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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Short Title: Genome-wide dysregulation of DNA methylation in MCL

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Brief description: For the first time we show a genome-wide marked reprogramming of the methylation profile of MCL cells compared to normal cells. Our results suggest that DNA hypermethylation might play a more important role than DNA hypomethylation in MCL lymphomagenesis. We identified a subset of MCL cases characterized by the accumulation of CpG hypermethylation highly associated with an increased proliferation signature and other aggressive clinicopathological features. These cases could benefit of new epigenetic treatments.

ABSTRACT

Mantle cell lymphoma (MCL) is a B-cell neoplasm with an aggressive clinical behaviour 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 methylation pattern dominated by DNA hypomethylation when compared to normal lymphoid samples. However, the methylation profile of the MCL samples was heterogeneous with 454 hypermethylated and 875 hypomethylated genes 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 has 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.

INTRODUCTION

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 haematological malignancies.[5] Few studies have addressed the contribution of DNA methylation in MCL lymphomagenesis. Early single gene analysis in MCL identified low frequency or 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 pre-germinal center tumors 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 still remains an open question.

In the current 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] The 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 (bMCL) (15%) variants. In four cases, two sequential samples with a median interval period of five years were available. All tumor samples included in the study had at least 80% tumor cells. Comparative genomic hybridization (CGH) 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 was available for 79 primary MCL (Gene Expression Omnibus (GEO) GSE21452 & 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). 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 was 54 years (range, 23 to 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 following the manufacturer's protocols. The Illumina BeadChips were scanned with an Illumina BeadArray Reader and then preprocessed by the Illumina GenomeStudio software. 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 in the detection p-value was applied. CpG probes that showed a detection p-value >0.05 in more than 10% of the samples were excluded. Afterwards, the array methylation data were color balance adjusted and quantile normalized following the pipeline implemented in the lumi package. Since 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 following the equation $Beta_i = 2^{Mi} / 2^{Mi} + 1$ for an i^{th} interrogated CpG site.[16] In order to avoid a gender specific methylation bias we removed all probes present on both sex chromosomes.

Pyrosequencing analysis

Methylation analysis by bisulfite pyrosequencing technology was performed with the PyroMark Q96 ID platform according to standard protocols (Qiagen). Pyrosequencing results were evaluated with the analysis software PyroMark-CpG Software 1.0. Briefly, DNA from GRANTA519, 16 MCL cases, and 4 normal controls were bisulfite converted using the EpiTect Plus DNA Bisulfite Kit (Qiagen), according to the manufacturer's instructions. Two amplicons to evaluate 3 and 6 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 TGGGTTGGGAGTTGG. For *SFRP1* analysis the forward and reverse primers for amplification were 5'-GGGTTAGTAGAAGTAGAAGAATTGTATGAT-3' and Biotin-5'-CAACTAAATACCCCTACTCAACAA-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 Inc., 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 ($\Delta\beta \geq 0.3$) and showed an adjusted p-value<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 was less than 5%. A likelihood ratio-test was performed to test the bimodal distribution of hypermethylated CpG in primary MCL.[19]

To correlate methylation data with gene expression, the probes of the HU133 plus 2.0 array were collapsed to HUGO gene symbol using the probe set that showed the higher median expression level. Functional annotation enrichment and pathway analysis was performed with the Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc., Redwood City, CA) and DAVID Bioinformatics Resources applications. The statistical evaluation of clinical variables was performed using nonparametric tests. Overall survival (OS) 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 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 Inc, 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 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 (see Material and Methods). We observed hypermethylation and hypomethylation in 154 and 538 CpG residues, respectively (Fig. 2A). Classic (cMCL) and blastoid/pleomorphic MCL (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).

