MicroRNA Expression, Chromosomal Alterations, and Immunoglobulin Variable Heavy Chain Hypermutations in Mantle Cell Lymphomas

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Abstract
The contribution of microRNAs (miR) to the pathogenesis of mantle cell lymphoma (MCL) is not well known. We investigated the expression of 86 mature miRs mapped to frequently altered genomic regions in MCL in CD5+/CD5− normal B cells, reactive lymph node samples, and purified tumor cells of 17 leukemic MCL, 12 nodal MCL, and 8 MCL cell lines. Genomic alterations of the tumors were studied by single nucleotide polymorphism arrays and comparative genomic hybridization. Leukemic and nodal tumors showed a high number of differentially expressed miRs compared with purified normal B cells, but only some of them were commonly deregulated in both tumor types. An unsupervised analysis of miR expression profile in purified leukemic MCL cells revealed two clusters of tumors characterized by different mutational status of the immunoglobulin genes, proliferation signature, and number of genomic alterations. The expression of most miRs was not related to copy number changes in their respective chromosomal loci. Only the levels of miRs included in the miR-17-92 cluster were significantly related to genetic alterations at 13q31. Moreover, overexpression of miR-17-5p/miR-20a from this cluster was associated with high MYC mRNA levels in tumors with a more aggressive behavior. In conclusion, the miR expression pattern of MCL is deregulated in comparison with normal lymphoid cells and distinguishes two subgroups of tumors with different biological features.

Introduction
MicroRNAs (miR) are noncoding regulatory small RNAs generated from larger precursors with a hairpin-like structure (1, 2). These small RNAs bind to specific mRNA transcripts leading to their degradation and/or translational blocking (3, 4). Different studies have shown their contribution to the modulation of cellular processes such as differentiation, proliferation, and apoptosis that play an important role in oncogenesis (5, 6). miRs are located throughout the genome and are often found in chromosomal loci altered in different neoplasms (7). Genomic alterations including gains/amplifications, losses, and translocations of these regions could be a causal event of miR deregulation in cancer (8, 9).

Mantle cell lymphoma (MCL) is genetically characterized by the t(11;14)(q13;q32) translocation resulting in rearrangement and overexpression of the CCND1 gene (10). In addition to this primary genetic alteration, most MCL carry a high number of recurrent secondary gains and losses as well as uniparental disomies, homozygous deletions, and high-level DNA amplifications (11, 12). In a recent review of the potential genes that could be targeted by recurrent MCL chromosomal aberrations, we noticed the presence of a relatively high number of miRs in these regions (13). Interestingly, some of the miRs involved have been shown to modulate genes related to the regulation of cell cycle and survival pathways, two mechanisms that have an important role in the progression of MCL (14). However, the miR expression profile in MCL and their potential involvement in the pathogenesis of these tumors have not been examined previously.

To determine the role of miR deregulation in the pathogenesis of MCL, we have investigated the expression of a panel of miRs located in commonly altered chromosomal regions in a series of primary tumors and cell lines and their relationship to the clinicopathologic characteristics of the patients.

Materials and Methods
Case selection and cell lines. Peripheral blood samples from 17 patients with classic leukemic MCL and 50 nodal MCL, 36 classic and 14 blastoid variants, were obtained from the Department of Pathology of the Hospital Clinic and Hospital del Mar and Institute of Pathology of the University of Würzburg. The leukemic cases were selected based on the sample availability for tumor cell purification and the nodal cases based on the high content of tumor cells (>85%). Tumor cells were purified in leukemic samples as described previously (15). In addition, CD5− and CD5+ fractions of normal B cells were obtained by cell sorting from equal cell amounts from four tonsil samples using FACSVantage SE cell sorter (BD Biosciences) of the Cytomic Unit Facility of Institut d’Investigacions Biomèdiques August Pi i Sunyer and with antibodies against CD19-FITC (Beckman Coulter) and CD5-PE (BD Biosciences). Twelve reactive lymph node samples were pooled and used as an additional normal tissue sample for comparison with nodal MCL.

