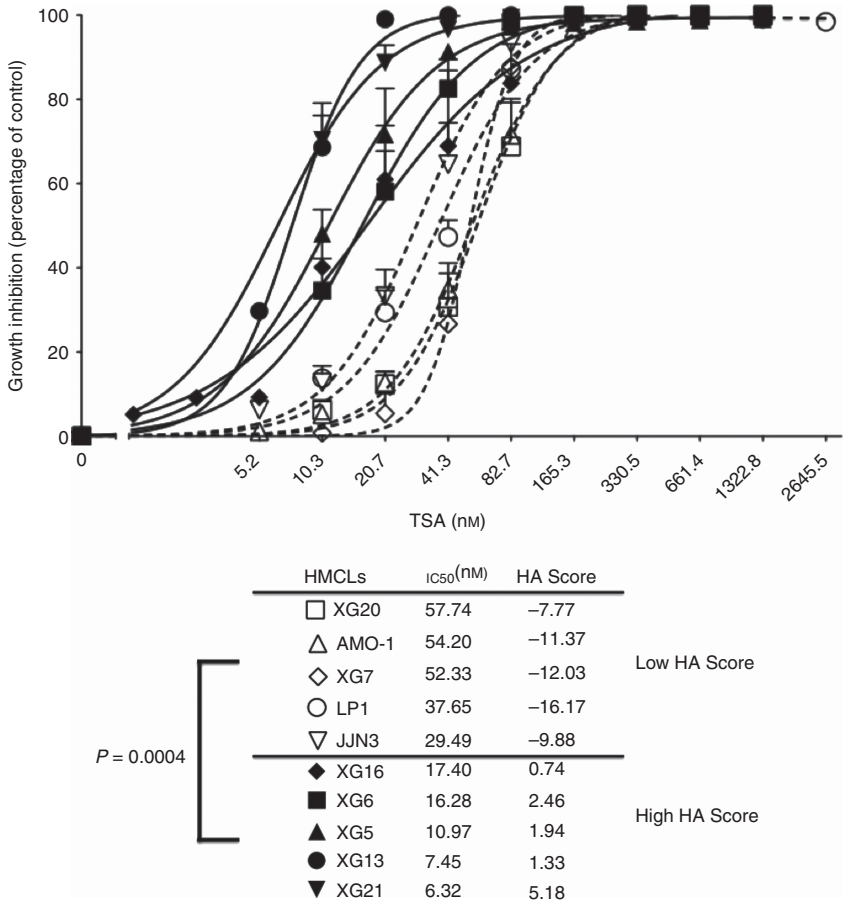

Figure 4. Histone acetylation score predicts for sensitivity of HMCLs to TSA. HMCLs with high HA Score ($N=5$) exhibit significant higher TSA sensitivity compared with HMCLs with low HA Score ($N=5$). Human myeloma cell lines were cultured for 4 days in 96-well flat-bottom microtitre plates in RPMI 1640 medium, 10% fetal calf serum, 2 ng ml^{-1} IL-6 culture medium (control) and graded TSA concentrations. Data are mean values ± standard deviation (s.d.) of five experiments determined on sextuplet culture wells.

Table 2. Characteristics of HDACi-resistant or -sensitive HMCLs

HMCL name	IL-6 dependence[a]	Origin[b]	Disease[c]	Patient sample[d]	Gender	Isotype	t(14q32 or 22q11)	Target genes	*Ras*	*TP53*	CD45	HMCL classification
TSA-resistant HMCLs												
XG7	+	MN	MM	PB	F	Ak	t(4;14)	*MMSET*	*mut*	*wt*	+/−	MS
XG20	++	MN	PCL	PB	M	I	t(4;14)	*MMSET*	*wt*	*abn*	−	MS
AMO1	−	CO	PCT	AF	F	Ak	t(12;14)	Unknown	*wt*	*wt*	+	CD-2L
JJN3	−	CO	MM	PE	F	Ak	t(14;16)	*c-Maf*	*mut*	*abn*	+/−	MF
LP1	−	CO	MM	PB	F	GI	t(4;14)	*MMSET/ FGFR3*	*wt*	*abn*	−	MS
TSA-sensitive HMCLs												
XG5	++	MN	MM	PB	F	I	t(11;14)	*CCND1*	*wt*	*abn*	−	CD-1
XG6	++	MN	MM	PB	F	GI	t(16;22)	*c-Maf*	*wt*	*wt*	+	CTA/MF
XG13	++	MN	PCL	PB	M	GI	t(14;16)	*c-Maf*	*wt*	*abn*	+	MF
XG16	++	MN	PCL	PB	M	k	None	None	*mut*	*abn*	+	CTA/FRZB
XG21	++	MN	MM	PE	M	I	t(11;14)	*CCND1*	*wt*	*wt*	+	CD-1