Due 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 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 cell (data not shown).[20]

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 corresponding to 875 genes were selected as hypomethylated (Fig. 3A, 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* hypermethylated 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 since 25% of the WNT antagonist were hypermethylated 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 gene set enrichment analysis (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 significantly 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 genes which methylation 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 Fig. S2B-C). On the contrary, the study of 839 hypomethylated genes interrogated by the expression array did not evidence an inverse relationship between gene expression and the methylation degree, since 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 group (LMP), whereas 40 cases with a number of hypermethylated CpG higher than 150 were classified as the high methylation profile group (HMP). 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 ($\Delta\beta \geq 0.3$, adjusted P-value<0.001). Interestingly, the HMP group had also a five-fold 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 HMP (n=24) tumors (Supporting Information Table S5A, Fig. 4C). Interestingly, among the genes upregulated in the HMP group, there was an outstanding enrichment in cell cycle related genes since 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). In order to determine whether the degree of DNA hypermethylation observed in the HMG group was associated with a deregulated expression of genes involved in DNA methylation, we analyzed the differential expression of DNA (cytosine-5-)-methyltransferases and polycomb repressor genes between HMP and LMP cases. Interestingly, *DNMT1* (P=0.005) and *DNMT3B* (P=0.034) and two members of the polycomb repressive complex 2, *EZH2* (P<0.0001) and *EED* (P=0.03), were significantly overexpressed in HMP cases (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) (Fig. 5A,B). Moreover, 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 with the proliferation signature in a bivariate COX regression analysis, only the proliferation signature remained as a significant predictor for poor overall survival (relative risk (RR): 1.086; 95% CI: 1.054-1.119; P<0.001).

Regardless 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 seems 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 due to the relatively low number of cases investigated in these studies that may not capture the complex biological behavior of MCL.[12,21]

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.[22-24] The genes that displayed *de novo* hypermethylation were significantly enriched in homeobox genes and genes targeted by EZH2 or carrying inactivating histone marks in normal B-cells or in embryonic stem cells.[20] These results are consistent with previous reports in other B-cell lymphomas[21,24,25] 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.[26-29] 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.[25,30]

The association between the hypermethylation and hypomethylation phenomena that we observed in MCL have already been observed in solid tumors[31] and in different B-cell lymphomas.[24] 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 blastoid/pleomorphic 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.[32] 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.[33] In a previous study of a reduced number of genes we identified a subset of MCL tumors that accumulated DNA methylation.[34] 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*.[35-37]. 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, the HMP cases showed significantly higher mRNA levels of *E2F1* and *E2F2* (Supporting Information Table S5A), DNA methyltransferases (*DNMT1* and *DNMT3B*), and polycomb repressor genes (*EZH2* and *EED*). Furthermore, we have observed that the majority of HMP cases (63%) show hypermethylation of the *CDKN2A* promoter or 9p21 deletion. Interestingly, *CDKN2A* inactivation in breast tumors has been associated with a strong dysregulation of DNA methylation ending in gene hypermethylation.[37] These observations may support a model in which the disruption of the *CCND1/CDK4/CDKN2A/RB1* pathway may be important for both 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 work, 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 deregulation in MCL pathogenesis. In addition, we have found that a number of known TSG, like *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 significantly 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.[38] In that sense, treatment with vorinostat, a HDAC inhibitor, has shown important response rates in relapsed MCLs.[39] Interestingly, it has been shown that MCL cell lines are sensible to DNMT inhibitors and show synergy with HDAC inhibitors[12,34]. Now, our results would suggest that these epigenetic treatments could be tailored to MCL patients with high proliferation signature since 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.[40]

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, since 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.

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FIGURE LEGENDS

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 and CG content, 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 as targets of the EZH2 protein or as carriers of repressive histone marks in normal B-cells; **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 High Methylation Profile (HMP) and cases with lower than 120 CpG were defined as Low Methylation Profile (LMP). Five cases with a number of CpG

hypermehtylated between 121-150 remaining unclassified ; **B)** Bar graphs of the number of CpG with differential methylation ($\Delta\beta \geq 0.3$) 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. Grey indicates unclassified samples. The proliferation signature, together with gene expression levels of *DNMT1*, *DNMT3B*, *EZH2* and *EED* are displayed 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 starts 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).