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

Meena Kanduri[1], Birgitta Sander[2], Stavroula Ntoufa[3,4], Nikos Papakonstantinou[3,4], Lesley-Ann Sutton[5], Kostas Stamatopoulos[3,4], Chandrasekhar Kanduri[6], and Richard Rosenquist[5,*]

[1]Department of Clinical Chemistry and Transfusion Medicine; Institute of Biomedicine; Gothenburg University; Gothenburg, Sweden; [2]Department of Laboratory Medicine; Division of Pathology; Karolinska Institutet and Karolinska University Hospital; Huddinge, Sweden; [3]Hematology Department and HCT Unit G. Papanicolaou Hospital; Thessaloniki, Greece; [4]Institute of Applied Biosciences; CERTH; Thessaloniki, Greece; [5]Department of Immunology, Genetics and Pathology; Uppsala University; Uppsala, Sweden; [6]Department of Medical and Clinical Genetics; Department of Biomedicine; The Sahlgrenska Academy; Gothenburg University; Gothenburg, Sweden

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The chromatin modifier EZH2 is overexpressed and associated with inferior outcome in mantle cell lymphoma (MCL). Recently, we demonstrated preferential DNA methylation of HOX genes in MCL compared with chronic lymphocytic leukemia (CLL), despite these genes not being expressed in either entity. Since EZH2 has been shown to regulate HOX gene expression, to gain further insight into its possible role in differential silencing of HOX genes in MCL vs. CLL, we performed detailed epigenetic characterization using representative cell lines and primary samples. We observed significant overexpression of EZH2 in MCL vs. CLL. Chromatin immune precipitation (ChIP) assays revealed that EZH2 catalyzed repressive H3 lysine 27 trimethylation (H3K27me3), which was sufficient to silence HOX genes in CLL, whereas in MCL H3K27me3 is accompanied by DNA methylation for a more stable repression. More importantly, hypermethylation of the HOX genes in MCL resulted from EZH2 overexpression and subsequent recruitment of the DNA methylation machinery onto HOX gene promoters. The importance of EZH2 upregulation in this process was further underscored by siRNA transfection and EZH2 inhibitor experiments. Altogether, these observations implicate EZH2 in the long-term silencing of HOX genes in MCL, and allude to its potential as a therapeutic target with clinical impact.

Introduction

Mantle cell lymphoma (MCL) is a clinically aggressive B-cell malignancy with a poor prognosis. The genetic hallmark of MCL is the chromosomal rearrangement caused by the t(11;14)(q13;q32), which results in overexpression of cyclin D1 and deregulated cell cycle control, an essential part of MCL pathobiology.[1] Limited knowledge exists regarding the role of epigenetic modifications and their potential impact on MCL pathogenesis. In MCL, the chromatin modifier EZH2 is overexpressed in proliferating cells and associated with poor outcome,[2,3] similar to other malignancies.[4,5] EZH2 is a core member of polycomb repressive complex (PRC) 2, mediating repressive H3 histone K27 lysine tri methyltransferase activity (H3K27me3) of the chromatin.[6] On the other hand, along with histone methyltransferase activity, EZH2 has also been reported to directly control DNA methylation through its association with and regulation of the activity of DNA methyltransferases.[7]

Using methylation microarrays, we recently reported differential methylation of HOX genes (n = 13) in MCL compared with the more indolent chronic lymphocytic leukemia (CLL).[8] Notably, despite their differential methylation status in MCL and CLL, HOX genes were not expressed in either entity, thus indicating that both DNA methylation-dependent and independent mechanisms may operate to silence HOX genes. HOX genes are a highly conserved group of genes which encode homeodomain containing transcriptional factors that are essential during early embryonic development and regulate cell differentiation and hematopoiesis in adult cells.[9,10] Inappropriate or deregulated expression of HOX genes has been implicated in the development of several cancers, including hematologic malignancies.[11,12] However, HOX genes have both tumor suppressor and oncogenic activity and these contrasting actions occur in a tissue-dependent fashion.[13,14]

Epigenetic mechanisms, such as DNA methylation and the activity of the polycomb and trithorax group of proteins, including EZH2, have been implicated in the deregulation of HOX genes in human cancers.[15,16] For instance, hypermethylation and transcriptional silencing of the complete *HOXA* cluster has been linked to tumor progression in breast cancer.[13] Methylation changes in *HOXA* have also been proposed as biomarkers for grading gliomas.[17] Furthermore, hypermethylation of *HOXA* cluster genes has been shown to correlate with disease progression in leukemia;[18] however, it is not clear whether methylation is a primary determinant of gene silencing or if it occurs as a consequence of silencing mediated by other mechanisms.

