The hydroxymethylome of multiple myeloma identifies FAM72D as a 1q21 marker linked to proliferation

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Received: March 15, 2019.
Accepted: June 19, 2019.
Pre-published: June 20, 2019.
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SUPPLEMENTAL DATA

SUPPLEMENTAL METHODS

Human Myeloma Cell Lines (HMCLs)
XG human myeloma cell lines were obtained as previously described.1 1 HMCLs were cultured in the presence of recombinant IL-6. HMCLs were authenticated according to their short tandem repeat profiling and their gene expression profiling using Affymetrix U133 plus 2.0 microarrays deposited in the ArrayExpress public database under accession numbers E-TABM-937 and E-TABM-1088.1

Construction of HMCLs overexpressing FAM72D
FAM72D cDNA was cloned in the pLenti4-mGFP-Tagged cloning vector (Origene). HMCLs were transduced with FAM72D lentiviruses (MOI = 2) and stable transduced cells were obtained after selection using cell sorter. HMCLs transduced with control lentiviruses were used as control.2,3
Cell growth assay

HMCLs were cultured for 4 days in RPMI 1640 medium, 10% FCS, and 2 ng/ml IL-6 (control medium) in the presence or absence of graded concentrations of FDI-6 (Sigma). HMCLs overexpressing or not FAM72D were then IL-6- and serum-starved for 2 hours and cultured for 4 days in 96-well flat-bottom microtiter plates in serum-free culture medium without cytokine (control) or with graded concentrations of IL-6 as described.\textsuperscript{4,5} Cell growth was evaluated by quantifying intracellular ATP amount with a Cell Titer Luminescent Assay (Promega, Madison, WI, USA) using a Centro LB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Sensitivity of primary myeloma cells to HDACi/DNMTi combination

Primary myeloma cells of 17 patients were cultured with or without 2 μM 5azacitidine and 300 nM SAHA (Sigma). MMC cytotoxicity was evaluated using anti-CD138-PE monoclonal antibody (Immunotech, Marseille, France) as described.\textsuperscript{6}

Identification of genes deregulated by the HDACi/DNMTi combination

HMCLs were treated with 0.5 μmol/L decitabine (Sigma, St Louis, MO) for 7 days in RPMI 1640, 10% fetal bovine serum supplemented with IL-6 for IL-6-dependent HMCLs. During the last 24 h, 0.33 μmol/L trichostatin A (TSA; Sigma) was added as described.\textsuperscript{6} Whole-genome GEP was assayed with Affymetrix U133 2.0 plus microarrays (Affymetrix).

Knock-down of FAM72D in MCF-7 cells and mitotic defect analysis

For siRNA experiments, MCF-7 cells were plated in 6-well plates (250,000 cells/well) in DMEM supplemented with 10% fetal calf serum and antibiotics. After 24 hours, cells were switch to opti-MEM medium and transfected for 6 hours with 10 nM of control or FAM72D siRNAs (Cohesion Biosciences, ref CRJ8695) diluted in opti-MEM and oligofectamine (Thermo Fischer Scientific). After transfection, cells were further cultured for 48 hours in DMEM supplemented with 10% serum before processing either for RT-qPCR or mitotic defect analysis. Total RNAs were extracted with TRizol (Invitrogen) and reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) and Pd(N)6 random hexamers (Amersham Pharmacia Biosciences). Analysis of the efficiency of the siRNAs to knock-down FAM72D was assessed by qPCR using SYBR green master mix (Biorad) and the following oligonucleotides: FAM72D forward: 5'-tggttagttacctgttagttcct-3', FAM72D reverse: 5'-actctctttctcttgcaagt-3',
FOXM1 forward: 5'-gccatcaacagcactgagag-3', FOXM1 reverse: 5'-tgggtgaatggtccagaag-3', NUF2 forward: 5'-gcccgggtgaatgactttgag-3', NUF2 reverse: 5'-tttcacggcatgcttctctg-3'. For phenotypic analysis, cells grown on coverslips were fixed with 4% paraformaldehyde for 10 minutes and permeabilized for 15 minutes in 0.2% Triton X-100 in PBS. Cells were next mounted in Vectashield medium (VECTOR Laboratories) containing DAPI. All experiments were run in triplicates and repeated 3 times. A total of 180 anaphases were monitored for lagging chromatids, 180 metaphases for misaligned chromosomes, and 1,800 cells for micronuclei in both control and FAM72D siRNA conditions.

