microRNA-29c and microRNA-223 down-regulation has in vivo significance in chronic lymphocytic leukemia and improves disease risk stratification

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Aberrant expression of miRNAs has been recently associated with chronic lymphocytic leukemia (CLL) outcome. Although disease evolution can be predicted by several prognostic factors, a better outcome individualization in a given patient is of utmost interest. Here, we showed that miR-29c and miR-223 expression levels decreased significantly with progression from Binet stage A to C were significantly lower in poor prognostic subgroups (defined by several prognostic factors) and could significantly predict treatment-free survival (TFS) and overall survival (OS). Furthermore, we developed a quantitative real-time polymerase chain reaction (qPCR) score combining miR-29c, miR-223, ZAP70, and LPL (from 0 to 4 poor prognostic markers) to stratify treatment and death risk in a cohort of 110 patients with a median follow-up of 72 months (range, 2-312). Patients with a score of 0/4, 1/4, 2/4, 3/4, and 4/4 had a median TFS of greater than 312, of 129, 80, 36, and 19 months. The online version of this article contains a data supplement.

Introduction

In the last few years, a new class of RNAs, called miRNAs (or miR), has been described as important players in posttranscriptional gene regulation. Indeed, these small noncoding RNAs of approximately 22 nucleotides, which are cleaved from a 60-110 nucleotide precursor, could regulate gene expression by hybridizing with a target mRNA sequence (with total or partial complementation) resulting in translational repression or degradation. Their role has been underlined in many biologic processes, such as cell proliferation and cell death, not only during development, but also during oncogenesis. Deregulation of the normal microRNA expression profile could play a critical role in human disease, particularly in solid tumors, but also in hematologic malignancies. Recent reports have highlighted the importance of microRNAs in chronic lymphocytic leukemia (CLL) biology and prognosis. This type of leukemia is the most common form in Western countries and up to now remains incurable and displays 2 kinds of clinical evolution; namely some patients will rapidly progress and die, while others remain asymptomatic for many years. In last decades, several prognostic factors have been proposed to better assess CLL outcome, including classical staging system, such as that proposed by Rai and Binet; serum markers, such as β2-microglobulin (β2-M) or soluble CD23 (sCD23); a proliferation marker, such as lymphocyte doubling time (LDT); cytogenetic markers; surface markers, such as CD38 molecule (CD38); immunoglobulin heavy chain (IgVH) mutational status and its surrogate markers, ζ-associated protein-70 (ZAP70) and lipoprotein lipase (LPL); and finally, a microRNA signature. Based on microarray studies published by Calin et al in 2005, a signature of 13 microRNAs (differentially expressed between IgVH mutated/ZAP70+ and IgVH unmutated/ZAP70− patients) is able to predict the time from diagnosis to initial treatment in CLL patients. In 2007, Fulci et al found 3 microRNAs differentially expressed between IgVH mutated and unmutated cases using a quantitative technology based on microRNA cloning and quantitative real-time polymerase chain reaction (qPCR) of mature microRNAs. Interestingly, these 2 studies, which are based on different technologies, share only 2 microRNAs, miR-29c and miR-223. Therefore, we investigated the association of these 2 microRNAs, measured by qPCR, with other classical prognostic factors and their ability to predict individual treatment free-survival (TFS) in addition to overall survival (OS). We applied this score to Binet stage A patients.
the additional power of this qPCR score in poor or good prognosis subgroups defined by other classical prognostic markers.

## Methods

### Patients, sample collection, and RNA extraction

This study, which was approved by the Bordet Institute Ethics Committee, was based on peripheral blood samples collected (from June 1977 to June 2008) at diagnosis from CLL patients with informed consent obtained in accordance with the Declaration of Helsinki. The 68 male and 42 female patients had a typical CD19+CD5+CD23- phenotype and blood leukocyte counts between 10^5 and 250 × 10^5 cells/μL. The median age at diagnosis was 65 years (range, 37-89 years). The median follow-up duration was 72 months (range, 2-312 months). Table 1 summarizes other patient features. Complete prognostic data were not available for all patients due to a lack of biologic material (Table 1). Binet stages, ZAP70, LPL, and TFS and OS were calculated from the time of diagnosis until date of first treatment and date of death, respectively. Peripheral blood mononuclear cells were isolated by density-gradient centrifugation over Linfosep (Biomedics, Madrid, Spain). B cells were purified with a CD19+ magnetic-bead system (Miltenyi Biotec, Bergish Gladbach, Germany), according to the manufacturer’s instructions. Mean B-cell purity was greater than 99% as measured by flow cytometry (FC). Total RNA was extracted from purified CD19+ cells in a single step using TriPure Isolation Reagent (Roche Applied Science, Vilvoorde, Belgium).
Assessment of prognostic factors