*Correspondence to: Richard Rosenquist; Email: richard.rosenquist@igp.uu.se
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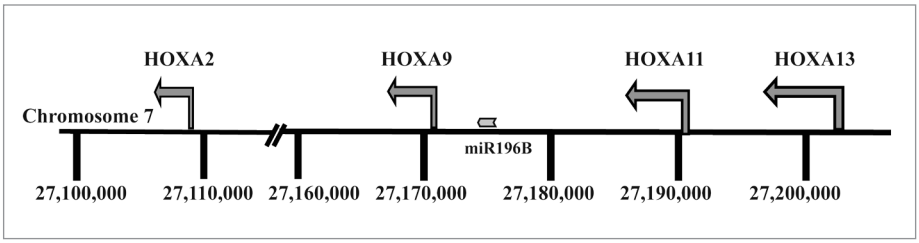

Figure 1. Physical map showing the location of the *HOXA* and *miR196B* genes on chromosome 7.

Since EZH2 has been shown to regulate HOX gene expression,[15,16] one possible scenario is that *HOXA* genes could be targets of EZH2 in MCL. To gain insight into the mechanisms involved in silencing of *HOXA* genes in MCL and CLL, we investigated the functional roles of repressive chromatin modifications, such as H3K27me3, as well as EZH2 in the recruitment of the DNA methylation machinery. Importantly, while HOX genes were silenced by H3K27me3 histone trimethylation in CLL, EZH2 overexpression with subsequent recruitment of methyltransferases to the *HOXA* promoter was critical for long-term silencing of these genes by DNA methylation in MCL. The central role for EZH2 in gene silencing was further evidenced by *EZH2* siRNA experiments and by applying an EZH2 inhibitor, ultimately highlighting EZH2 as a target of potential therapeutic interest in MCL.

Results and Discussion

Overexpression of *EZH2* in MCL compared with CLL

In line with previous studies in MCL,[2,3] we observed a significantly higher *EZH2* expression level in MCL (n = 20) compared with CLL (n = 116) using RQ-PCR (fold difference (FD) 1.97 and $P < 0.0001$). When *EZH2* expression levels were compared separately with favorable-prognostic *IGHV*-mutated CLL samples (n = 61), the FD was even more pronounced (FD 2.77, $P < 0.0001$), whereas the comparison against poor-prognostic *IGHV*-unmutated CLL samples (n = 55) rendered a slightly lower FD (FD 1.35, $P < 0.02$). These results are also in agreement with previous reports in diffuse large B cell lymphoma (DLBCL) and follicular lymphoma, thus indicating that EZH2 overexpression in B-cell lymphomas might be considered as a sign of clinical aggressiveness.[4]

The entire *HOXA* gene cluster is differentially methylated in MCL vs. CLL

An important finding in our previous genome-wide methylation array study of MCL and CLL concerned the identification of 13 differentially methylated HOX genes. While these genes were hypermethylated in MCL and predominantly hypomethylated in CLL, none were expressed in either entity.[8] In order to gain insight into the epigenetic mechanisms involved in HOX gene silencing in each disease, we selected for further analysis genes from the *HOXA* cluster located on chromosome 7, i.e., *HOXA2, HOXA9*, and *HOXA13* (**Fig. 1**).[8] Similar to our recent finding that the methylation status of the *HOXA13* gene differed between MCL and CLL,[8] by analyzing 9 MCL and 12

