microRNA Expression Profiles Identify Subtypes of Mantle Cell Lymphoma with Different Clinicobiological Characteristics

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Abstract

Purpose: microRNAs (miRNA) are posttranscriptional gene regulators that may be useful as diagnostic and/or prognostic biomarkers. We aim to study the expression profiles of a high number of miRNAs and their relationship with clinicopathologic and biologic relevant features in leukemic mantle cell lymphomas (MCL).

Experimental Design: Expression profiling of 664 miRNAs was investigated using a high-throughput quantitative real-time PCR platform in 30 leukemic MCLs. Statistical and bioinformatic analyses were conducted to define miRNAs associated with different clinicopathologic parameters. Gene expression profiling was investigated by microarrays in 16 matching cases to study the potential genes and pathways targeted by selected miRNAs. The prognostic value of miR-34a was investigated in 2 independent series of 29 leukemic and 50 nodal MCLs.

Results: Robust consensus clustering defined 2 main MCL subgroups with significant differences in the immunoglobulin (IGHV) mutational status, SOX11 expression, genomic complexity, and nodal clinical presentation. Supervised analyses of IGHV and SOX11 categories identified 17 and 22 miRNAs differentially expressed, respectively. Enriched targets of these miRNAs corresponded to relevant pathways in MCL pathogenesis such as DNA stress response, CD40 signaling, and chromatin modification. In addition, we found 7 miRNAs showing prognostic significance independently of IGHV status and SOX11 expression. Among them, miR-34a was also associated with poor prognosis in 2 independent series of leukemic and nodal MCL, and in cooperation with high expression of the MYC oncogene.

Conclusion: We have identified miRNAs and target pathways related to clinical and biologic variants of leukemic MCL, and validated miR-34a as a prognostic marker in MCL. Clin Cancer Res; 19(12); 3121–9. ©2013 AACR.

Introduction

microRNAs (miRNA) are noncoding small RNAs that bind to specific mRNA target transcripts leading to their degradation and/or translational blocking and, consequently, acting as negative regulators of gene expression (1, 2). Posttranscriptional miRNA regulation seems to be focused on gene transcripts involved in physiologic differentiation, proliferation, and apoptosis processes (3, 4).

Concordantly, miRNA expression deregulation has also been described in many types of neoplasm and has proven to be useful as biomarkers for diagnosis (5, 6). Some cancer-related miRNAs are also causally involved in oncogenesis due to their impact in the deregulation of oncogenes and tumor suppressor genes, and may have prognostic significance as has been previously shown in certain lymphoid neoplasms (7). Mantle cell lymphoma (MCL) is considered an aggressive entity genetically characterized by the t(11;14)(q13;q32) translocation resulting in the overexpression of the CCND1 gene (8). In addition to this primary genetic alteration, most MCL carry a high number of recurrent secondary chromosomal aberrations that contribute to the additional oncogenic events necessary for the progression of the disease (9). These secondary alterations result in alterations of coding genes involved in cell-cycle regulation, DNA damage response, and survival signaling pathways among other oncogenic relevant mechanisms (10). Several genes of these pathways may also be deregulated posttranscriptionally by different miRNAs in MCL, and concordantly, their altered expression has been related to their biologic and prognostic
Mantle cell lymphoma (MCL) is an uncommon lymphoid neoplasm with heterogeneous biologic and clinical manifestations. microRNA (miRNA) expression could be used to improve the understanding of the diversity of this lymphoma. In particular, peripheral blood presentation could be the unique manifestation of this lymphoma in some patients, but present knowledge on miRNA expression profiles is mainly restricted to nodal samples. Therefore, our study focuses on defining which miRNAs may be biomarkers of leukemic MCL subtypes related to relevant biologic parameters such as immunoglobulin mutational status, SOX11 expression, or degree of genomic alterations, and clinicopathologic aspects such as nodal presentation or prognosis. We also investigate the molecular pathways in which these miRNAs may be involved. Validation of the prognostic value of miR-34a expression is studied in independent series of leukemic and nodal MCL.

