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Genome-wide methylation analyses identify a subset of mantle cell lymphoma with a high number of methylated CpGs and aggressive clinicopathological features

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Abstract

Mantle cell lymphoma (MCL) is a B-cell neoplasm with an aggressive clinical behavior characterized by the t(11;14)(q13;q32) and cyclin D1 overexpression. To clarify the potential contribution of altered DNA methylation in the development and/or progression of MCL, we performed genome-wide methylation profiling of a large cohort of primary MCL tumors ($n = 132$), MCL cell lines ($n = 6$) and normal lymphoid tissue samples ($n = 31$), using the Infinium HumanMethylation27 BeadChip. DNA methylation was compared to gene expression, chromosomal alterations and clinicopathological parameters. Primary MCL displayed a heterogeneous methylation pattern dominated by DNA hypomethylation when compared to normal lymphoid samples. A total of 454 hypermethylated and 875 hypomethylated genes were identified as differentially methylated in at least 10% of primary MCL. Annotation analysis of hypermethylated genes recognized WNT pathway inhibitors and several tumor suppressor genes as frequently methylated, and a substantial fraction of these genes (22%) showed a significant downregulation of their transcriptional levels. Furthermore, we identified a subset of tumors with extensive CpG methylation that had an increased proliferation signature, higher number of chromosomal alterations and poor prognosis. Our results suggest that a subset of MCL displays a dysregulation of DNA methylation characterized by the accumulation of CpG hypermethylation highly associated with increased proliferation that may influence the clinical behavior of the tumors.

Keywords

mantle cell lymphoma; genome-wide; hypermethylation; hypomethylation; pathogenesis

Mantle cell lymphoma (MCL) is a B-cell neoplasia with a relatively short median survival.¹ This lymphoma is genetically characterized by the t(11;14)(q13;q32) translocation and cyclin D1 overexpression promoting cell-cycle dysregulation. Besides this primary oncogenic event, MCL cells may carry a high number of secondary molecular alterations that seem to contribute to the aggressive clinical course of the disease.² Increasing evidence confirms that acquired epigenetic abnormalities, like the genome-wide decrease in global DNA methylation (hypomethylation) and the concomitant increase of methylation (hypermethylation) in tumor suppressor genes (TSG), may be considered an important hallmark of human cancer,^{3,4} including hematological malignancies.⁵ Few studies have addressed the contribution of DNA methylation in MCL lymphomagenesis. Early single-gene analysis in MCL identified low frequency or the absence of methylation events in critical TSG commonly inactivated by mutation or deletion in MCL.^{6–9} Moreover, recent studies of DNA methylation in a limited number of genes have described that pregerminal center tumours such as MCL display significantly less methylation than lymphomas derived from germinal center cells.^{10,11} In addition, a genome-wide analysis performed in a reduced number of MCL concluded that DNA hypomethylation was more predominant than

hypermethylation.¹² Despite these studies, the role of DNA methylation in MCL pathogenesis and its clinical relevance remains an open question.

In our study, we sought to identify the potential role of *de novo* DNA methylation changes in the pathogenesis of MCL and to clarify their clinical relevance by performing a comprehensive genome-wide CpG island methylation profiling study, and correlating the DNA methylation status with gene expression, genomic alterations and clinical data in a large cohort of primary MCL tumors.

Material and Methods

MCL primary tumors, cell lines and normal lymphoid samples

Tumor tissue specimens from 132 MCL patients were obtained from the Tumor Bank of the Department of Pathology of the Hospital Clínic/IDIBAPS of Barcelona, the Institutes of Pathology of the University of Würzburg and the Robert-Bosch Krankenhaus in Stuttgart, the Institute of Human Genetics/Pathology Department of Kiel, and from the Lymphoma/Leukemia Molecular Profiling Project (LLMPP) consortium. The MCL diagnosis was established according to the classification criteria of the 2008 World Health Organization and all cases had the t(11;14)(q13;q32) and/or cyclin D1 overexpression.¹ Our study was approved by the Institutional Review Board of the respective institutions. All patients gave informed consent to participate in the study according to the guidelines of the local Ethic Committees.

The primary MCL samples studied included 112 classic (cMCL) (85%) and 20 blastoid/pleomorphic MCL (bMCL) (15%) variants. In four cases, two sequential samples with a median interval period of 5 years were available. All tumor samples included in the study had at least 80% of tumor cells. Comparative genomic hybridization data had been published previously for 83 cases.^{13,14} Microarray expression data generated with the Affymetrix GeneChip Human Genome U133 Plus 2.0 array were available for 79 primary MCL (Gene Expression Omnibus (GEO) GSE21452 and GSE36000). Clinical and follow-up information was available in 127 MCL patients. We also studied six well-characterized MCL cell lines (UPN1, JEKO1, HBL2, GRANTA519, JVM2 and MINO) and 31 control samples corresponding to different normal lymphoid cells and tissues. These control samples included CD19-positive cells purified from peripheral blood ($n = 6$) or tonsils ($n = 3$), peripheral blood naïve B cells ($n = 4$), reactive lymph nodes ($n = 10$) and spleen samples ($n = 8$). The mononuclear cell fraction from tonsil or peripheral blood was isolated using Ficoll (Boehringer, Mannheim, Germany). Normal CD19⁺ B and naïve B lymphocytes were obtained by magnetic cell sorting using CD19 human microbeads and Naïve B-cell isolation Kit II, respectively, and autoMACS separator following the manufacturer's protocol (MACS, Miltenyi Biotec, Madrid, Spain). The median age of patients with the primary MCL cases was 64 years (range, 37–92 years), and the median age of the individuals from whom the normal samples were obtained was 54 years (range, 23–83 years). Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