Abbreviations: AF = ascitic fluid; BM = bone marrow; CO = collected; F = female; HDACi = histone deacetylase inhibitor; HMCL = human myeloma cell line; IL = interleukin; M = male; MM = multiple myeloma; MN = Montpellier or Nantes; PB = peripheral blood; PCL = plasma cell leukemia; PCT = plasmacytoma; PE = pleural effusion; TSA = trichostatin A.
[a] ++, if growth is strictly dependent on adding exogenous IL-6; +, if dependent on adding exogenous IL-6; −, if not.
[b] Origin of the HMCL: MN, CO.
[c] Disease at diagnosis: MM, PCL, PCT.
[d] Origin of the sample: AF, BM, PE, PB.

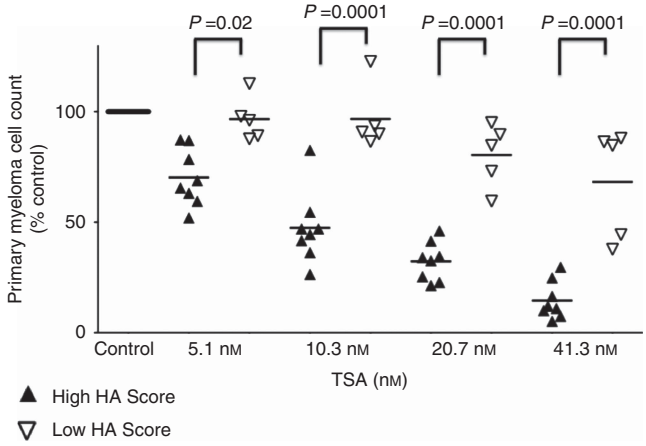

Figure 5. Histone acetylation score predicts for TSA sensitivity of primary myeloma cells of patients. Mononuclear cells from tumour samples of 13 patients with MM were cultured for 4 days in the presence of IL-6 (2 ng ml^{-1}) with or without graded TSA concentrations. At day 4 of culture, the count of viable MMCs was determined using CD138 staining by flow cytometry. Black color represents patients with high HA Score ($N = 8$) and white represents patients with low HA Score values ($N = 5$).

DISCUSSION

In this study, we have identified a gene expression-based HA Score, which is predictive for patients' survival and for the *in vitro* sensitivity of HMCLs or patients' primary myeloma cells to the pan-HDACi, TSA and also to other three clinical-grade HDACi. Histone deacetylase inhibitors have been investigated for treating patients with MM, either as a single agent (Richardson *et al*, 2008; Niesvizky *et al*, 2011; Neri *et al*, 2012) or in combination with current drugs used in MM, such as dexamethasone, lenalidomide or bortezomib (Badros *et al*, 2009; Harrison *et al*, 2011; Neri *et al*, 2012). Panobinostat, used as a single agent, demonstrated limited activity in patients with MM, refractory to at least two lines of treatment (Wolf *et al*, 2012). In association with melphalan, panobinostat yielded a 33% overall response rate in a phase I study, including 12 patients with relapsed/refractory MM (Offidani *et al*, 2012). Association of panobinostat and bortezomib could be promising as proteasome inhibition affects the UPR pathway, leading to increased HDAC-mediated aggregosome formation (Hideshima *et al*, 2011). Phase IB and II studies have shown that association of panobinostat to bortezomib and dexamethasone could yield to objective response in relapsing patients refractory to bortezomib therapy (Siegel *et al*, 2008; San-Miguel *et al*, 2009; Richardson *et al*, 2011). Given the encouraging *in vitro* and *in vivo* data, association of panobinostat, bortezomib and dexamethasone is now being evaluated in a large phase III randomised trial (San-Miguel *et al*, 2011). Combination of vorinostat and bortezomib was also investigated in a phase II trial, including patients with MM refractory or ineligible to bortezomib or IMiD therapy (Siegel *et al*, 2011) and showed a 17% overall response rate and 6 months median response duration. These data also prompt investigating the interest of a combination of vorinostat and bortezomib in phase III trial, including 637 patients with relapsed/refractory MM (Dimopoulos *et al*, 2011). Final results of the trial remain awaited, but interim results did not demonstrate a marked therapeutic benefit of vorinostat (Dimopoulos *et al*, 2011). These trials suggest that HDACi could have some benefit for MM. However, their efficacy could be underestimated because it was limited to a subgroup of patients. The current HA Score could be promising to investigate whether the best response to HDACi is found in patients with MMCs displaying a high HA Score.