REFERENCES


SUPPLEMENTAL FIGURES S1 to S10 (pages 5 to 14)
motif analysis for the presence of transcription factor binding motifs in their vicinity. Sets of significantly enriched (p<0.05) motifs were compared to motifs enriched in lymph node NBCs and PBs through a Venn diagram.

Figure S1: SCL-exo and SCL identify active genomic regions in MM patients. (A) Aggregated 5hmC positive regions (40,586 CpGs for oxBS-450K; 86,591 CpGs for SCL-exo; 64,424 regions for SCL-seq) were annotated using GREAT with default settings. (B) C’1 and C’2 subgroup-specific sets of 5hmCpGs obtained through heatmap clustering were analyzed for the presence of transcription factor binding motifs in their vicinity. Sets of significantly enriched (p<0.05) motifs were compared to motifs enriched in lymph node NBCs and PBs through a Venn diagram.
Figure S2: SCL-exo and SCL identify active genomic regions in MM patients. (A) Association of the MM 5hmCpGs with chromatin states (ChromHMM) from the GM12878 lymphoblastoid cell line. (B) Venn diagram analysis of the distribution of MM 5hmCpGs in genomic regions identified as active enhancers and active super enhancers (SEs) in MM1.S cells. (C) GAS2 expression levels in normal cells: naive B cells (NBCs), centroblasts (CBs), centrocytes (CCs), memory B cells (MBCs), pre-plasmablasts (prePBs), plasmablasts (PBs), early plasma cells (early PCs), and bone marrow plasma cells (BMPCs). Graphs were generated by GenomicScape (http://genomicscape.com/). (D) GAS2 expression levels in MMPCs from patients of the Arkansas cohort (n=414) classified in the following molecular groups: proliferation (Prolif), low bone disease (LBD), MMSET, hyperdiploid (Hyper), CCND1, CCND2, and MAF.
Figure S3: The 1q21.1 region is enriched in 5hmCpG-associated genes. (A) Heatmap of the 5hmC signal at 41S MM-specific 5hmCpGs. (B) Integrated genome browser (IGB) view of the 5hmC signal at the BMP6 and FAM72D loci in PB (orange), MM35174 (dark blue) and E10025 (grey) samples. (C) Venn diagram comparing the list of genes associated with 5hmC and those located at 1q21.1 and 1q21.2. (D) Genomic organization of the 1q21.1 cytoband. Genes associated with 5hmC are indicated in red font.
Figure S4: Expression levels of identified poor and good prognosis genes in different states of B cell differentiation.
Box plots show gene expression in naive B cells (NBCs), centroblasts (CBs), centrocytes (CCs), memory B cells (MBCs), pre-plasmatocytes (prePBs), plasmablasts (PBs), early plasma cells (early PCs), and bone marrow plasma cells (BMPCs). Graphs were generated by GenomicScape (http://genomicscape.com/).
**Figure S5:** (A) FAM72 expression in patients from the Arkansas cohort with 2, 3 or 4 and more (4+) copies of 1q21. (B) Overall survival of patients from the Arkansas cohort with 2, 3 or 4 and more (4+) copies of 1q21. (C) Correlation analysis of FAM72D hydroxymethylation (RPKM: reads per kb per million reads) and FAM72 expression levels in MM patients. (D) Integrated genome browser (IGB) snapshots showing input-seq, un-normalized and normalized SCL-exo signals, along the full chromosome 1 (chr1) and 3 (chr3), as well as close up views of 1q21.1 and the FAM72D locus. (E) IGB snapshot showing H3K4me1 signal form normal plasma cells (PC H3K4me1), the presence of highly significant MM 5hmCpGs, and the SCL-exo signal from E10025 at the FAM72D locus. (F) BMP6 expression levels in the different molecular subgroups from the Arkansas cohort. (G) FAM72 expression levels in the different molecular subgroups from the Arkansas cohort.