DNA methylation microarrays

The Infinium Assay HumanMethylation27 BeadChip from Illumina (San Diego, CA) was used to quantify the DNA methylation of the samples by following the manufacturer's protocols. The Illumina BeadChips were scanned with an Illumina BeadArray Reader and then preprocessed by the Illumina GenomeStudio software. Raw data are accessible through GEO (GSE45253). The output files were processed with the Bioconductor lumi package to get the M -values as a measurement of the methylation levels.¹⁵ A quality-control filtering based on the detection p -value was applied. CpG probes that showed a detection p -value of >0.05 in more than 10% of the samples were excluded. Afterward, the array methylation data were color balance adjusted and quantile normalized following the pipeline implemented in the lumi package. As the biological interpretation of the traditional B -value is more intuitive than the M -values, we generated the final B -values from the M -values by following the equation $Beta_i = 2^{M_i} / (2^{M_i} + 1)$ for an i^{th} interrogated CpG site.¹⁶ To avoid a gender-specific methylation bias, we removed all probes present on either sex chromosomes.

Pyrosequencing analysis

Methylation analysis by bisulfite pyrosequencing technology was performed with the PyroMark Q96 ID platform according to the standard protocols (Qiagen). Pyrosequencing results were evaluated with the analysis software PyroMark-CpG Software 1.0. Briefly, DNA from GRANTA519, 16 MCL cases and four normal controls were bisulfite converted using the EpiTect Plus DNA Bisulfite Kit (Qiagen), according to the manufacturer's instructions. Two amplicons to evaluate three and six CpG sites in *SOX9* and *SFRP1* genes, respectively, were designed using the PyroMark Assay Design Software 2.0 and amplified using the Pyromark PCR Kit (Qiagen). The forward and reverse primers for *SOX9* amplicon were 5'-GGTGAGGAGGAGTATTTATTAAAGAGAT-3' and Biotin-5'-CCTTCTCTTCTCCTCCTACAA-3', respectively. The *SOX9*'s pyrosequencing primer was TGGGTTGGGAGT TGG. For *SFRP1* analysis, the forward and reverse primers for amplification were 5'-GGGTTAGTAGAAGTAGAAGAATTGTATGAT-3' and Biotin-5'-CAACTAAATACCCCTACTCAAC AA-3', respectively. The primer for *SFRP1* pyrosequencing was 5'-AGATGGGTAGGTTTAGGTA-3'.

Bioinformatic and statistical analysis

Hierarchical clusterings with the β -values were performed using Rank Spearman Dissimilarity and the Ward method implemented in the Partek Genomics Suite software (Partek, St. Louis, MO). A principal component analysis (PCA) also was performed with Partek. To identify highly significant differential methylation events between groups, we established that the average geometric difference of β -value should be at least 0.3 ($\beta - 0.3$) and showed an adjusted p -value of <0.001 . The Human 133 Plus 2.0 arrays were normalized using the MAS5 algorithm. Clustering analysis with gene expression data was performed with the Pearson correlation metric and the centroid linkage method using the D-CHIP application.¹⁷ Differential expression analysis among groups was performed by a multivariate permutation test implemented in the BRB-tool application.¹⁸ We used the multivariate permutation test to provide 90% confidence that the false discovery rate (FDR) was $<5\%$. A likelihood ratio test was performed to test the bimodal distribution of

hypermethylated CpG in primary MCL.¹⁹ The molecular proliferation signature value of the tumors was calculated as described by Rosenwald *et al.*²⁰ using the 18 genes of the proliferation signature that were present in the Affymetrix GeneChip Human Genome U133 Plus 2.0 as we described previously.²¹

To correlate methylation data with gene expression, the probes of the HU133 plus 2.0 array were collapsed to HUGO gene symbol using the CollapseDataset tool implemented in the gene set enrichment analysis (GSEA) application using the method defined by default: Max-probe. Functional annotation enrichment and pathway analysis was performed with the Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA) and DAVID Bioinformatics Resources applications. GSEA was performed using the default parameters. A gene set of WNT inhibitors was built based on the KEGG WNT signaling pathway (hsa04310) and following a bibliography search of genes described as WNT inhibitors in different models.^{22–26} The statistical evaluation of clinical variables was performed using nonparametric tests. Overall survival was estimated using the Kaplan–Meier method and compared by means of the log-rank test. Cox proportional-hazards model was used to analyze prognostic factors. The association between two variables was computed using Pearson's correlation (r). The level of significance was set at 0.05 for clinical variables. All calculations were performed with the SPSS software (version 18; SPSS, Chicago, IL).

Results

Genome-wide DNA methylation profiling analysis in primary MCL

A hierarchical clustering analysis of all samples identified two main groups, one containing the cell lines and the majority of primary MCL (80%), and the second comprising all the normal samples and a group of primary MCL (20%) (Fig. 1). The cell lines showed a CpG methylation pattern opposite to normal cells, whereas primary MCL evidenced a pattern similar to cell lines, but with a lower number of hypermethylated and hypomethylated CpG sites. Interestingly, the four sequential tumor samples obtained with a median interval of 5 years (range, 5–10 years) clustered together with their respective initial diagnostic specimens. Normal lymphoid samples had a more homogeneous methylation pattern than primary MCL (average correlation coefficient among normal samples $r = 0.97$; range = 0.05 vs. primary MCL $r = 0.88$, range = 0.37). The tumor methylation heterogeneity was confirmed by the dispersion of the samples in a PCA analysis that in addition revealed a clear separation between the normal samples and the majority of primary MCL (Supporting Information Fig. S1).