We measured ZAP70 and LPL by qPCR, CD38 expression by FC, sCD23, and β2-M by enzyme-linked immunosorbent assay (ELISA) immunoassay, and we performed IgVH gene mutational analysis as previously described.14-17 LDT was assessed according to Montserrat et al.18 Classical cytogenetics by standard karyotype analysis were done for 81 patients. Additional interphase fluorescent in situ hybridization (FISH) was performed to screen for most common aberrations in 47 of these patients using Chromoprobe Multiprobe-CLL System (Cytocell; Amplitexc, Compiegne, France). Patients were classified according to Döhner’s recommendations.19

Results

miR-29c and miR-223 down-regulation is associated with higher tumor burden, disease aggressiveness, and poor prognostic factors

The median TFS of this cohort was 57 months (range, 2-226 months), while the median OS was 237 months (range, 2-312 months). Expression of miR-29c and 223, as determined by qPCR on purified CD19+ cells, was significantly associated with markers of tumor burden (Binet stage, LDT, sCD23, and β2-M; Table 1 and Figure 1). The level of these 2 microRNAs decreased significantly with progression from Binet stage A to C (miR-29c, P = .001; miR-223, P = .018). Moreover, patients with a LDT less than 1 year or who were positive for sCD23 or β2-M expressed significantly lower levels of miR-29c and miR-223. Similar results were obtained for several classical prognostic markers commonly used to prognosticate CLL evolution (IgVH mutational status, ZAP70, LPL, and CD38 expression, cytogenetic abnormalities; Table 1 and Figure 1). All these factors were proven to be significant predictors of TFS and OS in our CLL cohort (Figure S3).

miR-29c and miR-223 are powerful prognostic factors

A requirement for treatment and patient death are clearly associated with miR-29c and miR-223 expression (Table 1). Using ROC curve analysis to maximize the concordance with the IgVH mutational status, we defined the miR-29c and miR-223 cutoffs. ROC curves are provided in Figure S4. Although these cutoffs were not optimized for TFS/OS prediction, they were sufficient to observe the following statistical differences: among our 110 patients, 61 (55%) and 37 (34%) were deemed positive, and 49 (45%) and 73 (66%) were deemed negative, for miR-29c and miR-223, respectively. Using these cutoffs, miR-29c and miR-223 were predictors of TFS and OS. The median TFS of miR-29c+ and miR-223+ patient subgroups were 101 and 107 months, respectively, while both values were only 24 months for the negative subgroups (miR-29c, P = .002; miR-223, P < .001). OS was also statistically associated in the log-rank tests with miR-29c (P = .023) and miR-223 (P < .001). The 2 positive subgroups had a median OS greater than 312 months, while this value fell to 183 months (miR-29c) and 137 months (miR-223) for the negative subgroups (Figure 2). We also used Cox regression to evaluate the impact of these dichotomized data (using Table 1 cutoffs) on TFS and OS. miR-29c and miR-223 were both univariate predictors of TFS and OS; miR-29c negative patients had a 2.3-fold higher risk (P = .002) to be treated and a 3.8-fold higher risk to die (P = .035). For miR-223 negative patients, these values were 2.7 (P < .001) and 4.9 (P = .002), for TFS and OS, respectively (Figure S5). In a multivariate analysis that included the classical prognostic markers currently used (ZAP70, LPL, CD38, mutational status) and the 2 microRNAs (miR-29c and miR-223), only ZAP70 and miR-29c were significant predictors of TFS (n = 99; ZAP70, P = .006; miR-29c, P = .036). OS prediction by multivariate analysis was not informative due to the limited number of events. However, ZAP70 and miR-29c displayed the lowest P value for OS prediction (Figure S5).