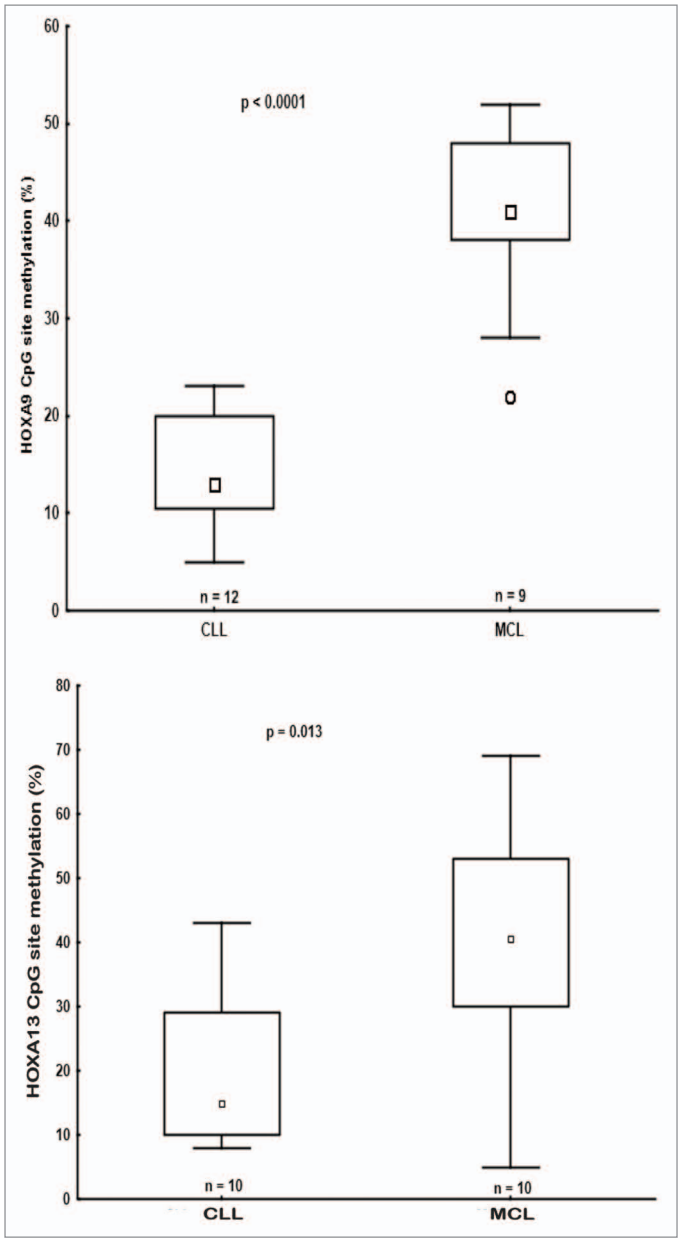

Figure 2. *HOXA9* and *HOXA13* genes were differentially methylated in CLL and MCL primary samples. Box plots showing DNA methylation levels of the *HOXA9/HOXA13* genes in CLL and MCL primary samples, as quantified by pyrosequencing.

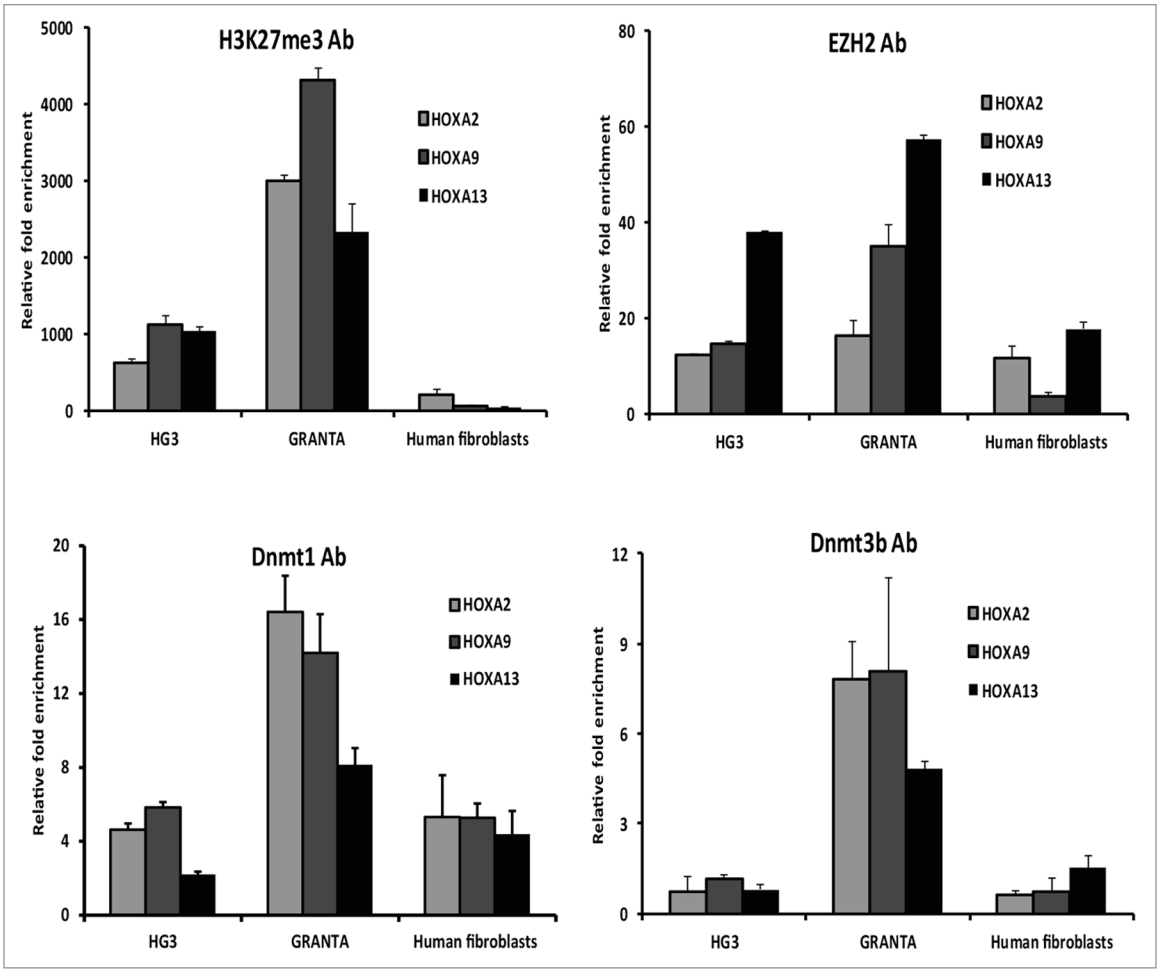

Figure 3. Differential EZH2 levels correlated with DNMT1 recruitment in CLL vs. MCL cell lines. Enrichment of H3K27me3, EZH2, DNMT1, and DNMT3b levels at the *HOXA* gene promoters in the Granta 519 and HG3 cell lines was measured using ChIP with the respective antibody. The data represents values from triplicates plotted over IgG. A human fibroblast cell line was used as a positive control.