In two MCL patients, paired peripheral blood and tumor lymph node samples were available and the CD19+CD5+ tumor cells were purified from both. MCL-derived cell lines GRANTA519, REC1, Jeko1, UPN1, HBL2, MINO, MAYER1, and JVM2 were also studied and grown as described...
previously (16). EBV infection status of these cell lines has been described previously (17).

All MCL of the study were positive for cyclin D1 expression. Ki-67 proliferation antigen expression was studied by immunohistochemistry on formalin-fixed, paraffin-embedded material of nodal MCL cases (18). The gene expression profile of the leukemic MCL was examined previously using Affymetrix U133 Plus 2.0 microarrays and tumor proliferation signature was calculated according to Rosenwald and colleagues (19) as the normalized mean expression of the 20 genes included in this signature.\(^7\)

The immunoglobulin variable heavy chain genes (IgVH) mutational status was studied as described previously (20). All patients gave informed consent and the whole study was previously approved by the Hospital Clinic of Barcelona Institutional Review Board.

**miR selection.** The complete list of the miRs analyzed is shown in Supplementary Table S1. This set was selected to cover the highest number of known miRs mapped at the most frequent chromosomal altered regions in MCL (21, 22) but limited by the presence of homologous mature forms in different genomic locations and the reagent availability (Applied Biosystems). A total of 85 miRs were finally included corresponding to 3 located at 1q13, 24 at 1q32, and 58 at loci involved in regions of chromosomal gains/amplifications or losses in MCL (11). Additionally, miR-181a was also included to explore its possible clinical relevance in MCL, because it had been characterized in chronic lymphocytic leukemia (CLL) as the most significant miR showing high levels associated with short time from diagnosis to initial therapy (23).

This miR was excluded for the correlation analysis between the expression and genomic status because their two variants map to two different chromosomal loci (see Supplementary Table S1) and could not be differentiated in the expression analysis of the resulting mature miR variants.

**RNA isolation and reverse transcriptase–looped quantitative PCR.** Total RNA was isolated from all samples using Trizol reagent (Invitrogen). The miRNA levels of the mature miRs previously selected were investigated by reverse transcriptase–looped quantitative PCR as described previously (24). The CD5+/CD5− normal B-cell samples were used as calibrators. As a normalization of RNA input, a noncoding RNA (RNU19/ snoRA74A; Applied Biosystems) was selected at its location at 5q31.2, an infrequently altered region in MCL, and their low variation amplification values. miR quantifications in relative units were calculated with software package SDS 2.1 (Applied Biosystems) based on 2\(^{-\Delta\Delta C_{T}}\) method and converted to log_{20} scale for more convenient data representation.

Reverse transcriptase–looped quantitative PCR of miR-17-5p and miR-20a was done in 35 additional nodal MCL samples. MyC mRNA expression in the 50 nodal MCL cases had been previously investigated by reverse transcriptase–looped quantitative PCR (25).

**Genomic analysis: single nucleotide polymorphism arrays.** The detection of DNA copy number changes and uniparental disomy was investigated in the cell lines and leukemic MCL using the GeneChip mapping 100K single nucleotide polymorphism array (Affymetrix) as described previously (11). The genomic profile of 12 of these MCL cases has been published previously (11), and 5 additional cases were investigated for the present study using the same protocol and criteria. The genomic alterations in the nonpurified nodal MCL samples had been previously analyzed using comparative genomic hybridization (21). miR genomic loci status was defined by their physical position on public databases overlapping with chromosomal regions defined as altered in the studied samples.

**Statistical and bioinformatics analysis.** Nonsupervised clustering analysis of miR expression data was done using an Euclidean-related metrics (Manhattan distance) and a complete clustering algorithm (TIGR MeV software package; ref. 26). The differential miR expression analysis between MCL groups was done using unpaired/paired significance analysis of microarrays (SAM) method as implemented in TIGR MeV software package (26), adjusting delta parameter to ensure the lowest false discovery rate (q ≤ 0.01; refs. 27, 28). Differentially expressed miRs from MCL samples compared with each of the considered controls were established using 95% confidence interval of the difference of means between samples and controls for each miR. In all comparisons, only miRs with detectable expression in >50% of the samples in each group were considered.