miR-29c and miR-223 improve risk stratification when combined in a qPCR score with ZAP70 and LPL: cross-validated model in a 110-patient cohort

miR-29c and 223 are significant prognostic factors, but they are still not superior to ZAP70, for example. Therefore, to obtain a
refinement in the assessment of TFS and OS in CLL patients, we combined these 2 microRNAs with 2 other well-validated prognostic factors, ZAP70 and LPL. These markers are easily measurable by qPCR, and thus no other technique, such as flow cytometry or FISH analysis, are needed. This qPCR score varied from 0 to 4, according to the number of unfavorable factors (ie, low expression of miR-29c or 223 and high expression of ZAP70 or LPL) and was applied to a 110-patient cohort with a median follow-up of 72 months (range, 2-312). The presence of a poor prognostic marker corresponds to an increase of 1 unit in the final qPCR score. We gave thus the same weight to all 4 factors. According to this qPCR score, the patients were thus stratified into 5 groups (0/4, 1/4, 2/4, 3/4, and 4/4), and the HR of the 5 groups (named as 0, 0.25, 0.5, 0.75, and 1) was calculated by univariate Cox analysis. In other words, HR represents the hazard ratio between group 0/4 and 4/4, taking into account the intermediate groups, such that 0/4/H_1, 1/4/H_2, 2/4/H_3, 3/4/H_4.

Patients with a score of 0/4, 1/4, 2/4, 3/4, and 4/4 had a median TFS of greater than 312, of 129, 80, 36, and 19 months.

Figure 1. Correlation of miR-29c and miR-223 expression with other prognostic factors. The mean miR-29c and miR-223 levels measured by qPCR were plotted with their SEM according to Binet stages (A,I), untreated/treated patients (B,M), alive/dead patients (C,N), LDT (D,O), sCD23 (E,P), IgVH mutational status (G,R), ZAP70 (H,S), LPL (I,T), CD38 (J,U), cytogenetic abnormalities (K,V). miR values are expressed in fold of target miR expression in the calibrator cell line (Namalwa). Statistical differences were assessed using the Kruskal-Wallis test for Binet stages, and the Mann-Whitney nonparametric test was used for other variables. Statistical details can be found in Table 1.
respectively (HR = 17.00, \(P < .001\)). For OS prediction, we divided our patient cohort only in 3 subgroups due to the limited number of events (n = 20): patients with a score of 0-1/4, 2-3/4, and 4/4 had a median OS of greater than 312, of 183, and 106 months, respectively (HR = 13.69, \(P < .001\); Figure 3A-D). Concerned by the risk of overfitting, we performed a prevalidation study\(^2\) and observed a significant HR for the prediction of TFS (HR = 10.88, \(P < .001\)) and OS (HR = 8.12, \(P = .002\)) reinforcing our previous results (Figure S2). Within the 10-fold cross-validation, the computation of this score was stable (concordance of 82%) as reported in Figure S2. Finally, in Binet stage A patients (n = 77), this score remained relevant and significant for TFS and OS prediction (HR = 18.56, \(P < .001\) and HR = 12.5, \(P = .007\), respectively; Figure 3E-H). All the cross-comparisons of groups can be found in Figure S6.

**Figure 2.** miR-29c and miR-223 can predict TFS and OS. The representative TFS and OS curves for miR-29c (A,B) and for miR-223 (C,D). miR-29c and miR-223 were measured by qPCR, and cut-offs were optimized to maximize IgVH mutational concordance (see Table 1) using ROC curve analyses. Statistical differences between curves were calculated using the log-rank test.

**Figure 3.** qPCR score combining miR-29c, miR-223, ZAP70, and LPL stratifies CLL patients in terms of TFS and OS. TFS, according to our qPCR score, was plotted with Kaplan-Meier methods for all Binet stages (A) and only Binet stage A (E). Tables B and F provide the number of patients and treatment events per subgroup. OS, according to our qPCR score, was plotted with Kaplan-Meier methods for all Binet stages (C) and only Binet stage A (G). Tables D and H provide the number of patients and treatment events per subgroup. The HR was calculated with univariate Cox regression.
Additional impact of our qPCR score on other prognostic factors