Translational Relevance

Mantle cell lymphoma (MCL) is an uncommon lymphoid neoplasm with heterogeneous biologic and clinical manifestations. microRNA (miRNA) expression could be used to improve the understanding of the diversity of this lymphoma. In particular, peripheral blood presentation could be the unique manifestation of this lymphoma in some patients, but present knowledge on miRNA expression profiles is mainly restricted to nodal samples. Therefore, our study focuses on defining which miRNAs may be biomarkers of leukemic MCL subtypes related to relevant biologic parameters such as immunoglobulin mutational status, SOX11 expression, or degree of genomic alterations, and clinicopathologic aspects such as nodal presentation or prognosis. We also investigate the molecular pathways in which these miRNAs may be involved. Validation of the prognostic value of miR-34a expression is studied in independent series of leukemic and nodal MCL.

Gene expression and genomic alterations

The gene expression profiling of 16 cases with additional RNA available was investigated for further correlation analysis with the miRNA expression data. The gene expression was studied using hybridization to Affymetrix HG133Plus 2.0 (Affymetrix) microarrays as previously described (13), and normalized and processed as detailed in Supplementary Materials and Methods. The raw data have been deposited in the Gene Expression Omnibus database (GSE36000). In addition, SOX11 mRNA expression measured by quantitative real-time PCR (qRT-PCR) and the genomic profile studied by single-nucleotide polymorphism arrays were available in all and 23 cases, respectively, from a previous study (16). Expression quantification of additional genes is detailed in Supplementary Materials and Methods.

Statistical and bioinformatic analyses

The miRNA expression data were analyzed with the consensus clustering method as implemented in the Consensus Cluster Plus package from Bioconductor (20). Consensus clustering is a previously validated approach to improve the classical hierarchical clustering methods to obtain reliability in the number of subgroups present in the dataset and the group memberships of cases (ref. 21; see Supplementary Materials and Methods for details). To define the miRNAs differentially expressed in relation to IGHV status and SOX11 expression in the whole series and in relation to the clusters identified in the previous analysis, we used a supervised method based on the empirical Bayes moderated t-statistic from the Bioconductor package limma (http://cran.r-project.org). The potential genes and pathways regulated by the miRNAs found differentially expressed as well as the associations between miRNA expression and clinical features were analyzed using different bioinformatic and statistical methods detailed in Supplementary Materials and Methods.
Results

miRNA expression profiling and relationship to IGHV mutational status and SOX11 expression

A total of 583 miRNAs of the 664 examined showed detectable expression by qRT-PCR in at least one sample of the initial leukemic MCL series. We selected a subset of 286 miRNAs showing an expression variation more than 50th percentile of the variation degree among samples and with detectable expression in more than 25% of cases (Fig. 1A and Supplementary Table S2). An unsupervised consensus clustering analysis was conducted trying different partitions (from $k = 2$ to $k = 6$). The empirical cumulative distribution function showed that approximate maximum stability of partitioning was reached at $k = 3$, indicating that the optimal number of robust clusters in this data set was 3 (Supplementary Fig. S1A), as can also be observed in the consensus matrix plots (Supplementary Fig. S1B). Only 2 samples (MCL#29 and MCL#30; Supplementary Table S3A) showed less robust cluster membership even at $k = 3$ (Supplementary Fig. S1C), and therefore were excluded to define the representative samples for each of the core clusters (named as A, B, and C) used in subsequent analyses (Fig. 1B).

The clinical and biologic characteristics of the patients in the 3 core clusters are summarized in Table 1. Patients in clusters A and B had significant differences in several parameters, whereas patients in cluster C had intermediate features between them. Cluster A included patients with nodal presentation (100%) and tumors with unmutated IGHV (86%), high number of chromosomal alterations (mean 8.3; range 2–19), and high levels of SOX11 expression (mean 25.65; range 11.3–42.1 relative units; RU).