The correlation between miR loci copy number changes and their expression levels was analyzed using the Kendall \(\tau\)-b statistic. Genomic alterations were divided into loss and gain/amplification. Only miR loci with alterations present in >15% of the cases were included in the analysis. Uniparental disomy was considered separately and analyzed using Kruskal-Wallis test. For statistical correction of multiple comparisons, \(P\) values were adjusted using the Benjamini and Hochberg false discovery rate method (ref. 29; R package).\(^8\) Adjusted \(P\) values < 0.05 were considered significant. Categorical data were compared using Fisher’s exact test, and Mann-Whitney nonparametric test was used for comparisons of continuous variables.

The survival analysis was done using the Kaplan-Meier analysis, and survival curves were compared with the log-rank test (SPSS software 13.0) on the patients groups categorized by miR-17-5p/miR-20a and MYC expression levels. These groups were defined into high (above cutoff) or low (at or below cutoff), and given that no clear criteria of gene overexpression in tumors without increased gene dosage could be defined, expression cutoff was set at the 70th percentile expression. This cutoff was the highest to (a) maximize the number of patients in all the groups analyzed and (b) define a high expression group including patients with gain/amplification of these genes.

**Results**

**miR expression profile in leukemic MCL.** The expression of the selected miRs was initially investigated in leukemic and cell line MCL samples and compared with CD5+ and CD5− B–cell pools. Leukemic MCL showed a miR expression profile different from both B-cell controls (Fig. 1A; Supplementary Table S2A-C), including 17 and 9 miRs, respectively, which were not detectable in these normal samples but were expressed in 12% to 100% of the tumors (Supplementary Table S2A). Significant differences were also detected in miRs with detectable expression in both B-cell controls and tumors. Thus, 17 miRs showed a higher mean fold change (MFC, 1.36–8.59), whereas 34 miRs were down-regulated in the tumors compared with CD5+ B cells (MFC, 0.61–0.02; Fig. 1A; Supplementary Table S2B). Similarly, 2 miRs had significantly higher levels in the tumors (MFC, 3.36–6.83), whereas 49 miRs were significantly down-regulated (MFC, 0.59–0.01) in the tumors compared with CD5+ B cells (Fig. 1A; Supplementary Table S2C).

The unsupervised hierarchical clustering analysis of the miR expression in tumor samples and cell lines showed three distinctive clusters corresponding to the cell lines and two groups of leukemic MCL. These three clusters were observed using either normal CD5+ or CD5− B cells as reference calibrators (clusters A and B; Fig. 1A; Supplementary Fig. S1A).

To determine the significance of the two subgroups of MCL, we studied several clinical and biological characteristics of these tumors (Fig. 1A). Interestingly, the mutational status of the IgVH genes was significantly different in the two clusters of MCL. Thus, 7 of 9 tumors in the cluster B had a hypermutated IgVH gene (>95% homology), whereas 7 of 8 cases included in cluster A were found unmutated (>98% homology; \(P = 0.012\), Fischer’s exact test).

The number of genomic alterations detected by the single nucleotide polymorphism array analysis was also significantly different between cluster A (mean, 6.5 ± 5.07) and cluster B (mean, 1.56 ± 2.35;
In addition, the mean proliferation signature obtained from the microarray expression analysis of these tumors was also significantly higher in cluster A (mean, 0.17 ± 0.49) than in cluster B (mean, −0.15 ± 0.61; *P* = 0.043, Mann-Whitney test; Supplementary Table S3). Although patients in cluster A show a shorter median overall survival (15 months) than cluster B patients (median overall survival not reached), the differences were not significant.
Table 1. miR signature with significant differential expression between clusters A and B of leukemic MCL cases

<table>
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<th>Cytoband</th>
<th>Score (d)*</th>
<th>Fold change</th>
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*A SAM statistical score.

To determine the miRs differentially expressed between these two groups of MCL, we performed an unpaired SAM analysis. A group of 28 miRs, all with high SAM statistical scores (>2), showed a significant overexpression in cluster A compared with cluster B (MFC, 4.12; range, 2.02-11.86; Table 1). An additional supervised unpaired SAM analysis between IgVH mutational classes was done. A total of 29 miRs showed a significant overexpression in unmutated versus hypermutated IgVH MCL cases (MFC, 3.41; range, 1.57-16.26; Supplementary Table S4) and included 17 (61%) miRs of the differentially expressed miRs found between MCL clusters A and B.

