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Supplemental Material to:

Meena Kanduri, Birgitta Sander, Stavroula Ntoufa, Nikos Papakonstantinou, Lesley-Ann Sutton, Kostas Stamatopoulos, Chandrasekhar Kanduri, and Richard Rosenquist

A key role for EZH2 in epigenetic silencing of HOX genes in mantle cell lymphoma

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Supplementary Materials and Methods

RQ-PCR assays

Primers for the p16 assay were designed using the Primer3 software (Broad institute, Cambridge, MA) and are listed in Supplementary Table 2. RQ-PCR analysis was performed using the Maxima1 SYBR Green/ROX qPCR master mix (Fermentas) and the 7900HT fast real time PCR system instrument (Applied Biosystems, Foster City, CA, USA). Differences in expression were calculated using the MxPro QPCR software (Stratagene), with beta-actin as a reference gene and the ΔCt method.

Quantification of EZH2 mRNA levels was similarly achieved by RQ-PCR, using the RT² qPCR Primer Assay for the human EZH2 gene (SABiosciences, USA), according to the manufacturer's instructions. The ABL gene was used as a reference (housekeeping gene). Data were analyzed using the 2-ΔΔCt algorithm.

Western blot analysis

Total cell lysates were prepared using modified RIPA buffer (150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, 1mM ethylenediamine tetraacetic acid (EDTA), 1mM phenylmethylsulfonyl fluorite (PMSF), 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecylsulfate, 5 mg /ml aprotinin and 5mg /ml leupeptin). Briefly, cells were collected by centrifugation and washed twice with PBS and resuspended in 100 µL of RIPA lysis buffer. Cell lysates were transferred to 1.5 mL tubes and centrifuged at 14,000 rpm for 30 min to purify samples from insoluble cellular debris. Concentrations of soluble proteins in samples of purified supernatants were determined using Bradford assays. Samples were heat-denatured at 70°C for 10 min in protein denaturing sample buffer (Invitrogen). Cell lysates were run on a NuPAGE 4–12% Bis-Tris gel (Invitrogen), along with a molecular weight marker, and transferred onto a membrane filter (Amersham BioSciences, HybondN+), which

was incubated with the primary antibody. Primary antibodies used were the same as for the ChIP assay and are listed in the material and methods section of the paper. All the HOXA antibodies were purchased from Abcam, Cambridge, UK. (HOXA2 (AB119126); HOXA13 (AB26084) and HOXA9 (AB83480)). Secondary antibodies used were anti-mouse IgG (NA 931) and anti-rabbit IgG (NA934) (GE Health Care). The blots were washed and developed using enhanced chemiluminescence reagent as substrate (Amersham, ECL plus western blotting detection system) and visualized by exposure to an X-ray film. Membranes were blotted with a beta-actin antibody and GAPDH antibody to control for equal loading of protein samples on gels and transfer onto membranes.

Pyrosequencing and Bisulfite sequencing

Pyrosequencing was performed using the Pyromark kit and software according to the manufacturer's instructions. Briefly, genomic DNA was bisulfite-converted for both assays using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) according to manufacturer's protocol. The PyroMark^{TM} (Qiagen, Hilden, Germany) software was used to design three pyrosequencing primers for each gene; forward and reverse primers for PCR amplification of the desired product (one biotin labelled in the 5' end) and one sequencing primer (Supplementary Table 2). The sequenced target region contains the specific CpG site targeted by the 27K Illumina methylation array (applied in our previous paper[8]) along with nearby CpG sites. The bisulfite-converted DNA was PCR amplified under the following conditions were: initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec followed by final extension at 72°C for 3 min. The PCR product was immobilized with Streptavidin Sepharose High Performance (GE Healthcare, Uppsala, Sweden), followed by annealing of the sequencing primer. The analysis was

performed using PyroMark^{TM} Q24 pyrosequencer instrument (Qiagen), and for each CpG site methylation percentage was calculated using the PyroMark Q24 software.