Figure S6: FAM72 is part of FOXM1 network. (A) IGB view of FOXM1 ChIP-seq signal at the FAM72D locus in GM12878 lymphoblastoid cells. (B) Correlation analysis of FAM72D hydroxymethylation (RPKM: reads per kb per million reads) and FOXM1 expression levels in MM patients. (C) Venn diagram showing the overlap between genes coregulated (correlation coefficient above 0.5) with FOXM1 or with FAM72 in patients from the Proliferation group. (D) Functional annotation of the top-50 genes coregulated with FAM72 in the proliferation group. Benjamini indicates the corrected p-values of these annotations. (E) Expression levels of FAM72 in bone marrow plasma cells (BMPCs), monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma cells (MMCs) and human myeloma cell lines (HMCLs).
Figure S7: Top gene sets significantly associated with high FAM72 expression in MM (TT2 cohort). GSEA enrichment plots with the absolute enrichment $P$ value and the normalized enrichment score of the gene set.
Figure S8: Overexpression of FAM72D::GFP in the multiple myeloma cell line XG21. (A) Expression levels of FAM72 in human myeloma cell lines (HMCLs). Graphs in (A) and (B) were generated by GenomicScape (http://genomicscape.com/). (B) Fluorescence acquired cell sorting of XG21 cells expressing FAM72D::GFP. (C) RT-qPCR analysis of FAM72D expression in XG21 and XG21-FAM72D cells. (D) Cell proliferation assay with control and FAM72D-overexpressing XG23 cells in the presence of increasing concentrations of IL-6. (E) Impact of increasing concentration of FDI-6 on XG21 and XG21-FAM72D HMCLs.
Figure S9: Alterations of FAM72D levels in breast cancer. (A) FAM72D alterations in various cancer samples (http://www.cbioportal.org/). (B) Box plot analysis of FAM72D gene expression as a function of TP53 mutations in 981 breast cancer patients from the TCGA database (breast invasive carcinoma cohort, The Cancer Genome Atlas). (C) IGB view of the 5hmC signal at the FAM72D locus in PBs, MM E10025, and MCF-7 samples. (D) Inactivation of FAM72D in MCF-7 cells. From left to right: RT-qPCR analysis of FAM72D, FOXM1 and NUF2 expression in MCF-7 cells transfected with control or FAM72D siRNAs; quantification of chromatin bridges and lagging chromatids in anaphases of control and FAM72D siRNA transfected MCF-7 cells; quantification of misaligned chromosomes in metaphases of control and FAM72D siRNA transfected MCF-7 cells; quantification of cells with micronuclei in control and FAM72D siRNA transfected MCF-7 cells. Images exemplify mitotic defects which are indicated by white arrowheads.
**Figure S10: MM cells with high FAM72 expression show distinct sensitivity to drugs.**

(A) High FAM72 expression is associated with a shorter overall survival (OS) in a cohort of 188 patients at relapse treated with bortezomib monotherapy (Mulligan cohort). (B) OS in a cohort of 76 patients at relapse treated with dexamethasone monotherapy (Mulligan cohort). Cut-points for FAM72 expression were similar in (A) and (B). (C) 10 HMCLs were cultured with graded concentrations of Panobinostat for 4 days and IC50 were calculated with mean values of five experiments determined on sextuplet culture wells. A trend between high FAM72 expression (Affymetrix microarrays) and a higher sensitivity to Panobinostat was identified. (D) FOXM1 expression predicts 5azacitidine/SAHA combination sensitivity of primary myeloma cells of patients. Mononuclear cells from tumor samples of 17 patients with MM were cultured for 4 days in the presence of IL-6 (2 ng/ml) with or without 2 μM 5azacitidine and 300 nM SAHA. At day 4 of culture, the count of viable MMCs was determined using CD138 staining by flow cytometry.