The third cluster in the unsupervised analysis corresponded to the MCL cell lines. No significant differentially expressed miRs were found between EBV+ (GRANTA519 and JVM2) and the remaining EBV- MCL cell lines. A SAM analysis comparing the expression profile of the cell lines and primary tumors revealed 29 miRs differentially expressed between these groups (Supplementary Table S5). Only 4 miRs were not detectable in the cell lines but were expressed in 12% to 65% of the tumors (Fig. 1A; Supplementary Table S2A).

miR expression profile in nodal MCL. The expression miR profile was next examined in nodal MCL in reference also to CD5+/CD5− B-cell pools. Nodal MCL samples showed a high number of differentially expressed miRs (Supplementary Tables S5A and B). Thus, 73 miRs were significantly overexpressed compared with CD5+ B cells (MFC, 3.18-1153.71). Similarly, 66 miRs were significantly overexpressed (MFC, 1.92-2052.29), but only 3 miRs were down-regulated (MFC, 0.29-0.12) in tumors compared with CD5− B cells. In addition, the proportion of common deregulated miRs between nodal and leukemic MCL samples (marked with asterisks in Supplementary Tables S5A and B and S2B and C) was noticeable low in reference to either CD5+ B cells (20.6% overexpressed and 29.4% down-regulated miRs, respectively) or CD5− B cells (7.4% overexpressed and 9.8% down-regulated miRs, respectively). Noticeably, miR-337 was detected in the pool of reactive lymph nodes but was not expressed in the CD5+/CD5− normal B cells or any tumor sample.

Unsupervised hierarchical clustering analysis of the relative miR expression in the nodal tumors in reference to either normal CD5+/CD5− B cells generated two miR signatures that separate 4 and 8 tumors (Fig. 1B; Supplementary Fig. S1B) but were not related to significant differences in histologic variants, Ki-67 proliferation index, IgVH mutational status, or genomic complexity of the tumors (Fig. 1B).

miR expression and microenvironment of tumor cells. The different pattern of miR expression in purified leukemic cells and nodal lymphomas prompted us to evaluate the possible influence of the nodal microenvironment on the miR expression profile. Thus, we were able to study purified (>98%) MCL cells from matched samples obtained simultaneously from leukemic and nodal samples in two patients (identified as A-PB/A-Node and B-PB/B-Node samples in Figs. 1A and 2). Most miRs were expressed at similar levels in both samples in the two patients. However, 11 miRs had a marked significant overexpression (3.6- to 21.9-fold change) in the nodal sample compared with the leukemic cells of both cases, suggesting that they may be modulated by influences of the tissue microenvironment (Fig. 2).

miR expression and genetic alterations. The t(11;14)(q13;q32) translocation is the primary genetic alteration in MCL (14). Several miRs have been mapped in the chromosomal subregions involved, including 7 miRs at 11q13 and 60 miRs at 14q32 (30). To determine whether this translocation may deregulate them, we have compared their expression in the cell lines and leukemic MCL with those

![Figure 2. Heat map representation of the significant miR expression differences in paired samples of purified tumor cells from simultaneous matched peripheral blood (PB) and tumor lymph nodes (NODE) of two patients (A and B).](image-url)
in the normal CD5+/CD5− B cells. The analyzed miRs included 3 mapped at 11q13 (1 centromeric and 2 telomeric to the CCND1 gene) and 24 at 14q32 (all centromeric to the IgH gene; Supplementary Fig. S2) covering several individual and polycistronic miR regions (31). These miRs had a highly variable expression among the MCL samples (range, 0.16-2.24 relative units). The 3 11q13 miRs showed significant higher levels in MCL samples compared with CD5+ normal B cells but were down-regulated in reference to the CD5− normal B cells irrespective of their location in the derivative chromosomes and thus suggesting an absence of IgH enhancer influence in their expression (Supplementary Fig. S2). The expression of most miRs located at 14q32 did not show significant