CLL samples using pyrosequencing we also observed differential methylation between MCL vs. CLL for the *HOXA9* gene (**Fig. 2**). Hence, using a more quantitative methodology such as pyrosequencing, significantly higher methylation levels of *HOXA* genes were validated in MCL vs. CLL.

In addition to HOX genes, a CpG island flanking the *miR196B* is located in the *HOXA* cluster between the *HOXA9* and *HOXA10* genes (**Fig. 1**). Previous studies have indicated that methylation of CpG islands regulates not only the expression of HOX genes but also the nearby microRNA *miR196b* promoter.[19] Using pyrosequencing to measure the methylation level at the *miR196B* promoter site, as for the *HOXA* genes, a significant difference in the level of methylation was observed between disease entities, i.e., hypermethylation in MCL (n = 8) and hypomethylation in CLL (n = 10) ($P = 0.0004$, **Fig. S1A**), in line with earlier studies in other malignancies.[19,20] However, similar to *HOXA* genes, the expression levels of *miR196b* were extremely low in both MCL and CLL samples/cell lines as compared with normal human fibroblasts (~100-fold lower expression; **Fig. S1B**).

Therefore, we conclude that the entire *HOXA* cluster, including *miR196B,* is silenced in both MCL and CLL, however this is accomplished via different mechanisms, with DNA methylation likely playing a more prominent role in MCL. That said, since the methylation level of *HOXA* cluster genes was < 50% in MCL samples (**Fig. 2**), we cannot exclude that this could either be due to differential methylation of the *HOXA* alleles or due to other cell types present in the samples (although all MCL samples contained >70% tumor cells).

Differential enrichment of H3K27me3, EZH2, and DMNT1/3b at *HOXA* promoters in MCL vs. CLL

Since HOX genes are regulated by EZH2, we wanted to investigate if *HOXA* genes are targets for silencing in MCL and CLL. First, we investigated if the *HOXA2*, *HOXA9*, and *HOXA13* genes carry other epigenetic modifications in their upstream regulatory regions. For this reason, we performed ChIP assays on MCL and CLL cell lines (Granta 519 and HG3) using antibodies for the repressive histone mark H3K27me3 and EZH2, respectively. Normal human fibroblast cells were used as a negative control for histone methylation, since *HOXA13* is highly expressed in these cells.[8] Overall, results from the ChIP assays indicate clear differences in H3K27me3 modification between MCL vs. CLL cell lines, with the Granta 519 cell line showing