To evaluate the impact of our qPCR score, we applied this score to good and poor prognostic subgroups defined by the 11 prognostic factors stated in Table 1. We thus obtained 22 subgroups (11 of good and 11 of poor prognosis). We divided each of these subgroups according to the qPCR score, and we calculated the median TFS and the median OS for all the subgroups generated. The univariate Cox HR was calculated as described earlier. Interestingly, we observed that this qPCR score allowed identification of patients with a higher median TFS or OS in poor prognostic subgroups and of patients with lower TFS or OS in good prognosis subgroups (Figure S7). For instance, in unmutated IgVH patients with a global median TFS of 24 months, our qPCR score was able to identify groups with a median TFS of greater than 312 (score 0/4), of 60.9 (score 1/4), 24 and 18.6 months (score 3/4 and 4/4, respectively; \( P < .0001 \)). Furthermore, in patients with a cytogenetically favorable prognosis (with a median TFS of 157 months), we found patient groups with a TFS of greater than 312 (score 0/4 and 1/4), of 157 (score 2/4), 75.7 (score 3/4), and 21.4 months (score 4/4; \( P = .002 \)). Independently from the chosen subgroups, all patients with any poor or good prognostic factor, but with a score of 0-1/4, had a median OS greater than 312 months. All comparisons are reported in the table of Figure S7. It should be noted that all HRs for groups with a score of 0-1/4 were not significant, probably because of the limited number of patients and the absence of the 5 qPCR-score groups per prognostic subgroup. Therefore, we analyzed the impact of our qPCR score more globally; each TFS and OS for all the poor prognosis subgroups and all the good prognosis subgroups were plotted together (Figure 4). A clear trend of median TFS and OS decrease is observed, and a Kruskal-Wallis test demonstrated that this qPCR score could find at least 2 distinct subgroups with significantly different TFS or OS. The univariate Cox HR of our qPCR score was clearly significant for the prediction of TFS (HR = 12.88, \( P < .0001 \)) and OS (HR = 29.98, \( P = .001 \)). Figure 5 forest plot also shows qPCR strength compared with the analyzed prognostic markers. When we introduced the classical factors currently used in CLL prognosis (ZAP70, LPL, CD38, IgVH mutational status) in a multivariate Cox regression analysis with our qPCR score (n = 99), qPCR score was the sole independent predictor for TFS (\( P = .004 \)). Analysis of OS prediction by multivariate analysis was not informative probably due to the limited number of events (n = 16). However, ZAP70 and our qPCR score displayed the lowest \( P \) value with the highest HR (Figure S8).

miR-29c down-regulation is associated with higher level of Tcl1 oncogene

Tcl1 has been previously described as a target of miR-29 family.\(^{21}\) To assess the correlation between Tcl1 and miR-29c expression, we quantified Tcl1 by Western blot analysis on CLL samples with different miR-29c status. A representative Western blot analysis for 10 CLL patients was shown in Figure 6A; a majority of patients deemed as miR-29c expressed higher levels of Tcl1 compared with miR-29c\(^{-}\) patients. Protein bands were quantified by 2-D densitometry and normalized using actin band. Tcl1/actin ratios obtained for 20 patients indicated that miR-29c\(^{-}\) patients expressed significantly higher levels of Tcl1 compared with miR-29c\(^{+}\) patients (\( P = .007 \); Figure 6B).

Discussion

The aim of the present study was to evaluate the clinical value of miR-29c and miR-223, previously described in 2 studies,\(^{10,13}\) their relation with other well-established prognostic factors (IgVH mutational status, ZAP70, LPL, CD38, cytogenetic abnormalities), and indicators of tumor burden (Binet stage, sCD23, \( \beta \)2-M, LDT) to refine CLL prognosis. Indeed, managing the treatment course of
miR-29c and miR-223 for CLL Risk Stratification

Cancer, while miR-223 is down-regulated in hepatocellular carcinoma, explained, at least for miR-223, by an epigenetic silencing.