Figure 3. Correlation analysis of the miR locus copy number changes and expression levels in cell lines and leukemic MCL. A, representation of the correlation Kendall τ-b statistic results as a bar chart for each miR ordered by chromosomal physical position. Only miR loci with copy number alterations in >15% of cases were included. A few miRs (crosses) showed a statistical significant correlation (P < 0.05). miRs at 13q31 showed a significant correlation after correction for multiple comparison (asterisks; adjusted P = 0.042). The percentage of cases with gains/amplifications (red) and losses (green) in leukemic MCL (PB-MCL) and cell lines is represented as heat maps for each considered miR. B, heat map comparing the expression levels and gene copy number status for the miRs with a significant correlation between these two parameters. For each miR, cell line and leukemic MCL results are mixed. The different cases are grouped by presence (red) or absence (black) of genomic alteration of the miR locus, and inside these categories, miR expression levels are shown ordered decreasingly from left to right.
differences between tumor samples and both subsets of normal B cells (Supplementary Fig. S2).

To determine the possible influence of DNA copy number changes in the expression of the mature miRs, we performed a correlation analysis between the miR expression levels in the cell lines and leukemic MCL and the genomic data of the respective chromosomal loci obtained in the single nucleotide polymorphism array study. A significant positive correlation between increased expression levels and high DNA copy number was found for the miRs located at 8q24 (miR-30d), 11q13 (miR-139), 13q31 (miR-17-3p, miR-17-5p, miR-18, miR-19a, and miR-20a), and 18q21 (miR-122a; Fig. 3; Supplementary Table S6). Interestingly, the relationship between expression levels and copy number changes of the 13q31 miRs was observed even after stringent adjustment for multiple testing (adjusted \( p = 0.042 \); Supplementary Table S6). The single nucleotide polymorphism array analysis detected also regions of partial uniparental disomy in all cell lines and 31% of the primary tumors. These uniparental disomies contained the genomic loci of 24% and 7% of the examined miRs in the cell lines and primary tumors, respectively. However, no significant association between the genetic alteration and the expression levels was observed, suggesting that the uniparental disomies do not deregulate the expression of the studied miRs.

To confirm the influence of copy number changes on miR expression in primary tumors, we expanded the study to the 12 nodal MCL in which a comparative genomic hybridization analysis had been done previously. These cases included 8 tumors with 13q31 genomic alterations (3 amplifications and 5 losses), 3 cases with 8q24 gains/amplifications, 2 tumors with gains of 11q13, and 1 case with 18q21 gain. A significant correlation was observed between the 13q31 copy number changes and the expression levels of the studied mature miRs of the \textit{miR-17-92} cluster (Fig. 4; Supplementary Table S7). No other significant correlations were observed in this set of tumors.

\textbf{MYC and 13q31 miR expression.} Experimental studies have shown that the \textit{miR-17-92} cluster may cooperate with \textit{MYC} in the development and progression of tumors (32). To determine whether the overexpression of these genes could influence the outcome of MCL patients, we analyzed the survival effect of the concomitant overexpression of \textit{MYC} and miR-17-5p and/or miR-20a miRs in 50 patients with nodal MCL. Concomitant high levels of \textit{MYC} and miR-17-5p or MYC and miR-20a were found in 8 patients who noticeably had a significant shorter overall survival (median overall survival, 17 months) than the 17 patients with high expression of \textit{MYC} or miR-17-5p/miR-20a alone (median overall survival, 48 months) and the remaining 25 patients with low expression of all these genes (median overall survival, 69 months; \( p = 0.025 \), log-rank test; Fig. 5). The mean Ki-67 proliferation index in these three groups was 45.2 ± 30, 38.9 ± 23.7, and 29.8 ± 15.6, respectively. Although the proliferation tended to be higher in the tumors with higher levels of \textit{MYC} and miR-17-5p/miR-20a, the differences were not statistically significant (\( p = 0.681 \), Kruskal-Wallis test).