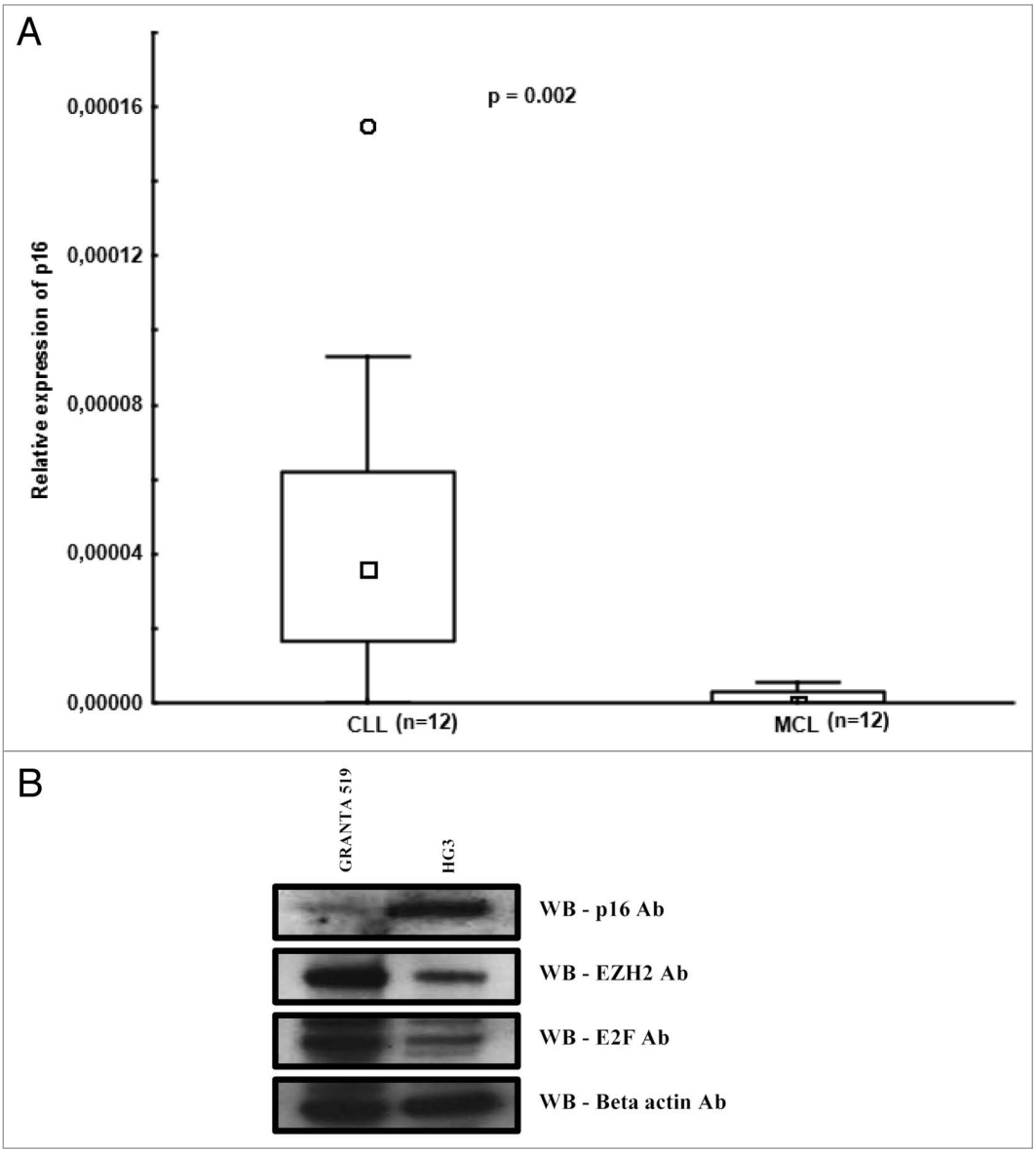

Figure 4. Differential expression of *p16* in CLL and MCL. (**A**) Box plots showing relative *p16* expression levels in CLL and MCL primary samples quantified using RQ-PCR. (**B**) Western blot analysis of p16, EZH2, E2F, and β actin in the HG3 and Granta 519 cell line.

several-fold higher enrichment of H3K27me3 for all three *HOXA* genes (i.e., *HOXA2, HOXA9*, and *HOXA13*) compared with the HG3 cell line (**Fig. 3**). Furthermore, the degree of H3K27me3 enrichment correlated well with EZH2 binding, i.e., high in MCL and low in CLL (**Fig. 3**).

Next, we investigated if the differential methylation status of *HOXA* genes could be related to differences in recruitment of DNA methyltransferases (DNMTs) to the *HOXA* promoters. To this end, we performed ChIP assays with DNMT1 and DNMT3b antibodies and showed that both DNMT1 and DNMT3b binding levels at the HOX gene promoters were associated with the DNA methylation levels, i.e., higher in the MCL cell line compared with the CLL cell line (**Fig. 3**). Nevertheless, the HOX genes also showed some background levels of H3K27me3, EZH2, and DNMTs in the control human fibroblast cell line. When extending these ChIP analyses to primary patient material (3 MCL and 3 CLL samples), similar results were obtained for DNMT1, DNMT3b, and EZH2, although the differences in fold enrichment were less pronounced as compared with the more homogeneous cell line data (**Fig. S2**).

Hence, a higher EZH2 occupancy at the *HOXA* promoters in MCL may lead to increased enrichment of H3K27me3 and recruitment of DNMT1/3b that in turn execute DNA methylation. Yet, what could be the reasons behind higher EZH2 binding in MCL?

Regulation of EZH2 expression by the cyclin D1/p16-pRB-E2F pathway in MCL

The frequent loss of the $p16^{INK4}$ tumor suppressor gene (which is an inhibitor of cyclin D1) by genomic deletion or promoter hypermethylation has been shown to be associated with high