Bisulfite sequencing was performed by amplification of the target HOX gene using the same primers used above (Supplementary Table 2). The PCR mix contained 1.5mM $MgCl_2$, 200μM dNTP mix, 0.2μM primers (Sigma Aldrich, St. Louis, MO), 1x PCR Taq buffer, 1U Platinum Taq (Invitrogen, Carlsbad, CA, USA), and ~50 ng of bi-sulfite-treated DNA. PCR conditions were the same as described above for the pyrosequencing amplification. The PCR product was cloned using the Invitrogen TOPO TA cloning kit-2.1 TOPO vector (Invitrogen, Carlsbad, CA, USA), and 12-15 clones per sample were selected for verification of the correct sized insert via PCR using vector specific M13 forward and reverse primers (provided by the cloning kit). PCR products containing the correct sized insert were purified using EXO/SAP IT treatment (Fermentas, Burlington, Canada) at 37^{o}C for 45 minutes and 80^{o}C for 15 minutes. Purified products were then sequenced using the same forward and reverse BSP separately as mentioned above and the BigDye Terminator Cycle Sequencing Reaction Kit (Perkin-Elmer, Foster City, CA). All sequence reactions were analyzed using an automated DNA sequencer (ABI 377, Applied Biosystems, Foster City, CA). Sequences were aligned and analyzed using the bisulfite sequencing web-based tool BiQ Analyzer software (Max-Planck Institute for information, Saarbrucken, Germany) and the degree of methylation represented via a lollipop grid.

Chromatin immunoprecipitation assay

Cells were cross-linked using 1% formaldehyde in phosphate-buffered saline (PBS) solution for 10 min at room temperature. Cross-linking was inhibited by 125 mM glycine. Cells were washed in ice-cold PBS, treated with cell lysis buffer provided according to the manufacturer's instructions (Shearing module kit, Diagenode, Liège, Belgium). Following

this, cells were sonicated at 2×10 min (30 sec ON/30 sec OFF) using Bioruptor® (Diagenode, Liège, Belgium) in shearing buffer provided in the kit. Chromatin immune precipitation was performed using OneDay ChIP Kit™ (Diagenode, Liège, Belgium) according to the manufacturer's protocol. Precipitated DNA was analyzed by real-time quantitative PCR (RQ-PCR) using the Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). Primer sequences are provided in Supplementary Tables 2A & 2B. The PCR conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The PCR analysis was performed using 7900HT fast real time PCR system instrument and software (Applied Biosystems, Foster City, CA, USA). The normalized percentage of input and fold enrichment values for ChIP data was calculated using ΔΔCt method from the Excel based ChIP-qPCR analysis template.

Supplementary Table 1A. MCL samples investigated in the current study.

MCL sample	Tissue type	Gender	Age at diagnosis
MCL1*	Spleen	F	69
MCL2*	Spleen	F	79
MCL3*#	Lymph node	M	75
MCL5#	Lymph node	M	80
MCL7*#	Lymph node	M	68
MCL8*#	Lymph node	F	81
MCL9#	Lymph node	M	72
MCL12*#	Lymph node	M	56
MCL14#	Lymph node	M	76
MCL15*	Spleen	M	70
MCL16*	Lymph node	M	76
MCL17#	Lymph node	M	84
MCL18*#	Tonsil	M	62
MCL19*	Lymph node	M	61
MCL20*#	Lymph node	F	67
MCL21*	Lymph node	M	75
MCL22*	Blood	M	63
MCL23*	Lymph node	M	76
MCL24*	Lymph node	M	48
MCL25*	Lymph node	M	64
MCL26*	Blood	M	35
MCL28*	Lymph node	M	51
MCL29*	Lymph node	M	69
MCL30*	Spleen	M	56
MCL31*	Lymph node	M	50
MCL32*	Blood	M	66
MCL33*	Lymph node	M	78
MCL34*	Blood	F	44
MCL35*	Lymph node	F	75
MCL36*	Blood	M	62
MCL37*	Spleen	M	63
MCL38*	Blood	M	69
MCL39*	Lymph node	M	66
MCL40*	Lymph node	M	81
MCL41§	Lymph node	M	88
MCL42§	Blood	M	69
MCL43§	Spleen	M	69

MCL samples from 1-20 were also included in our previous methylation array paper[8].

* indicates MCL samples used for mRNA expression analysis

# indicates MCL samples used for DNA methylation analysis

§ indicates MCL samples used for ChIP assay

Supplementary Table 1B. CLL samples included in the current study.