Only upregulated genes were identified in the HMCLs treated with TSA compared with untreated HMCLs. This may be explained by the 24-h treatment of cell lines with HDACi according to usual protocols. This short treatment makes it possible to release the transcriptional suppressor activity of HDAC yielding to overexpressed genes. However, it is likely too short to

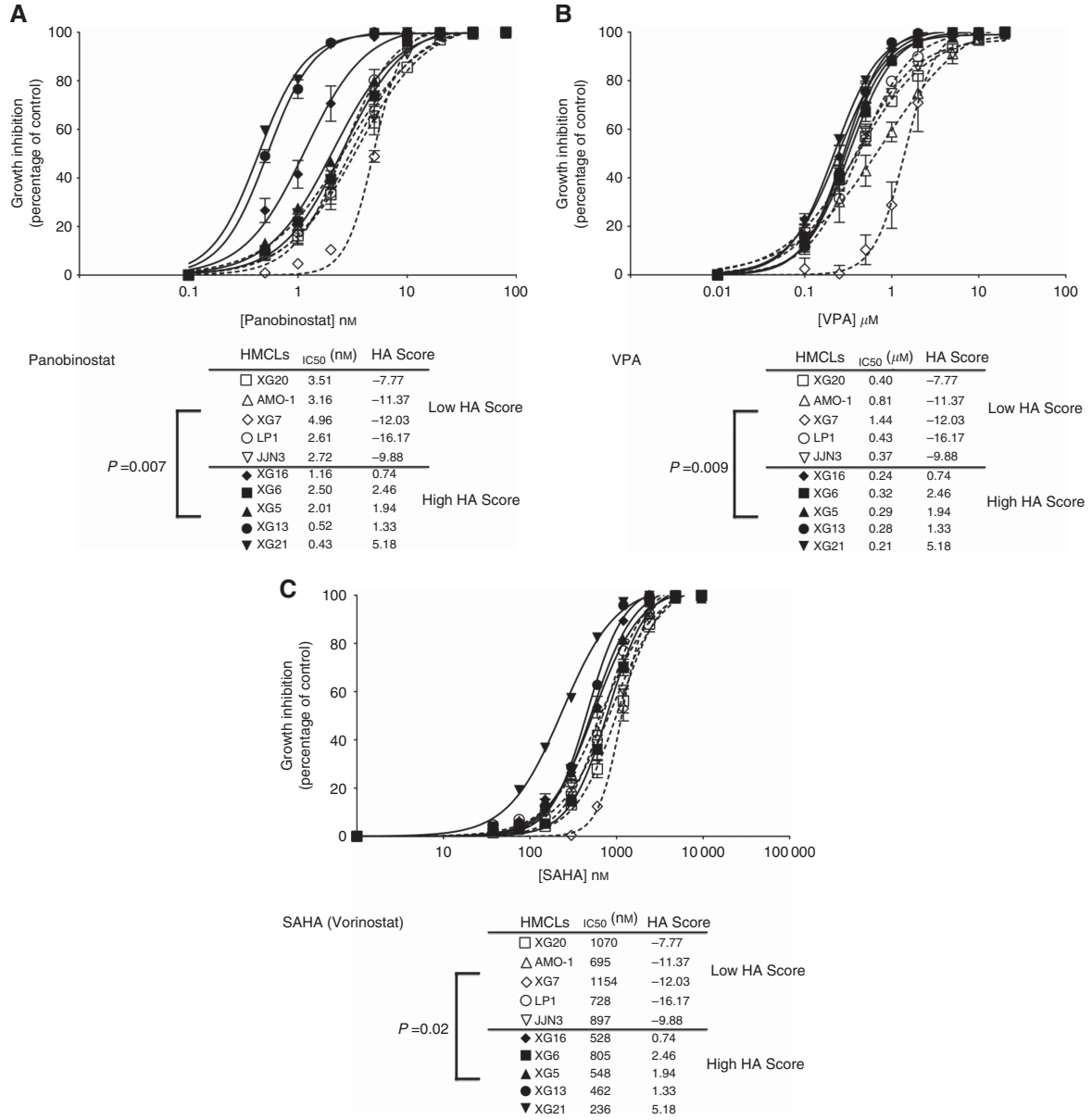

Figure 6. Histone acetylation score predicts for sensitivity of HMCLs to panobinostat, valproic acid (VPA) or SAHA HDACi. Human myeloma cell lines with a high HA Score ($N=5$) exhibit significant higher sensitivity to panobinostat, VPA or SAHA compared with HMCLs with a low HA Score ($N=5$). Human myeloma cell lines were cultured for 4 days in 96-well flat-bottom microtitre plates in RPMI 1640 medium, 10% fetal calf serum, 2 ng ml^{-1} IL-6 culture medium (control) and graded concentration of panobinostat (**A**), VPA (**B**) or SAHA (vorinostat) (**C**). Data are mean values ± standard deviation (s.d.) of five experiments determined on sextuplet culture wells.

get an indirect repression of genes because of the HDACi-induced overexpression of an inhibitor of these genes. Among the current 95 genes deregulated by TSA treatment in HMCLs, 24 genes were commonly identified by Heller *et al* (2008) (Supplementary Table S4). Seven out of 37 HA Score genes code for proteins that have been described as lysine acetylation target proteins and 18 out of 37 HA Score genes have been identified as HDACi targets (Choudhary *et al*, 2009; Niesen and Blanck, 2009; Bantscheff *et al*, 2011; Iwahashi *et al*, 2011) (Supplementary Tables S5 and S6).