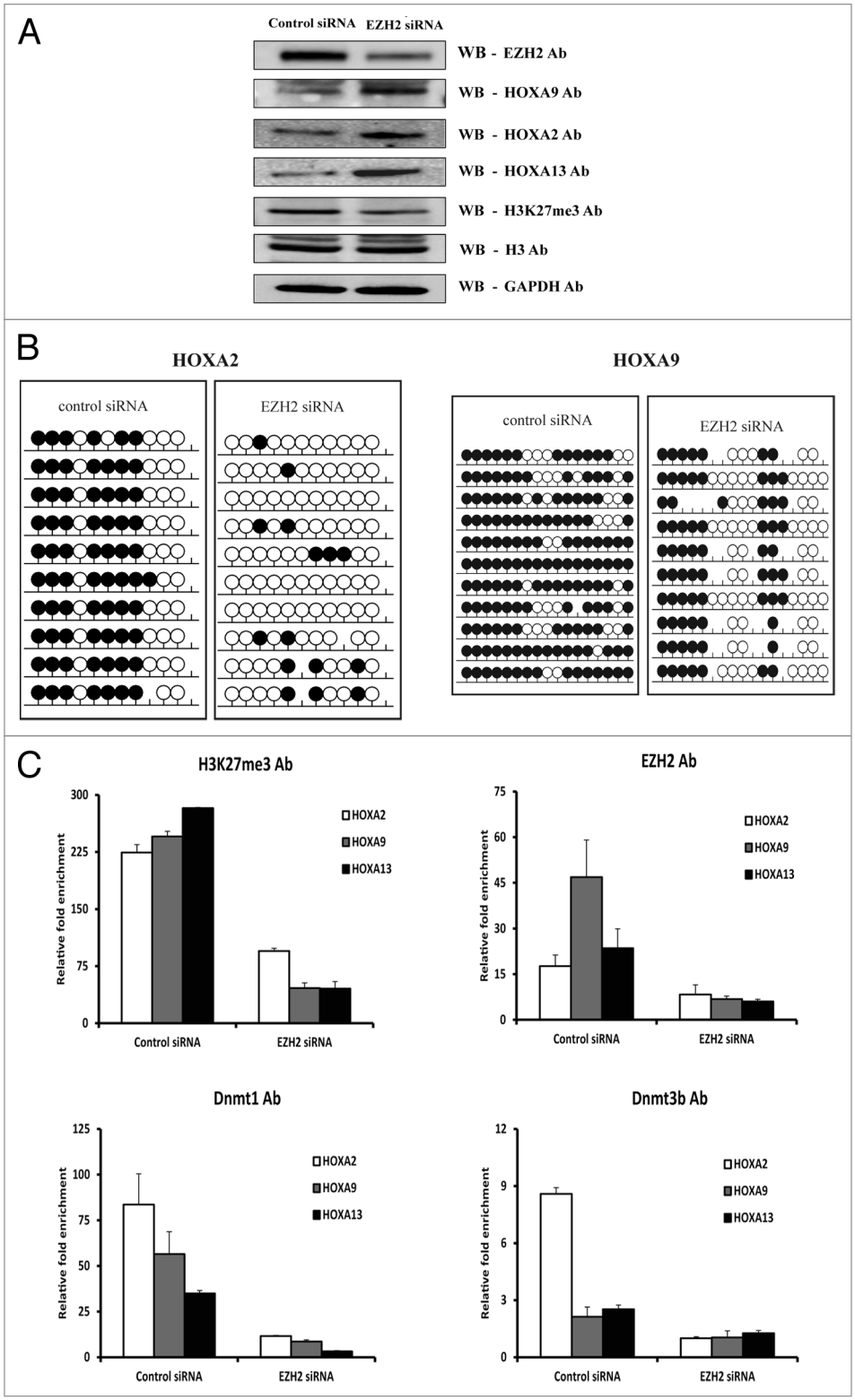

Figure 5. Downregulation of EZH2 using siRNA results in loss of DNA methylation and re-expression of HOXA genes in MCL. (**A**) Western blot analysis of EZH2, all three HOXA proteins (HOXA2, HOXA9, and HOXA13) and H3K27me3 expression after treatment with control siRNA or EZH2 siRNA in the Granta 519 MCL cell line. Histone H3 and GAPDH were used as internal loading controls. (**B**) Bisulfite sequencing analysis of the *HOXA2* and *HOXA9* genes in the Grant 519 cell line after treatment with EZH2 siRNA and control siRNA. The methylation status of 10 clones is presented for each sample; each circle represents one CpG site indicating either cytosine (open circles), methyl cytosine (filled circles) or non-CpG site (missing circles). (**C**) Enrichment of H3K27me3, EZH2, DNMT1, and DNMT3b levels at the *HOXA* gene promoters in the Granta519 MCL cell line using control siRNA and EZH2 siRNA treated. The data represents values from triplicates plotted over IgG.

proliferation and shorter survival in MCL.[21,22] Interestingly, in a human mammary breast cancer cell line, it was demonstrated that downregulation of the *p16*^{INK4} gene resulted in overexpression of the E2F transcriptional factor and polycomb PcG complex proteins, such as EZH2, leading to DNA hypermethylation of the *HOXA9* gene promoter.[23] In order to investigate the *p16*^{INK4} expression levels, we analyzed 12 MCL and 12 CLL patient samples by RQ-PCR and observed considerably higher expression levels in CLL compared with MCL (P = 0.002) (**Fig. 4A**). Furthermore, we analyzed protein levels of p16, E2F, and EZH2 in the CLL and MCL cell lines using western blotting. p16 protein expression was low and E2F and EZH2 proteins were high in the MCL (Granta 519) cell line as compared with the CLL cell line (HG3) (**Fig. 4B**). The lower expression of p16 in the Granta MCL cell line was expected since this cell line has been shown to carry a homozygous deletion of p16.[24] Also, due to the presence of both a mutation in *ATM* and del(17p), this cell line is more likely to represent aggressive MCL. Hence, reduced p16 expression may lead to increased EZH2 expression in MCL.