De novo hypermethylated and hypomethylated genes in primary MCL

To identify CpGs with a differential methylation pattern between primary MCL and normal samples, we performed a supervised analysis (Material and Methods section). We observed hypermethylation and hypomethylation in 154 and 538 CpG residues, respectively (Fig. 2a). Classic (cMCL) and bMCL had a similar number of hypomethylated CpG (cMCL = 516, bMCL = 699) when compared to normal samples. However, bMCL showed a significantly higher proportion of hypermethylated CpG than cMCL (cMCL = 138, bMCL = 380; $p < 0.0001$) (Fig. 2b).

Owing to the strong heterogeneity observed among the primary MCL samples, we followed another algorithm to identify CpGs that could be defined, at least in a small subset of MCL, as *de novo* hypermethylated or hypomethylated CpGs compared to normal samples. Thus, we considered that a CpG was *de novo* hypermethylated in a primary MCL when it displayed a β -value higher than 0.7 in at least 10% of primary MCL and an average β -value below 0.4 in at least four out of the five categories of normal controls (Fig. 3a). Following these criteria, a total of 551 CpG probes (including 93% of the CpG found in our previous approach) corresponding to 454 genes were considered *de novo* hypermethylated in MCL (Supporting Information Table S1). These hypermethylation events mainly occurred in CpG sites within CpG islands (95%, $p < 0.0001$) and were located in promoters with high CG content (81%, $p < 0.0001$) (Fig. 3b). In addition, we observed a significant enrichment of genes targeted by EZH2 or showing histone repression marks in mature normal B cells (40%, $p < 0.0001$ and 47%, $p < 0.0001$, respectively) (Fig. 3c) or in embryonic stem cells (data not shown).²⁷

CpG dinucleotides were considered *de novo* hypomethylated when displaying a β -value lower than 0.3 in at least 10% of primary MCL and an average β -value higher than 0.6 in at least four out of the five normal sample groups. A total of 947 CpG probes (including 63% of the CpG probes found in our previous algorithm) corresponding to 875 genes were selected as hypomethylated (Fig. 3a and Supporting Information Table S1). Contrary to the hypermethylated CpG, the hypomethylation in primary MCL mainly occurred in CpG outside CpG islands (83.5%, $p < 0.0001$) and mapped to promoters with a low CG content (68%, $p < 0.0001$) (Fig. 3b). Moreover, the set of hypomethylated genes did not show any enrichment of genes targeted by EZH2 or showing histone repression marks in mature normal B cells (Fig. 3c) or embryonic stem cells (data not shown).

The clustering analysis of the cases using *de novo* hyper-methylated and hypomethylated CpG revealed samples characterized by the accumulation of a higher number of hypermethylated and hypomethylated CpGs (Fig. 3a). Accordingly, to this observation, a positive correlation between the number of hypermethylation and hypomethylation events ($r = 0.737$ and $p < 0.001$) was identified in primary MCL (Fig. 3d).

WNT-pathway is frequently targeted by *de novo* DNA methylation in primary MCL

We searched for significantly enriched functions among *de novo* hypomethylated and hypermethylated genes. The inflammatory and defense-response pathways were overrepresented among the hypomethylated genes ($p < 0.005$), whereas the hypermethylated list showed an enrichment in homeobox domain containing genes ($p < 0.0001$) and in genes related to transcription factor activity ($p < 0.0001$) (Supporting Information Table S2). Interestingly, in the IPA analysis, the WNT canonical pathway was significantly enriched ($p < 0.025$) with several WNT antagonist genes hypermethylated. To further investigate this association, we assembled a homemade gene set containing 20 genes described as WNT inhibitors (Supporting Information Table S3). This gene set showed a highly significant enrichment in the IPA analysis as 25% of the WNT antagonists were hyper-methylated in primary MCL ($p < 0.0001$). In fact, more than 78% of primary MCL showed hypermethylation of at least one WNT inhibitor gene and 30% of primary MCL showed

hypermethylation of four or more genes (Supporting Information Table S3). We verified by pyrosequencing the methylation status of *SOX9* and *SFRP1* promoter regions on GRANTA519, MCL cases ($n = 16$), and normal samples ($n = 4$) (data not shown). A GSEA identified a modest enrichment of the WNT pathway ($p = 0.092$) that did not reach statistical significance, in cases with higher number of hypermethylated WNT inhibitor genes.

Correlation of gene methylation status with gene expression in primary MCL

To study the association between DNA methylation and gene expression, we analyzed a subset of 79 cases with microarray expression data. A significant inverse correlation between DNA methylation and gene expression was found more frequently than a positive correlation (Supporting Information Fig. S2A), consistent with the classical inverse association described between CpG methylation and gene expression. To identify which methylated genes might play a role in MCL tumorigenesis, we assessed which of the *de novo* hypermethylated genes had a significantly mRNA downregulation. The HU133 plus 2.0 array contained 98% of the *de novo* methylated genes in MCLs (444 out of 454). A total of 103 genes (22%) showed significant mRNA downregulation in hypermethylated cases (Supporting Information Table S4) but only 3% ($n = 13$) were upregulated in these cases. These results were in line with the widespread concept that increased CpG methylation is associated with decreased expression. Interestingly, this set of genes downregulated by DNA methylation was able to distinguish a cluster of samples characterized by lower gene expression levels and a significantly poor prognosis ($p = 0.0037$) (Supporting Information Figs. S2B and S2C). On the contrary, the study of 839 hypomethylated genes interrogated by the expression array did not evidence an inverse relationship between the gene expression and the methylation degree as a similar number of hypomethylated genes showed gene expression upregulation ($n = 68$) or downregulation ($n = 72$).