Why HA Score which is built using 37 HDACi-upregulated and prognostic genes could predict for the sensitivity of MMCs to HDACi? Patients with high HA score, and poor survival, are characterised by a higher expression of the 16 bad prognostic genes and a lower expression of the 21 good ones in MMCs (Figure 2B). Thus, one can speculate that primary MMCs of patients with high HA score have a high tumour metabolism and growth, which can be efficiently targeted by the upregulation of gene products encoded by genes upregulated by HDACi, in particular the 21 good prognostic genes. At the opposite, MMCs of patients with a low HA score could be in a more quiescent state and less sensitive to HDCAi. However, a full understanding of the reason why HA score could predict for HDACi sensitivity will be provided by an extensive study of the function of the products encoded by HDACi-regulated genes in promoting MMC survival and/or proliferation. Some genes could highlight pathways involved in MM and we comment below the putative roles of *NFKBIZ* (nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor zeta), *BASP1* (brain acid-soluble protein 1) or *QKI* (Quaking), whose expression in MMCs is induced by HDACi treatment and is associated with good prognosis. *NFKBIZ* is a member of IκB family

(Totzke *et al*, 2006), localised in the nucleus, where it interacts with and regulates nuclear NF-κB activity. Suppression of endogenous *NFKBIZ* renders cells more resistant to apoptosis, whereas its overexpression induces cell death (Yamazaki *et al*, 2001; Totzke *et al*, 2006). This is of interest because NF-κB pathway is frequently activated through various gene mutations in MM (Annunziata *et al*, 2007; Keats *et al*, 2007). More recently, it was demonstrated that *NFKBIZ* inhibits the transcriptional activity of STAT3, leading to cell growth inhibition and apoptosis induction mediated by the downregulation of a known STAT3 target, Mcl-1 (Wu *et al*, 2009). Mcl-1 is the major antiapoptotic protein for MMCs, involved in IL-6-mediated survival of MMCs (Derenne *et al*, 2002; Jourdan *et al*, 2003). *Brain acid-soluble protein 1* is repressed in Myc-transformed cells, and conversely has a strong potential to inhibit cell transformation induced by Myc (Hartl *et al*, 2009). The inhibition of Myc-induced fibroblast cell transformation by *BASP1* also prevents the transcriptional activation or repression of known Myc target genes. *Brain acid-soluble protein 1* appears to be a potential tumour suppressor in cancer (Hartl *et al*, 2009). Myc protein is frequently highly expressed in primary MMCs (Skopelitou *et al*, 1993) and a vicious circle involving IRF4 and Myc was identified yielding to deregulation of MMC growth (Shaffer *et al*, 2008). Histone deacetylase inhibitors could be useful to target NF-κB or Myc activation in MMCs through the upregulation of *NFKBIZ* and *BASP1* expression. The RNA-binding protein QKI belongs to the evolutionarily conserved signal-transduction and activator of RNA family. It has been demonstrated that overexpression of QKI induced the G1 cell cycle arrest in oligodendrocyte progenitor cells (Larocque *et al*, 2005). Furthermore, QKI inhibits colon cancer cell growth, acting as a tumour suppressor (Yang *et al*, 2010). It was demonstrated that QKI protein is directly transcribed by E2F1, which in turn negatively regulates the cell cycle by targeting multiple cell cycle regulators including p27, cyclin D1 and c-fos (Yang *et al*, 2011). These results demonstrated that a better understanding of the cellular response to epigenetic-targeted treatments will increase our knowledge of MM development and progression and will provide potential therapeutic advances. Epigenetic therapies could be combined with conventional therapies to develop personalised treatments in MM and render resistant tumours responsive to treatment. These advances may limit the side effects of treatment, improving compliance with dosing regimens and overall quality of life. Our methodology could be extended to other anti-MM treatments.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

MJ designed the research and wrote the paper; HD and GH collected BM samples and clinical data and participated in the writing of the paper; RT and VJL participated in the bioinformatics studies; LW and RG provided with technical assistance; and KB designed and supervised the research and wrote the paper.

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