The *HOXA* DNA methylation status is determined by the EZH2 levels

Since EZH2 can physically interact with DNMT1 and DNMT3b and recruit them to the gene promoters to silence HOX genes,[7,16,25] this may be the case for MCL. To corroborate the direct role of EZH2 in orchestrating DNA methylation of HOX genes, EZH2 expression was first abrogated using RNA interference. In this experiment, siRNA knockdown of EZH2 mRNA revealed a significant decrease in EZH2 protein levels in the MCL cell line as determined by western blot analysis. EZH2 knockdown also resulted in an expected decrease in the levels of H3K27me3, and a corresponding increase in the levels of the HOXA9, HOXA2, and HOXA13 proteins (**Fig. 5A**). These results were also validated at the mRNA level using RQ-PCR (**Fig. S3A**).

To investigate the impact of decreased EZH2 levels on the DNA methylation status of the *HOXA* genes, bisulfite sequencing was also performed, verifying the loss of DNA methylation at most CpG sites analyzed on both the *HOXA2* and *HOXA9* genes (**Fig. 5B**). Since the downregulation of EZH2 resulted in re-expression of *HOXA* genes, we also measured the degree of histone methylation and binding of DNMTs to *HOXA* gene promoters upon EZH2 depletion using ChIP. As expected, there was a significant loss of H3K27me3 as well as DNMT1 and DNMT3B at the *HOXA* promoters in response to EZH2 depletion (**Fig. 5C**).

To further investigate the role of EZH2 in the recruitment of DNMTs to *HOXA* gene promoters, EZH2 was depleted by treating the MCL cell line with the H3K27me3 histone methyl inhibitor 3-Deazaneplanocin A (DZNep), which is a cyclopentenyl analog of 3-deazaadenosine. Previously, this drug has been shown to deplete EZH2 levels and to inhibit H3K27me3 in acute myeloid leukemia[5] and multiple myeloma cells in a dose- and time-dependent manner.[26] When the MCL cell line was treated with increasing concentrations of DZNep, ranging from 0 to 10μM, a significant reduction in EZH2 as well as H3K27me3 levels was detected, followed by activation of the *HOXA9* gene (**Fig. 6**).

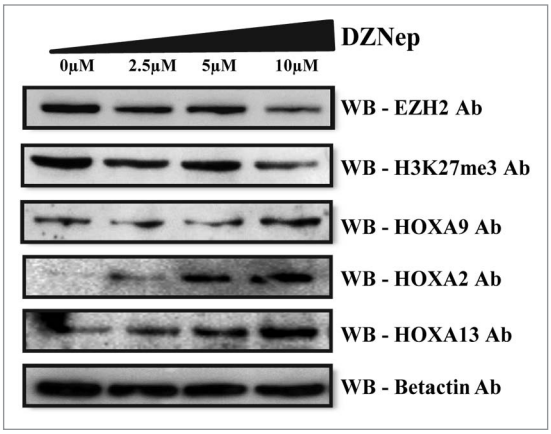

Figure 6. Re-expression of HOXA proteins after DZNep treatment. Western blot analysis of EZH2, H3K27me3, and HOXA proteins (HOXA2, HOXA9, and HOX13) levels in the Granta 519 cell line treated with increasing concentrations of DZNep ranging from 0–10 μM for 4 d. Beta actin levels are used as internal loading control.

EZH2: a potential novel target for epigenetic therapy?

In conclusion, this study for the first time highlights the epigenetic silencing mechanisms underlying differential HOX gene regulation in MCL vs. CLL. In the case of CLL, HOX genes appear to be silenced primarily by H3K27me3 histone methylation, which is catalyzed by EZH2, while in MCL EZH2 also binds to DNMTs thereby recruiting the DNA methylation machinery for efficient silencing of the *HOXA* genes. More specifically, *HOXA* gene promoters exhibited greater enrichment with EZH2 in MCL as compared with CLL, which in turn correlated with increased DNMT1 recruitment and CpG methylation, thus indicating that the level of EZH2 indeed determines the level of DNA methylation through DNMTs. This is in contrast to normal B cells which expressed HOXA genes at higher levels comparable to the expression levels in normal fibroblast cells (**Fig. S3**).[8]

Given that DNA methylation is a more stable epigenetic mark compared with histone modifications, EZH2-mediated specific methylation of the *HOXA* promoters could be a critical step in conferring an aggressive phenotype to MCL. This important function of EZH2 has also been reflected in B-cell activation and lymphomagenesis, where EZH2 acts as an epigenetic switch in promoting H3K27me3 toward DNA hypermethylation.[27] This transition toward DNA methylation reduces epigenetic plasticity and locks the target genes in a stable repressive state, and hence prevents any major transcriptional changes irrespective of external cues. Accordingly, our current data implies that EZH2-mediated DNA methylation ensures the stable repression of HOXA genes, a step that may be critical in the aggressive phenotype of MCL. Our novel observations also advocate that EZH2 plays a crucial role in HOX gene silencing in MCL, which was also confirmed by our siRNA and EZH2 inhibitor experiments, and that DNA methylation occurs as a secondary event following EZH2 recruitment to the HOX promoter. This is further supported by the fact that most MCL patient samples did not show complete methylation of *HOXA* cluster genes.