![Figure 1](https://www.aacrjournals.org/doi/figure/10.1158/1078-0432.CCR-12-3077)
Pathway enrichment analysis of predicted miRNA targets differentially expressed among subgroups of leukemic MCL

To determine the potential genes and pathways modulated by the relevant miRNAs differentially expressed in the MCL with mutated and unmutated IGHV, we compared the expression of these miRNAs with their putative target-coding genes included in high-throughput gene expression profiling arrays conducted in a common series of 16 MCL, and using the tools described in the Materials and Methods section. The top miRNAs differentially expressed in this set of 16 cases were representative of the whole series (Supplementary Table S6A). The obtained ranked target genes according to their inverse expression correlation with these miRNAs are shown in the Supplementary Table S7A. We conducted a gene set enrichment analysis analysis on these preranked genes using several gene sets and functional pathways involved in B-cell lymphomagenesis (see Supplementary Materials and Methods). This analysis showed that the target genes of the differentially expressed miRNAs were enriched in several pathways including “response to DNA stress” (GO:0006950; involving 19 genes) as the most significant (Supplementary Table S7B and Supplementary Fig. S3). Noticeably, among the genes in this pathway was ATM, the inactivation of which is associated with increased chromosomal instability in MCL tumors (22). ATM is a validated target of miR-181a/c (23, 24) and in our series was downregulated in U-MCL compared with M-MCL.
miR-455-5p expression and clinical outcome

In this series, MCL with unmutated IGHV or high SOX11 expression levels had a worse prognosis than tumors with mutated IGHV or with low or negative SOX11 expression ($P = 0.008$ and $P = 0.039$, respectively; Supplementary Fig. S5). To identify miRNAs that could add prognostic value to these parameters, we used Cox regression with these 2 variables and each individual miRNA. A total of 7 miRNAs showed a significant impact on overall survival (OS) with shorter survival associated with high expression in 2, and with low expression in 5 of them (positive and negative Cox coefficients, respectively; Table 2).

As proliferation is one of the most important prognostic factors in MCL, we analyzed the possible relationship between miRNA expression and gene expression proliferation signature (27), in the 16 cases with gene expression profiling analysis (Supplementary Table S3B). We found 36 miRNAs significantly correlated with this signature, some of them with known targets of cell-cycle regulation (Supplementary Tables S9 and S10). None of the 7 miRNAs that added prognostic value to SOX11 expression or IGHV mutational status were included among them.

Among the miRNAs with most prognostic significant impact ($P < 0.01$), miR-34a raised our interest because of its association with the control of several relevant genes of MCL pathogenesis, such as cyclin-dependent kinase (CDK)-4, CDK6, MYC, and CCND1, among others (28). The survival impact of this miRNA in MCL has not been previously investigated. Interestingly, we categorized miR-34a expression into high and low groups among the 30 leukemic MCL cases with high miR-34a expression included in the same subgroups by modulating this epigenetic regulator of gene expression.

### Table 2. Signficant results from the survival model including miRNA expression, IGHV mutational status, and SOX11 expression

<table>
<thead>
<tr>
<th>miRNA</th>
<th>$P$</th>
<th>Cox coefficients</th>
</tr>
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<tbody>
<tr>
<td>hsa-miR-190</td>
<td>0.002</td>
<td>-</td>
</tr>
<tr>
<td>hsa-miR-565</td>
<td>0.004</td>
<td>-</td>
</tr>
<tr>
<td>hsa-miR-34a</td>
<td>0.005</td>
<td>0.007</td>
</tr>
<tr>
<td>hsa-miR-34a*</td>
<td>0.006</td>
<td>0.008</td>
</tr>
<tr>
<td>hsa-miR-149</td>
<td>0.007</td>
<td>-</td>
</tr>
<tr>
<td>hsa-miR-649</td>
<td>0.008</td>
<td>0.003</td>
</tr>
<tr>
<td>hsa-miR-483-5p</td>
<td>0.009</td>
<td>0.006</td>
</tr>
</tbody>
</table>
observed inside the M-MCL ($P = 0.067$) and SOX11-negative MCL subgroups ($P = 0.085$; Supplementary Fig. S6). Finally, the association of miR-34a expression and survival was validated in an independent series of 29 leukemic MCL. In this series, the miR-34a low levels were also associated with worse prognosis (median OS: 25 months) compared with patients with higher miR-34a levels (median OS: 49 months; $P = 0.008$).