De novo CpG island hypermethylation shows a bimodal distribution in MCL

The identification of a higher proportion of hypermethylated CpG sites among blastoid cases, and the association between gene expression and DNA hypermethylation, but not with hypomethylation, led us to further analyze the hypermethylation phenomenon in our series. The histogram of the number of hypermethylated genes per sample revealed a statistically significant bimodal distribution ($p < 0.0001$) (Fig. 4a). Based on this bimodal distribution, 87 samples with a number of hypermethylated CpGs lower than 120 were classified as the low methylation profile (LMP) group, whereas 40 cases with a number of hypermethylated CpG higher than 150 were classified as the high methylation profile (HMP) group. When both groups were compared against normal samples, the HMP group showed, as expected, a clear global deregulation of DNA methylation with a high number of both hypermethylated and hypomethylated CpG dinucleotides ($\beta = 0.3$, adjusted p -value, < 0.001). Interestingly, the HMP group had also a fivefold increase in the proportion of hypermethylated CpG sites when compared to the LMP tumors ($p < 0.0001$), indicating that the HMP MCL were prone to accumulate preferentially hypermethylated CpG dinucleotides (Fig. 4b). Then, we investigated whether HMP and LMP MCL had a different gene expression profiling. We identified an important number of differentially expressed genes ($n = 300$, FDR = 5%) between the LMP ($n = 52$) and the HMP ($n = 24$) tumors (Supporting Information Table S5A and Fig. 4c). Interestingly, among the genes upregulated in the HMP group, there was

an outstanding enrichment in cell-cycle-related genes as 39% of all the overexpressed genes belonged to this ontology category ($p < 0.0001$, Supporting Information Table S5B), whereas the genes upregulated in LMP MCL showed an enrichment in T-cell differentiation and activation genes although they did not reach statistical significance ($p = 0.268$, Supporting Information Table S6). To determine whether the degree of DNA hypermethylation observed in the HMG group was associated with a dysregulated expression of genes involved in DNA methylation, we performed a GSEA analysis using a DNA methylation signature set containing the DNA (cytosine-5-)-methyltransferases, the members of the polycomb repressor complex 2 genes that have been described as directly involved in DNA methylation²⁸ and the UHRF1 protein involved in the recruitment of DNMT1 to DNA.²⁹ Interestingly, this DNA methylation signature showed a highly significant enrichment in HMP samples (FDR, $< 5\%$) (Fig. 4c).

CpG methylation profile and clinicopathological parameters

We analyzed whether the accumulation of hypermethylated or hypomethylated genes could have an impact on the clinicopathological features of the patients. A significant association was observed between the number of hypermethylated loci and high number of genomic alterations ($p = 0.003$) (Supporting Information Fig. S3A) ($p < 0.002$) and with the proliferation signature ($p < 0.001$) (Supporting Information Fig. S3B). In agreement with this, the HMP tumors displayed a significant higher number of chromosome alterations ($p = 0.005$) and higher proliferation signature ($p = 0.001$) (Figs. 5a and 5b). Moreover, blastoid MCL were found more often in the HMP than in the LMP group (58 vs. 28%, $p = 0.03$). Interestingly, the *CDKN2A* locus was targeted by hypermethylation (38 vs. 6%; $p < 0.001$) or 9p21 deletion (33 vs. 9%; $p < 0.001$) more frequently in HMP than in LMP cases. When both phenomena were considered, 63% of the HMP cases showed hypermethylation of *CDKN2A* locus or 9p21 deletion compared to 15% of LMP cases ($p < 0.0001$). Moreover, when we stratified patients in quartiles using the number of hypermethylated CpG, a significant association was observed with the overall survival of the patients ($p = 0.0012$) (Supporting Information Fig. S3C). Concordantly, we observed that HMP patients showed a worse prognosis ($p = 0.0001$) than LMP patients (Fig. 5c). When the methylation status (HMP vs. LMP) was compared to the proliferation signature in a bivariate COX regression analysis, only the proliferation signature remained as a significant predictor for poor overall survival (relative risk: 1.086; 95% confidence interval: 1.054–1.119; $p < 0.001$).

Regarding the correlation between hypermethylation and hypomethylation phenomena, the later was not associated with proliferation or genomic complexity and only patients with low number of hypomethylated genes had a better prognosis (Supporting Information Fig. S3C). Taken together, these findings suggest that hypermethylation on CpG of promoter regions had a stronger influence on the biological behavior of MCL than hypomethylation.