The key role for EZH2 in MCL lymphomagenesis is also underscored by the recent finding that the *MYC* oncogene upregulates *EZH2* in MCL, eventually leading to decreased expression of the tumor suppressor miRNA, *miR29*.[28-30] Furthermore, EZH2 inhibition was very recently pointed out as a potential treatment strategy for the germinal center subtype of DLBCL.[4,5,31-33] For all these reasons, as well as the well-established notion that EZH2 overexpression is associated with tumor invasion, tumor progression and poor prognosis in many different cancer types,[34-36] our novel observations highlight EZH2 as a potential novel target for epigenetic-based therapy in MCL. Nevertheless, systematic investigations of EZH2 target genes are required before this can be attempted in a clinical setting.

Methods

Patient material

In this present study, we included tumor samples from MCL and CLL patients collected from the biobanks at Uppsala University Hospital and Karolinska University Hospital, Sweden, and G. Papanicolaou Hospital, Thessaloniki, Greece. All MCL cases fulfilled the diagnostic criteria of the World Health Organization Classification.[8] All CLL samples (poor-prognostic subset #1 and favorable prognostic subset #4) were diagnosed according to recently revised criteria showing a typical CLL immunophenotype.[37] Clinical and molecular data are summarized in **Table S1**. Sorted $CD19^+$ B cells from healthy control was obtained from 3H Biomedical.

Cell lines and cell culture conditions

Two EBV transformed cell lines, one CLL (HG3)[38] and one MCL (Granta 519)[39] were used for ChIP assays and siRNA transfection assays. The cell lines were cultured in RPMI 1640 (Invitrogen) supplemented with glutamine (4 mM glutamine for Granta 519 and 2 mM Glutamine for HG3), 10% fetal bovine serum (FBS; Invitrogen), and 1× penicillin/streptomycin (Invitrogen).

Chromatin immunoprecipitation assay

Chromatin immune precipitation was performed using the Shearing module kit and OneDay ChIP Kit™ (Diagenode) according to the manufacturer's instructions. Antibodies used were: EZH2 polyclonal antibody (Diagenode, pAB-039-050), H3K27me3 polyclonal antibody (Diagenode, pAB-069-050), Dnmt1 monoclonal antibody (Imgenex, IMG-261A), Dnmt3b polyclonal antibody (Diagenode, pAB-076-005), and IgG (negative control, OneDay ChIP Kit™Diagenode). The detailed protocol is listed in the **Supplemental Materials**.

Gene and protein expression analysis

Total RNA was prepared using the TRIzol method (Invitrogen Life Technologies) or the AllPrep DNA/RNA kit (Qiagen) according to the manufacturer's instructions. The reverse transcription reaction was performed using MMLV-RT or Superscript II reverse transcriptase (Invitrogen) and random hexamers (Fermentas) according to the manufacturer's protocol. RQ-PCR analysis for the determination of the *p16* and *EZH2* mRNA levels was performed as described in the Supplementary Material. Results for *EZH2* mRNA expression in CLL that were used for comparison to MCL have been reported previously.[40]

Western blot analysis was performed using total cell lysates and the detailed protocol has been provided in the **Supplemental Materials**.

Pyrosequencing and bisulfite sequencing

Both pyrosequencing and bisulfite sequencing were performed as in our previous publications[8,41] and detailed protocols are provided in the Supplementary Material.

siRNA transfections and DZNep treatment assay

Granta 519 cells were transfected with predesigned siRNA against *EZH2* using Stealth RNAi siRNA, containing a mixture of three oligos (HSS103462; HSS176652, and HSS176653) in equal concentrations (Invitrogen). The siRNA negative control (Invitrogen) was used as control siRNA. Transient transfection was performed on an Amaxa Nucleofection Device (Lonza Cologne AG) according to the manufacturer's instruction. In brief, Granta 519 cells were split at a density of 5×10^5/ml in the medium 48 h before transfection. Thereafter, 4×10^6 cells were collected and resuspended in 100 μl human cell line nucleofector solution C with 100 pmol of EZH2 siRNA mix or control siRNA using the X-01 electroporation program.

The Granta 519 cells were treated with the EZH2 inhibitor, 3-Deazaneplanocin A (DZNep) (Cayman chemicals) for three days using different concentrations ranging from 0–10 μM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contributions

Kanduri M designed and performed the research, analyzed the data and wrote the paper. Papakonstantinou N, Ntoufa S, and Sutton L performed research. Sander B, Stamatopoulos K, and Kanduri C analyzed data and wrote the paper. Rosenquist R supervised the research and wrote the paper.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/26546

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