CLL samples[1]	*Gender*	*Age at diagnosis (years)*	*Rai*	*Binet*	*IGHV identity (%)*	*IGHV gene*	*Genomic aberrations*
S1-1#	M	77	IV	C	100	5-a	del(11q)
S1-3#	M	50	I	A	100	1-3	del(13q)
S1-4#	M	58	ND	ND	100	1-3	none
S1-6*	M	77	ND	ND	100	1-2	ND
S1-7#	M	75	ND	ND	100	1-3	none
S1-8*	F	76	0	A	99.7	1-18	+12
S1-9*	M	75	0	A	100	1-2	+12
S1-10*	M	72	0	A	99.7	1-2	+12
S1-11*	M	57	ND	ND	100	1-2	del(11q)
S1-15#	M	51	IV	C	99.6	1-3	ND
S4-18#*	F	64	0	A	96.2	4-34	none
S4-19#	M	43	0	A	91.2	4-34	none
S4-21*	F	46	0	A	95.9	4-34	none
S4-22*	F	54	0	A	93.2	4-34	del(13q)
S4-23*	F	57	ND	ND	93.0	4-34	none
S4-24*§	F	44	I	A	93.1	4-34	del(13q)
S4-25*	M	64	I	A	94.1	4-34	none
S4-26*	M	48	0	A	94.7	4-34	del(13q)
S4-27#	F	43	0	A	91.7	4-34	del(13q)
S4-29#	F	59	0	A	93.3	4-34	none
S4-30#	M	45	0	A	94.4	4-34	del(13q)
SCAN335§	F	56	1	A	96.6	4-34	ND

All CLL samples were used in our previous methylation array paper[8].
S1= stereotyped subset #1, S4 = stereotyped subset #4.
*indicates CLL samples used for mRNA expression analysis, # indicates CLL samples used for DNA methylation analysis, § indicates CLL samples used for ChIP assays.

Supplementary Table 2A – Pyrosequencing primer sequences

Gene	Amplicon length	Number of CpG sites analyzed	Primer sequences (5'-3')
HOXA9	175 bp	8	GTTTTGGATTGGAAGTTGT (Forward primer)
			CCACCACTAAAACCCTAAACAACTA (Reverse primer, Biotin labeled at 5'end)
			TTGAAGTAGGGGTGTT (Sequencing primer)
HOXA13	203 bp	8	ATTGGTATATTTAGGTAGTTAGGTATG (Forward primer)
			CACCCCAACCCCATCAAATC (Reverse primer, Biotin labeled at 5'end)
			GTTAGGTATGGGTTGA (Sequencing primer)
miR196b	213 bp	4	TGTGATTAGGTGGAGGTGTG (Forward primer)
			CAACACCACACTACCTTCATTACTTCAA (Reverse primer, Biotin labeled at 5'end)
			AAAGAGTTGAGGAGAAAGAT (Sequencing primer)

Supplementary Table 2B – ChIP and RQ-PCR primer sequences

Gene	Primer type	Primer sequences (5' -3')
HOXA2	ChIP primers (175 bp)	TGGGAGTGTGTGTGTGTGTG (Forward primer)
		TAATGACTGCAGGCGTCAGA (Reverse primer)
HOXA9	ChIP primers (161 bp)	CTTGGACTGGAAGCTGCAC (Forward primer)
		CCCTGGGCAACTACTACGTG (Reverse primer)
HOXA13	ChIP primers (197 bp)	GAAGACCCAAGGGTTCGTG (Forward primer)
		CGCGGACAAGTACATGGATA (Reverse primer)
HOXA2	Bisulfite sequencing primers	GTTTTTTGATTTGTAAGGATTTATTTAGTG (Forward primer)
		AACTACAAACCTCAAACTAAAATACTTAAA (Reverse primer)
HOXA9	Bisulfite sequencing primers	GTTTTGGATTGGAAGTTGT (Forward primer)
		CCACCACTAAAACCCTAAACAACTA (Reverse primer)
P16INK4	RT-PCR primers	ACTAGATCTTCGCACGAGGCAGCATGG (Forward primer)
		GTTGTGGCGGGGGCAGTTGT (Reverse primer)
EZH2	RT-PCR primers	AGAATGGAAACAGCGAAGGA (Forward primer)
		CTGCTGTAGGGGAGACCAAG (Reverse primer)