Discussion

In this DNA methylation genome-wide analysis of a large series of MCL, we have identified a marked reprogramming of the methylation profile of the tumor cells compared to the normal lymphoid samples. This methylation pattern was characterized by extensive

hypomethylation targeting CpG dinucleotides outside CpG islands and the hypermethylation of CpG islands, particularly in a subset of primary tumors with aggressive clinical and biological features. Our results support a scenario characterized by a heterogeneous MCL methylation profile that is related to the biological behavior and contrasts with the relatively homogeneous methylation profile observed in different non-neoplastic lymphoid samples. Previous studies have found a more homogeneous pattern of methylation in primary MCL but probably these apparent different results may be owing to the relatively low number of cases investigated in these studies that may not capture the complex biological behavior of MCL.^{12,30}

The distribution of *de novo* hypermethylated sites in CpG islands and loss of methylation in promoters with low number of CpG dinucleotides observed here in MCL is concordant with the observations in solid tumors and other hematological neoplasms.^{31–33} The genes that displayed *de novo* hypermethylation were significantly enriched in genes targeted by EZH2 or carrying inactivating histone marks in normal B cells or in embryonic stem cells.²⁷ These results are consistent with the previous reports in other B-cell lymphomas^{33,34} and agree with the established idea that genes frequently methylated in cancer are targeted in stem cells by members of the polycomb family proteins, or show repressive histone marks.^{35–38} In agreement with these results, our list of genes displaying *de novo* hypermethylation was highly enriched in homeobox domain containing genes. A similar preferential methylation of homeobox genes in MCL has been described in a recent study where MCL methylation profile was compared to the DNA methylation pattern displayed by CLL.³⁰ Furthermore, our hypermethylated gene list comprises most of the genes recently reported as methylated in MCL, including *ARHGAP29* reported by Ripperger *et al.*,³⁹ four of the six genes found frequently methylated in MCL by Rahmatpanah *et al.*¹⁰ (*LHX2*, *NKX6-1*, *POU3F3* and *HOXC10*) and the four genes described and validated by Leshchenko *et al.*⁴⁰ (*CDKN2B*, *MLF1*, *PCDH8* and *HOXD8*). Moreover, we identified six out of the seven genes as hypermethylated (*CDH1*, *AHR*, *HOXA9*, *SOX9*, *NR2F2* and *NPTX2*) as we found methylated in a previous study.⁴¹ A recent study has compared the DNA methylation profile of MCL and CLL also by using the 27k Illumina platform.³⁰ In total, 50% of the genes found hypermethylated and 75% of the genes described as hypomethylated in MCL when compared to CLL were present in our hypermethylated and hypomethylated lists, respectively. These important overlaps with the previously published data reinforce the usefulness of the algorithm that we have followed. The similar methylation profile in two sequential samples with a relatively long interval from four patients suggests that the methylation pattern of primary MCL might be relatively stable during the course of the disease without clinical progression. This would be consistent with some epigenetic events occurring early in MCL pathogenesis, similarly to what was reported for follicular lymphoma and chronic lymphocytic leukemia.^{34,42}

The association between the hypermethylation and the hypomethylation phenomena that we observed in MCL has already been observed in solid tumors⁴³ and in different B-cell lymphomas.³³ However, our data reveal substantial differences between both phenomena in MCL, suggesting that DNA hypermethylation, although less frequent, might play a more important role in MCL lymphomagenesis. In that sense, we observed that DNA hypermethylation in MCL correlated with a significant downregulation of gene expression,

and with several important clinicopathological parameters associated with aggressive behavior including bMCL morphology, chromosomal instability, proliferation signature and, concordantly, shorter overall survival. Moreover, the DNA hypermethylation in our series, contrary to DNA hypomethylation, follows a bimodal distribution. These data would suggest that a subgroup of MCL (HMP) may display a CpG island methylator phenotype (CIMP), a phenomenon described in other neoplasms including hematological diseases.⁴⁴ Recently, a genome-wide analysis has shown that CIMP colon cancers are characterized by a high capacity for CpG island hypermethylation compared to non-CIMP tumors.⁴⁵ In a previous study of a reduced number of genes, we identified a subset of MCL tumors that accumulated DNA methylation.⁴¹ Now, using a genome-wide approach, we have recognized that this phenomenon may be more general and may characterize a subset of primary MCL prone to accumulate a high number of hypermethylated genes.

The group of MCL classified as HMP displayed a differential gene expression profile characterized by the upregulation of an extremely high number of cell-cycle-related genes compared to MCL samples considered LMP. In that sense, the HMP MCL showed a significant higher proliferation signature than LMP samples. Interestingly, disruption of the *CDKN2A/RB1/E2F* pathway, a key element of cell-cycle control frequently targeted in MCL, has been described as a mechanism to dysregulate DNA hypermethylation through the E2F-dependent upregulation of *DNMT1* and polycomb repressors genes, like *EZH2*.^{46–48} We hypothesize that highly proliferative MCL cases would overcome RB regulation and consequently display higher E2F activity that would lead to transcriptional upregulation of cell-cycle genes, DNMT and polycomb repressors genes. Consistent with this fact, the HMP cases showed significantly higher mRNA levels of *E2F1* and *E2F2* (Supporting Information Table S5A), DNA methyl-transferases (*DNMT1* and *DNMT3B*) and polycomb repressor genes (*EZH2* and *EED*). Furthermore, we have observed that the majority of HMP cases (63%) show *CDKN2A* hypermethylation or 9p21 deletion. Interestingly, *CDKN2A* inactivation in breast tumors has been associated with a strong dysregulation of DNA methylation ending in gene hyper-methylation.⁴⁸ These observations may support a model in which the disruption of the *CCND1/CDK4/CDKN2A/RB1* pathway may be important for both the cell-cycle dysregulation and the accumulation of DNA hypermethylation events in MCL (Fig. 6).