Although many microRNAs have been implicated in regulating cancers, very few of their target genes have been identified. miR-29c regulates the Tcl1 oncogene highly expressed in aggressive CLL. These results were confirmed in this study; we indeed observed that patients with low level of miR-29c expressed high level of Tcl1 oncogene. Although these observations are statistically significant, this association is not absolute probably because Tcl1 expression can be influenced by other regulators such as miR-181. Other studies had also shown that miR-29 family could regulate myeloid cell leukemia sequence 1 (Mcl1), an antiapoptotic Bcl-2 family member close correlated with adverse CLL prognosis. Recently, Sengupta et al also demonstrated that miR-29c could down-regulate extracellular matrix proteins, including multiple collagens and laminins, by targeting the untranslated region (UTR) of their RNA. These findings are particularly relevant in CLL biology, since CLL cell survival is promoted by microenvironment contact. Furthermore, miR-29c reverts aberrant methylation by targeting DNA methyltransferase (DNMT) 3A and 3B, 2 key enzymes frequently up-regulated in lung cancer. Thus, the down-regulation of miR-29 inversely correlates with DNMT expression. All these observations strengthen the relationship between low expression of miR-29c and the poor prognosis observed in CLL patients. A little is known about the role of miR-223 in cancer, but many studies have described its role in normal tissues. However, Wong et al suggested that miR-223 down-regulates the expression of stathmin1 (STMN1), an oncoprotein overexpressed in many cancer types.

We also demonstrated for the first time in a 110-patient cohort with a median follow-up 72 months (range, 2-312) that miR-29c and miR-223 could individually predict TFS and OS. Multivariate analysis showed that miR-29c could also refine a ZAP70 prognosis. Cutoff determination was based on ROC analysis optimizing concordance with the IgVH mutational status, one of the most robust molecular prognostic factors currently used. The choice of a cutoff remains a controversial question. Some investigators determined cutoff by maximizing selected log-rank or simply by choosing a cutoff found in the literature. We and others have preferred to use ROC curve analysis. Globally, all these procedures tend toward the same result; namely to optimize concordance with TFS and OS. We are the first to propose a threshold for miR-29c and miR-223 expression, and thus, it is

CLL patients cannot be planned without taking into account their prognosis.

We showed that the expression of miR-29c and miR-223 was dramatically decreased in poor prognostic patients independently of the prognostic marker classification used. Indeed, Binet stage C patients expressed 5 times less miR-29c and 2.5 times less miR-223 than Binet stage A patients. Similar results were observed for sCD23, β2-M, and LDT, indicating that a lower expression of these 2 microRNAs is clearly associated with higher tumor burden. Patients requiring treatment also presented a 2.9-fold and a 1.9-fold decrease of miR-29c and miR-223, respectively, compared with untreated patients. Similar results were obtained when we compared alive and dead patients, indicating that the lower expression of these 2 microRNAs is linked to disease aggressiveness. The same relation was found for other prognostic factor classifications (IgVH mutational status, ZAP70, LPL, CD38, and cytogenetic abnormalities). The down-regulation of these 2 microRNAs has been associated with other types of cancer; miR-29c is down-regulated in nasopharyngeal carcinoma and in nonsmall-cell lung cancer, while miR-223 is down-regulated in hepatocellular carcinoma and in acute myeloid leukemia, compared with their normal counterparts. The low level of these microRNAs could be explained, at least for miR-223, by an epigenetic silencing. Although many microRNAs have been implicated in regulating cancers, very few of their target genes have been identified. miR-29c regulates the Tcl1 oncogene highly expressed in aggressive CLL. These results were confirmed in this study; we indeed observed that patients with low level of miR-29c expressed high level of Tcl1 oncogene. Although these observations are statistically significant, this association is not absolute probably because Tcl1 expression can be influenced by other regulators such as miR-181. Other studies had also shown that miR-29 family could regulate myeloid cell leukemia sequence 1 (Mcl1), an antiapoptotic Bcl-2 family member close correlated with adverse CLL prognosis. Recently, Sengupta et al also demonstrated that miR-29c could down-regulate extracellular matrix proteins, including multiple collagens and laminins, by targeting the untranslated region (UTR) of their RNA. These findings are particularly relevant in CLL biology, since CLL cell survival is promoted by microenvironment contact. Furthermore, miR-29c reverts aberrant methylation by targeting DNA methyltransferase (DNMT) 3A and 3B, 2 key enzymes frequently up-regulated in lung cancer. Thus, the down-regulation of miR-29 inversely correlates with DNMT expression. All these observations strengthen the relationship between low expression of miR-29c and the poor prognosis observed in CLL patients. A little is known about the role of miR-223 in cancer, but many studies have described its role in normal tissues. However, Wong et al suggested that miR-223 down-regulates the expression of stathmin1 (STMN1), an oncoprotein overexpressed in many cancer types.