One of the shown miR-34a targets is MYC, the overexpression of which has been associated with poor outcome in MCL (28–30). Moreover, miR-34a has been previously described to affect the MYC transcriptional activity in solid tumors (31–33). To determine whether the poor prognosis of miR-34a downregulation and MYC overexpression are mutually related in MCL, we investigated these transcripts in 38 leukemic MCLs with additional RNA available and in an independent series of 50 nodal MCLs. In both groups of cases, the results showed that low expression of miR-34a was associated with a significantly shorter OS ($P = 0.001$) in patients who also had high MYC expression (median OS for leukemic and nodal cases: 9 and 21 months, respectively) in comparison with those patients with only one of these factors (median OS for leukemic and nodal: 58 and 49 months, respectively) or none of them (median OS for leukemic and nodal: not reached and 64 months, respectively; Fig. 3). These results support that downregulation of miR-34a and MYC overexpression cooperates in the aggressiveness of these tumors.

Discussion

In the present study, we have investigated the expression of a high number of miRNAs (664) in a series of leukemic MCL and have identified 3 robust subgroups of tumors with a different miRNA expression profile. Two of these clusters (A and B) included tumors with significant differences in their clinical and biologic characteristics, whereas the third cluster (cluster C) was composed of tumors with intermediate features. The clinical and biologic characteristics of the A and B subgroups are concordant with the 2 molecular subtypes of MCL that we have recently identified on the basis of the $IGHV$ mutational status and SOX11 expression (14). Tumors in miRNA cluster A were characterized by unmutated $IGHV$, high expression of SOX11, complex karyotypes, and a
nodal clinical presentation, whereas tumors in cluster B had mutated IGHV, negative or very low SOX11 expression, low genomic complexity, and non-nodal presentation. The differences in the miRNA expression profile between these 2 groups would support the hypothesis that they may correspond to different biologic subtypes of MCL.

Recently, Iqbal and colleagues have described 3 miRNA clusters in lymph node MCL based on the expression profiling of 377 miRNAs, all included in our study (18). These 3 clusters are different from the ones identify in our work. One of these clusters (A) was defined by miRNAs involved in control of cell proliferation and apoptosis, whereas the other 2 (B and C) included miRNAs related to growth inhibitory functions depending on the accompanying stroma compartment. Concordant with the different biologic significance of the clusters in both studies, we only observed 4 overlapping miRNAs between the clusters in the 2 studies, namely miR-363 that is upregulated in both clusters A, miR-1 that is upregulated in both clusters C, and 2 miRNAs associated with stroma inhibitory functions in Iqbal’s cluster B that were upregulated in our cluster C (miR-539 and miR-485-3p). Moreover, miRNAs found significantly related to the gene expression proliferation signature (27) are poorly overlapped between our leukemic samples and those described in Iqbal and colleagues (only miR-199a-5p and miR-485-3p). These differences may be due to, in part, the different subtypes of MCL and type of tumor samples (leukemic vs. nodal) investigated in the 2 works that reflect the biologic heterogeneity of MCL and the importance of the microenvironment in modulating the miRNA expression. In this sense, the differences in miRNA expression between leu-

A supervised analysis of the miRNAs differentially expressed between MCL with mutated and unmutated IGHV in our study showed 17 miRNAs overexpressed in U-MCL compared with M-MCL with the top 3 being miR-708 and miR-455-5p/3p. A similar supervised analysis in the tumors according to SOX11 expression showed that the miRNAs differentially expressed in these 2 subgroups virtually overlapped with those found related to the IGHV mutational status, reflecting the close relationship previously described between these 2 parameters (14). The comparison of the miRNA expression levels and their paired gene expression profiles followed by a pathway analysis identified that the putative miRNA targets of the differentially expressed miRNAs between U-MCL and M-MCL mainly regulated the "response to stress," CD40 signaling pathway, and genes related to epigenetic modifications.

ATM was one of the genes included in the "response to stress" pathway. This gene is a key regulator of the DNA damage response pathway (35) whose inactivation by deletion and/or mutation has been described in MCL in relation to increased chromosomal instability (22). In our leukemic MCL, ATM mRNA showed a highly inverse correlation with their predicted regulators miR-181a/c and a pronounced downregulation in U-MCL compared with M-MCL. As ATM has been validated experimentally as a target of miR-181 family with functional impact (23), and our results show that ATM downregulation was associated to high levels of miR-181c even in absence of ATM locus deletions. These findings suggest that miR-181 upregulation may be an alternative mechanism to 11q deletions to downregulate ATM.