The strong association observed between the accumulation of hypermethylated genes and the proliferation of the tumors should not lead to consider hypermethylation in MCL as a passenger phenomenon in the development of the tumors. Rather, our results support that DNA hypermethylation might be an important oncogenic mechanism for MCL lymphomagenesis targeting crucial gene pathways. In that sense, we found that a significant number of cases showed hypermethylation of several WNT inhibitor genes. We have seen in a GSEA analysis a modest enrichment of the WNT pathway in cases with higher number of hypermethylated WNT inhibitor genes. Although we have not been able to show nuclear B-catenin expression by immunohistochemistry in a limited number of cases in a previous study, other authors have shown the constitutive activation of the WNT pathway in primary MCL.³⁸ Further studies should clarify the presence and role of WNT dysregulation in MCL pathogenesis. In addition, we have found that a number of known TSG, such as *BCL11b*, *KLF11*, *KLF4*, *AHR*, *DAPK1*, *CDKN2A*, *DCC* and *SFRP1* among others, are methylated

with a concomitant reduction in their mRNA levels. Interestingly, we observed that primary MCL showing lower expression levels of genes downregulated by DNA methylation showed a significant poor prognosis. This observation supports the idea that these methylation events might be important in MCL lymphomagenesis.

In the last years, new therapeutic options targeting the molecular mechanisms of MCL have been tested, including DNA methyltransferases and/or histone deacetylases inhibitors.⁴⁹ In that sense, treatment with vorinostat, a HDAC inhibitor, has shown important response rates in relapsed MCLs.⁵⁰ Interestingly, it has been shown that MCL cell lines are sensitive to DNMT inhibitors and show synergy with HDAC inhibitors.^{12,41} Now, our results would suggest that these epigenetic treatments could be tailored to MCL patients with high-proliferation signature as they tend to display a broader range of hypermethylated genes. Consistent with this idea, it has been described that tumors with defective *RB1* or upregulated E2F pathway would be more sensitive to HDAC inhibitors.⁵¹

Conclusions

In summary, we have described that primary MCLs show a heterogeneous DNA methylation pattern dominated by DNA hypomethylation in comparison to normal samples. However, DNA hypermethylation might play a more relevant role in MCL lymphomagenesis as an important fraction of the hypermethylated genes, including WNT inhibitors and several TSG, displayed gene expression downregulation in methylated samples. Moreover, these methylation events accumulate in a group of primary MCLs characterized by a high-proliferation signature, increased number of chromosomal alterations and poor prognosis.

Supplementary Material

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References

1. Swerdlow SH, Campo E, Harris NL, et al., eds. WHO classification of tumours of haematopoietic and lymphoid tissues. Lyon: IARC, 2008.
2. Jares P, Colomer D, Campo E. Genetic and molecular pathogenesis of mantle cell lymphoma: perspectives for new targeted therapeutics. *Nat Rev Cancer* 2007;7:750–62. [PubMed: 17891190]
3. Jones PA, Baylin SB. The epigenomics of cancer. *Cell* 2007;128:683–92. [PubMed: 17320506]
4. Egger G, Liang G, Aparicio A, et al. Epigenetics in human disease and prospects for epigenetic therapy. *Nature* 2004;429:457–63. [PubMed: 15164071]
5. Esteller M Epigenetics in cancer. *N Engl J Med* 2008;358:1148–59. [PubMed: 18337604]
6. Pinyol M, Cobo F, Bea S, et al. p16(INK4a) gene inactivation by deletions, mutations, and hypermethylation is associated with transformed and aggressive variants of non-Hodgkin's lymphomas. *Blood* 1998;91:2977–84. [PubMed: 9531609]
7. Hutter G, Scheubner M, Zimmermann Y, et al. Differential effect of epigenetic alterations and genomic deletions of CDK inhibitors [p16(INK4a), p15(INK4b), p14(ARF)] in mantle cell lymphoma. *Genes Chromosome Cancer* 2006; 45:203–10.
8. Mertens D, Wolf S, Schroeter P, et al. Down-regulation of candidate tumor suppressor genes within chromosome band 13q14.3 is independent of the DNA methylation pattern in B-cell chronic lymphocytic leukemia. *Blood* 2002;99:4116–21. [PubMed: 12010815]
9. Chim CS, Wong KY, Loong F, et al. Absence of ATM hypermethylation in mantle cell and follicular lymphoma. *Leukemia* 2005;19:880–2. [PubMed: 15744354]
10. Rahmatpanah FB, Carstens S, Guo J, et al. Differential DNA methylation patterns of small B-cell lymphoma subclasses with different clinical behavior. *Leukemia* 2006;20:1855–62. [PubMed: 16900213]
11. Taylor KH, Kramer RS, Davis JW, et al. Ultradeep bisulfite sequencing analysis of DNA methylation patterns in multiple gene promoters by 454 sequencing. *Cancer Res* 2007; 67:8511–8. [PubMed: 17875690]
12. Leshchenko VV, Kuo PY, Shaknovich R, et al. Genomewide DNA methylation analysis reveals novel targets for drug development in mantle cell lymphoma. *Blood* 2010;116:1025–34. [PubMed: 20427703]
13. Bea S, Ribas M, Hernandez JM, et al. Increased number of chromosomal imbalances and high-level DNA amplifications in mantle cell lymphoma are associated with blastoid variants. *Blood* 1999;93:4365–74. [PubMed: 10361135]
14. Salaverria I, Zettl A, Bea S, et al. Specific secondary genetic alterations in mantle cell lymphoma provide prognostic information independent of the gene expression-based proliferation signature. *J Clin Oncol* 2007;25:1216–22. [PubMed: 17296973]
15. Du P, Kibbe WA, Lin SM. Lumi: a pipeline for processing Illumina microarray. *Bioinformatics* 2008;24:1547–8. [PubMed: 18467348]
16. Du P, Zhang X, Huang CC, et al. Comparison of beta-value and M-value methods for quantifying methylation levels by microarray analysis. *Biomed Chromatogr Bioinformatics* 2010;11:587.
17. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci USA* 2001;98:31–6. [PubMed: 11134512]
18. Simon R, Lam A, Li MC, et al. Analysis of gene expression data using BRB-array tools. *Cancer Inform* 2007;11–7. [PubMed: 19455231]
19. Holzmann H, Vollmer S. A likelihood ratio test for bimodality in two-component mixtures with application to regional income distribution in the EU. *Adv Stat Anal* 2008;92.1:57–69.
20. Rosenwald A, Wright G, Wiestner A, et al. The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. *Cancer Cell* 2003;3:185–97. [PubMed: 12620412]
21. Fernandez V, Salamero O, Espinet B, et al. Genomic and gene expression profiling defines indolent forms of mantle cell lymphoma. *Cancer Res* 2010;70:1408–18. [PubMed: 20124476]
22. Niehrs C. Function and biological roles of the Dickkopf family of Wnt modulators. *Oncogene* 2006;25:7469–81. [PubMed: 17143291]

23. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* 2009;17:9–26. [PubMed: 19619488]
24. Topol L, Chen W, Song H et al. Sox9 inhibits Wnt signaling by promoting beta-catenin phosphorylation in the nucleus. *J Biol Chem* 2009;284: 3323–33. [PubMed: 19047045]
25. Rothbacher U, Lemaire P. Creme de la Kremen of Wnt signalling inhibition. *Nat Cell Biol* 2002;4: E172–3. [PubMed: 12105428]
26. Gage PJ, Qian M, Wu D, et al. The canonical Wnt signaling antagonist DKK2 is an essential effector of PITX2 function during normal eye development. *Dev Biol* 2008;317: 310–24. [PubMed: 18367164]
27. Velichutina I, Shaknovich R, Geng H, et al. EZH2-mediated epigenetic silencing in germinal center B cells contributes to proliferation and lymphomagenesis. *Blood* 2010;116:5247–55. [PubMed: 20736451]
28. Vire E, Brenner C, Deplus R, et al. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 2006;439:871–4. [PubMed: 16357870]
29. Bostick M, Kim JK, Esteve PO, et al. UHRF1 plays a role in maintaining DNA methylation in mammalian cells. *Science* 2007;317:1760–4. [PubMed: 17673620]
30. Halldorsdottir AM, Kanduri M, Marincevic M, et al. Mantle cell lymphoma displays a homogenous methylation profile: a comparative analysis with chronic lymphocytic leukemia. *Am J Hematol* 2012;87:361–7. [PubMed: 22374828]
31. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;349:2042–54. [PubMed: 14627790]
32. Fernandez AF, Assenov Y, Martin-Subero JI, et al. A DNA methylation fingerprint of 1628 human samples. *Genome Res* 2012;22:407–19. [PubMed: 21613409]
33. Martin-Subero JI, Ammerpohl O, Bibikova M, et al. A comprehensive microarray-based DNA methylation study of 367 hematological neoplasms. *PLoS One* 2009;4:e6986. [PubMed: 19750229]
34. O’Riain C, O’Shea DM, Yang Y, et al. Array-based DNA methylation profiling in follicular lymphoma. *Leukemia* 2009;23:1858–66. [PubMed: 19587707]
35. Ohm JE, Baylin SB. Stem cell chromatin patterns: an instructive mechanism for DNA hypermethylation? *Cell Cycle* 2007;6:1040–3. [PubMed: 17457052]
36. Ohm JE, McGarvey KM, Yu X, et al. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat Genet* 2007;39:237–42. [PubMed: 17211412]
37. Widschwendter M, Fiegl H, Egle D, et al. Epigenetic stem cell signature in cancer. *Nat Genet* 2007;39:157–8. [PubMed: 17200673]
38. Schlesinger Y, Straussman R, Keshet I, et al. Poly-comb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nat Genet* 2007;39:232–6. [PubMed: 17200670]
39. Ripperger T, von NN, Kamphues K, et al. Promoter methylation of PARG1, a novel candidate tumor suppressor gene in mantle-cell lymphomas. *Haematologica* 2007;92:460–8. [PubMed: 17488656]
40. Leshchenko VV, Kuo PY, Shaknovich R, et al. Genome wide DNA methylation analysis reveals novel targets for drugdevelopment in mantle cell lymphoma. *Blood* 2010;116:1025–34. [PubMed: 20427703]
41. Enjuanes A, Fernandez V, Hernandez L, et al. Identification of methylated genes associated with aggressive clinicopathological features in mantle cell lymphoma. *PLoS One* 2011;6: e19736. [PubMed: 21603610]
42. Cahill N, Bergh AC, Kanduri M, et al. 450Karray analysis of chronic lymphocytic leukemia cells reveals global DNA methylation to be relatively stable over time and similar in resting and proliferative compartments. *Leukemia* 2013; 27:150–8. [PubMed: 22922567]
43. Suzuki K, Suzuki I, Leodolter A, et al. Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage. *Cancer Cell* 2006;9:199–207. [PubMed: 16530704]
44. Issa JP. CpG island methylator phenotype in cancer. *Nat Rev Cancer* 2004;4:988–93. [PubMed: 15573120]