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![Figure 5. Forest plot comparing univariate Cox HR of qPCR score with other prognostic factors for TFS and OS prediction. The HR of all variables were calculated by univariate Cox analysis and plotted with the 95% confidence interval (CI) on this forest plot. Panel A shows the different HR for TFS prediction and panel B for OS prediction. More details can be found in Figure S5.](image)
probably not yet optimized. This cutoff point should be validated on an extended patient cohort. To be consistent in the present study, we also applied the same procedure for the determination of the other prognostic factor’s cutoffs.

However, although a plethora of prognostic factors have been described, individual prognosis evaluation of early stages of CLL is always a challenge for physicians. Thus, we developed a simple qPCR score that included 2 well-documented and powerful markers (ZAP70 and LPL) in addition to miR-29c and miR-223 that we have discussed here. We chose these 4 markers for many reasons. First, these factors could be easily measured by qPCR. Indeed, we demonstrated a method to accurately measure miR-29c and 223, and we validated their prognostic power. The prognostic value of qPCR-measured ZAP70 and LPL has been previously described. Second, the patient prognostication using only one parameter could lead to a misclassification, and a 4-parameter score will reduce this risk. Finally, the fact that this score takes into account the expression of 2 mRNAs, which were up-regulated, and 2 microRNAs, which were down-regulated, allows the use of different cellular mechanisms underlying the observed clinical evolutions.

The prognostic power of ZAP70 and LPL has been abundantly described in the literature and has been further confirmed in our patient cohort. Despite their powerful prognostic value, the concordance between these 2 factors is not absolute. In multivariate analysis, some studies found ZAP70 as an independent prognostic factor, while others found LPL. By combining these 2 powerful prognostic markers together with miR-29c and miR-223, we developed a simple qPCR score (from 0 to 4) to refine CLL prognosis. This score allows a clear separation of patients into 5 groups with different median TFS and into 3 groups with different median OS. Interestingly, during the first 50 months after diagnosis, only 10% of the patients with a 0/4 score required treatment, compared with 100% of the 4/4 group. Furthermore, after 26 years of follow-up, patients with a 4/4 score had a 27-fold higher risk to be treated and a 31-fold higher risk to die compared with patients with a 0/4 score. This score was validated by a 10-fold cross-validation study (Figure S2). We also observed that our qPCR score permits the division of Binet stage A patients into 5 groups with median TFS from greater than 312 to 16.4 months and into 2 groups in terms of OS (with a median OS of > 312 and 152.5 months). Furthermore, we observed that in the patient groups with or without poor prognostic factors, our qPCR score allowed a new separation into 2 to 5 subgroups with different prognosis. These analyses reveal that this score had a powerful prognostic value that allows the identification of patients with a worse prognosis or a less indolent evolution defined by the other recently admitted prognostic factors. Serial measurements of miR-29c and miR-223 indicated that the level of these microRNAs remains quite stable and similar to the value obtained at diagnosis (Figure S9). The microRNA status (and our qPCR score) does not change along the time until the evolution of disease. Indeed, as it could be expected, miRNA expression could decrease after disease acceleration (Figure S9). Finally, we evaluated the relative power of the classical prognostic factors, including IgVH mutational status, ZAP70, LPL, and CD38, using a multivariate Cox regression model; this analysis revealed that our qPCR score was the sole independent prognostic factor for TFS prediction. All our observations suggest that this qPCR score is currently the most powerful biologic indicator for determining the prognosis of CLL patients, even in Binet A stage patients. Furthermore, its availability could be extended to nonspecialized laboratory or not qPCR-equipped because lysed cells or cDNA could be readily sent at room temperature. In addition to having a powerful prognostic value, this qPCR score is easily and accurately determined, reproducible, and reduces patient misclassification. This score will also help to identify patients who will need early therapy and thus require a closer follow-up.

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Authorship
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