CD40 signaling pathway (25) was also identified as a significant target of the miRNAs differentially expressed between U-MCL and M-MCL. Interestingly, this pathway was previously described to be targeted by miR-377, miR-378, and miR-204 in nodal MCL samples (11). In our analysis, some of this pathway were identified as targets inversely correlated with miRNAs highly expressed in U-MCL compared with M-MCL. This finding would seem consistent with the activation of the CD40 signaling pathway in memory cells compared with cells in the mantle zone area (25).

miR-708 and miR-455-5p/3p were among the highest miRNAs differentially expressed between IGHV/U-MCL and M-MCL and SOX11-positive and negative MCL. The target genes of these miRNAs are not well known, but recent studies have highlighted their potential role in hematologic malignancies (36, 37). In our work, the pathway analysis of the inversely correlated predicted targets of these miRNAs identified genes coding for histone-modifying proteins. Among them, MLL2 was validated by qRT-PCR as inversely expressed to miR-455-5p. Interestingly, this gene was found mutated and inactivated in a high proportion of follicular and diffuse large B-cell lymphomas (38). Further studies are required to confirm the relevance of these mechanisms in MCL.

The understanding of the heterogeneity in the clinical presentation and evolution of MCL requires the identification of biologic parameters related to the different evolution of the patients. Previous studies have shown the value of IGHV mutational status and SOX11 expression in defining subgroups of patients with MCL with different clinical evolution (13–17, 39). We have now found 7 miRNAs, the expression levels of which had a significant survival impact independent of the IGHV and SOX11 alterations, and were not correlated with the gene expression proliferation signature previously described as prognostic factor in MCL (27). None of these miRNAs had been previously found related with prognosis in MCL (11, 18), but these studies had evaluated only nodal MCL emphasizing the possible biologic and clinical differences of our cohort of patients. Among these miRNAs, miR-34a targets several relevant genes in MCL pathogenesis such as CDK4, CDK6, MYC, and CCND1 among others (28). We observed in 2 independent series of leukemic MCL that its decreased expression was associated with a poor survival, concordantly with its described role as tumor suppressor (28). Although the number of patients is limited, this prognostic value was also observed in the subgroups of U-MCL and MCL with...
high SOX11 expression. miR-34a had been previously described as a negative regulator of MYC-dependent transcription (31–33), and MYC overexpression has been associated with poor prognosis in MCL (29, 30). Therefore, we studied the expression of miR-34a and MYC for a possible cooperation between them in independent series of nodal and leukemic MCL. Our results showed that tumors with the concomitant highest levels of MYC and simultaneously the lowest of miR-34a had significantly shorter OS than cases with high expression of only one or none of these factors. These results would support the cooperation of this miRNA and one of its targets in tumor progression and suggest that the prognostic value of some miRNAs could be enhanced by the simultaneous study of their targets.

In summary, we have shown that miRNA expression profiles identify 2 main subgroups of leukemic MCL with clear differences in IGHV mutational status, SOX11 expression, nodal presentation, and number of chromosomal alterations. The differentially expressed miRNAs seem to regulate different pathways relevant for MCL pathogenesis including DNA damage response in U-MCL. On the other hand, a subset of miRNAs had prognostic implications independently of IGHV mutational status and SOX11 expression. Among them, miR-34a downregulation was validated in 2 independent series of leukemic and nodal tumors and associated with poor prognosis in cooperation with high MYC expression. All these results highlight the biologic and clinical significance of miRNA expression in MCL, supporting their involvement in the pathogenesis and prognosis of these tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: A. Rosenwald, E. Campo, L. Hernández Development of methodology: A. Navarro, M. Prieto, L. Hernández Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Navarro, C. Royo, E. Hartmann, I. Salaverria, S. Beá, A. Rosenwald, G. Ott, A. Wiens ter, W.H. Wilson, I. Hernández Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Navarro, G. Clot, M.C. Veglan t, V. Amador, E. Hartmann, S. Beá, J.I. Martin-Subero, A. Rosenwald, L. Hernández Writing, review, and/or revision of the manuscript: A. Navarro, C. Royo, M.C. Veglan t, V. Amador, E. Hartmann, S. Beá, J.I. Martin-Subero, A. Rosenwald, G. Ott, W.H. Wilson, E. Campo, I. Hernández Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Navarro, M. Prieto, C. Royo, I. Salaverria, S. Beá, A. Rosenwald, G. Ott
Study supervision: E. Campo, L. Hernández

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