\section*{Discussion}

In this study, we have examined the expression profile of 86 selected miRs in a series of leukemic and nodal MCL. The comparison of leukemic MCL miR expression profiles with normal \textit{CD5+/CD5} B cells showed that the tumors had a profile different from both controls and included the up-regulation of several miRs that were not detected in these normal counterparts. Interestingly, the unsupervised hierarchical clustering analysis of highly purified leukemic MCL cells revealed two distinctive subsets of tumors showing significant differences in the \textit{IgVH} mutational status, proliferation signature, and number of chromosomal alterations. Although the number of tumors in each group is limited, these findings suggested that these clusters may correspond to two subtypes of MCL with marked biological differences. Interestingly, previous studies have recognized a subtype of MCL characterized...
than patients with high expression of only one (fail 5; line A) have a significant shorter overall survival than patients with high expression of only one (n = 17; fail 5; line B) or none (n = 25; fail 10; line C) of these factors (P = 0.025, log-rank test).

This relationship between miR expression and different tumor subsets may be reminiscent of the situation in CLL in which the miR profile distinguishes two tumor subgroups related to the IgVH mutation status (23, 35). miR-15a, miR-195, miR-23b, and miR-142-3p, described previously as IgVH mutation-related miRs in CLL (23, 36), were also included in the present study, but any of them showed a significant difference expression related to the IgVH mutation status in MCL. Interestingly, several up-regulated miRs in the unmutated IgVH subset of MCL tumors have been involved in tumor growth promotion, proliferation, and cell survival regulation. Thus, the highest differentially expressed miR in cluster A, miR-181a, has been associated with more aggressive clinical behavior in CLL and promotes tumor growth of multiple myeloma cells (23, 37, 38). miR-181a also controls negatively the p53 activity by interfering with p300-CBP-associated factor, an upstream regulator of p53 stability (37).

The causes of miR deregulation in tumors are not well known but may include gene dosage alterations or rearrangements close to miR loci (9, 39–41). In this study, we investigated a series of miRs mapped to chromosomal regions frequently altered in MCL (14). We found a significant correlation of the corresponding mature miR levels with the gene dosage alterations for several miRs located at 8q24 (miR-30d), 11q13 (miR-139), 13q31 (miR-17-3p, miR-17-5p, miR-18, miR-19a, and miR-20a), and 18q21 (miR-122a). Interestingly, this relationship for the mature miRs of the miR-17-92 cluster has been shown previously and may involve several mechanisms including cooperation with MYC inducing a decreasing in apoptosis (32, 44–46). On the other hand, MYC overexpression has been recognized as a prognostic factor in MCL (25). To determine the potential cooperation of these two factors, we studied MYC and miR-17-5p/miR-20a expression in 50 nodal MCL. Our results showed that tumors with the concomitant highest levels of MYC and miR-17-5p/miR-20a had significant shorter overall survival than cases with high expression of only one or none of these factors. These results suggest that the cooperation in tumor progression observed in experimental studies may also occur in human MCL.

In this study, we observed marked differences in the expression levels of several miRs between leukemic and nodal MCL. Although nodal MCL were selected by high tumor content, these differences may be due in part to contamination with other nonneoplastic cell types. Nevertheless, miR-337 expression was not detected in any leukemic MCL or nodal MCL in spite of being detected in the pool of reactive nodal tissues, suggesting that the degree of cell contamination of the nodal MCL was not enough to allow the detection of this miR. To determine whether these differences could be due to the different topographic location of the tumor, we were able to purify tumor cells from simultaneous peripheral blood and lymph node sample in two patients. Interestingly, we detected 11 miRs that had a marked significant overexpression in the nodal sample compared with the leukemic cells in both cases, suggesting that they may be modulated by influences of the tissue microenvironment. The idea that miR expression may be regulated in different cell compartments is also supported by a recent study of miR expression in CLL in which the expression of BIC/pri-miR-155 was higher in cells of the proliferation growth centers, whereas miR-150 was mainly expressed by surrounding resting CLL cells (47).
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In summary, our results show that the miR expression profile in MCL is deregulated in comparison with normal lymphoid cells and distinguishes two subgroups of tumors that differ in the mutational status of the IgVH genes, genomic complexity and proliferation. The deregulation of some miRs is related to gene copy number changes, whereas others seem to be modulated by microenvironment influences. The concomitant high expression of MYC and miR-17-5p/miR-20a from the miR-17-22 cluster seems to be associated with tumors with a more aggressive behavior.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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