45. Xu Y, Hu B, Choi AJ, et al. Unique DNA methylome profiles in CpG island methylator phenotype colon cancers. *Genome Res* 2012;22:283–91. [PubMed: 21990380]
46. Jung JK, Arora P, Pagano JS, et al. Expression of DNA methyltransferase 1 is activated by hepatitis B virus X protein via a regulatory circuit involving the p16INK4a-cyclin D1-CDK 4/6-pRb-E2F1 pathway. *Cancer Res* 2007;67:5771–8. [PubMed: 17575144]
47. Pradhan S, Kim GD. The retinoblastoma gene product interacts with maintenance human DNA (cytosine-5) methyltransferase and modulates its activity. *EMBO J* 2002;21:779–88. [PubMed: 11847125]
48. Reynolds PA, Sigaroudinia M, Zardo G, et al. Tumor suppressor p16INK4A regulates polycomb-mediated DNA hypermethylation in human mammary epithelial cells. *J Biol Chem* 2006;281:24790–802. [PubMed: 16766534]
49. Parekh S, Weniger MA, Wiestner A. New molecular targets in mantle cell lymphoma. *Semin Cancer Biol* 2011;21:335–46. [PubMed: 21945517]
50. Kirschbaum M, Frankel P, Popplewell L, et al. Phase II study of vorinostat for treatment of relapsed or refractory indolent non-Hodgkin's lymphoma and mantle cell lymphoma. *J Clin Oncol* 2011;29:1198–203. [PubMed: 21300924]
51. Zhao Y, Tan J, Zhuang L, et al. Inhibitors of histone deacetylases target the Rb-E2F1 pathway for apoptosis induction through activation of proapoptotic protein Bim. *Proc Natl Acad Sci USA* 2005;102:16090–5. [PubMed: 16243973]

What's new?

Genome-wide epigenetic abnormalities may be an important hallmark of hematological malignancies, and their characterization could fuel the generation of new treatments. Here, the authors report marked genome-wide reprogramming of the methylation profile in mantle cell lymphoma (MCL) tumor cells compared with normal lymphoid cells. They identified a subset of MCL cases characterized by the accumulation of CpG hypermethylation, which was associated with an increased proliferation signature and other aggressive clinicopathological features. The results suggest that DNA hypermethylation could play a key role in MCL lymphomagenesis and that DNA methyltransferase and/or histone deacetylase inhibitors might be effectively tailored to MCL patients.

Figure 1. Unsupervised clustering of primary MCL and normal samples. Heat map of an unsupervised hierarchical clustering of 132 primary MCL, 4 sequential MCL and 6 MCL cell lines together with 31 normal samples. The β -values of the 25th percentile of CpG probes with the highest standard deviation were used.

Figure 2.

Differentially methylated CpGs in primary MCL versus normal samples. (a) Heat map of a hierarchical clustering performed using the CpGs differentially hypermethylated ($n = 154$) and hypomethylated ($n = 538$) between primary MCL and normal samples; (b) Venn diagrams representing the number of hypermethylated and hypomethylated CpG dinucleotides in blastoid and classical MCL when compared to normal samples.

Figure 3.

De novo hypermethylated and hypomethylated CpG in at least 10% of primary MCL. (a) Heat map of a hierarchical clustering using the CpGs identified as *de novo* hypermethylated ($n = 551$) and hypomethylated ($n = 947$); (b) Bar graphs of the distribution of *de novo* hypermethylated and hypomethylated CpG according to CpG island association (CpG location in green and CG content in blue), together with the distribution of the whole set of CpG present in the 27K Illumina array; (c) Bar graphs of the distribution of the genes found as *de novo* hypermethylated or hypomethylated, and the genes interrogated by the 27k Illumina array, according to their description in normal B cells as targets of the EZH2 protein or as carriers of repressive histone marks; (d) Scatter-plot showing the number of *de novo* hypermethylated and *de novo* hypomethylated events *per* sample.

Figure 4.

Distribution of CpG island hypermethylation events in primary MCL. (a) The histogram of the number of hypermethylated CpG per sample shows a bimodal distribution of MCL cases. The samples with more than 150 hypermethylated CpG were classified as HMP and cases with lower than 120 CpG were defined as LMP. Five cases with a number of CpG hypermethylated between 121 and 150 remaining unclassified. (b) Bar graphs of the number of CpG with differential methylation ($\beta \geq 0.3$) when LMP and HMP samples were compared to normal samples. (c) Heat map of a hierarchical clustering using the genes that were differentially expressed between LMP and HMP samples. Gray indicates unclassified samples. The proliferation signature, together with gene expression levels of *DNMT1*,

DNMT3B, *EZH2*, *EED* and *UHRF1*, is shown in the upper heat map. Genes highly expressed in HMP were significantly enriched in cell-cycle genes.

Figure 5.

Association of LMP and HMP samples with clinicopathological features. Box plots representing the median and range of the number of chromosome alterations (a) and the proliferation signature (b) for patients classified as HMP or LMP. Circles and stars represent outliers. (c) Kaplan–Meier survival curves for patients classified as HMP or LMP.

Figure 6.

A suggested model to explain the association between cell proliferation and DNA hypermethylation in MCL. The dysregulation of the *CCND1/CDK4/CDKN2A/RB1* pathway by frequent genetic alterations is critical in MCL lymphomagenesis. These alterations would promote E2F activation that would be responsible for cell-cycle progression. In addition to cell-cycle genes, E2F might promote dysregulation of epigenetic regulator genes that might be involved in the inactivation of TSGs and important pathways (i.e., WNT canonical pathway).