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miRNAs as Biomarkers in Chronic Myelogenous Leukemia

Kasuen Kotagama[1], **Yung Chang**[1,2], and **Marco Mangone**[1,3,*]

[1]Molecular and Cellular Biology Graduate Program, Arizona State University, Tempe, AZ, USA

[2]Center of Infectious Diseases and Vaccinology, The Biodesign Institute, Arizona State University, Tempe, AZ, USA

[3]Virginia G. Piper Center for Personalized Diagnostics, The Biodesign Institute, Arizona State University, Tempe, AZ, USA

Abstract

Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

Chronic myelogenous leukemia (CML) is a myeloproliferative neoplasm that is frequently characterized by the constitutive expression of the oncogenic protein BCR-ABL tyrosine kinase. Tyrosine kinase inhibitors (TKIs) targeting breakpoint cluster region-ABL are the first-line therapy for most CML patients and have drastically improved the prognosis of CML. However, some CML patients are unresponsive to TKI treatment, and a notable proportion of initially responsive patients develop drug resistance. Several molecular pathways have been correlated with resistance to TKI treatment, however, the exact mechanism of developing drug resistance remains ambiguous. Recently, microRNAs (miRNAs) have been implicated in the progression of CML and the development of resistance to TKI treatment based on their important regulatory function in cell homeostasis, and the deregulation observed in the initiation and progression of many leukemia subtypes. In this review, we summarize some of the major discoveries regarding miRNAs in CML, and their relevance as biomarkers for diagnosis, disease progression, and drug sensitivity.

Keywords

microRNA; chronic myelogenous leukemia; biomarkers; Imatinib; drug response

[*]Correspondence to: Marco Mangone, Molecular and Cellular Biology Graduate Program, Arizona State University, Tempe, AZ, USA. mangone@asu.edu.

CONFLICT OF INTEREST STATEMENT

None declared.

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INTRODUCTION

Chronic myelogenous leukemia (CML) is a chronic myeloproliferative disorder with an incidence of 1–2 cases per 100,000 individuals, and a predicted diagnosis rate of 6,000 new patients annually in the United States [Siegel et al., 2015]. Ninety-five percentage of CML patients possess a well-known chromosomal translocation, known as the Philadelphia chromosome (Ph) [Nowell, 1962], in which the breakpoint cluster region (*BCR*) gene on chromosome 22 is fused to a large part of the Abelson gene (*Abl-1*) on chromosome 9, resulting in a BCR-ABL fusion protein. This fusion leads to the constitutive activation of the ABL-1 kinase [McWhirter et al., 1993], which activates multiple proliferative signaling pathways, and drives the clonal expansion of myeloid cells.

The diagnosis and progression of CML is defined by the abundance of blast cells in full blood count tests. The prevalence of blast cells in blood and bone marrow increases proportionally with the advancement of the disease. The progression of CML is divided into three major stages based on the percentage of blast cells in a total cell count: (i) chronic phase (CP), with less than 10%; (ii) accelerated phase, 10–19%; and (iii) blast phase (BP) with greater than 20% blast cells [Hochhaus et al., 2008]. A majority of patients are diagnosed during the CP of the disease, which is often asymptomatic and can last from approximately a few months up to 3–5 years [O'Brien et al., 2009]. The exact staging of CML has important clinical relevance due to its correlation with treatment response and prognosis. Additional chromosomal abnormalities beyond the Ph are often observed in blast crisis (BC) patients, suggesting that additional mutations can contribute to disease progression. However, the mechanistic basis underlying the CP to BC transition is still poorly understood [Radich et al., 2006; Hanfstein et al., 2012].

The recommended treatment for CML is the administration of tyrosine kinase inhibitors (TKIs), such as Imatinib, which inhibit the kinase activity of the BCR-ABL oncoprotein [Hochhaus et al., 2008; O'Brien et al., 2009]. Imatinib was the first rationally designed anti-cancer drug, and has been extremely effective at slowing disease progression and modulating symptoms [Druker et al., 2006]. Eighty-seven percent of patients administered with Imatinib for 60 months exhibit a complete cytogenetic response, with no detectable Ph [Druker et al., 2006]. However, Imatinib resistance may develop over time, in part, due to duplications or mutations in *BCR-ABL*, which results in elevated kinase activity or the inability of Imatinib to bind to its target. To combat Imatinib resistance, second and third generations of TKIs were developed. These new TKIs have demonstrated better clinical efficacy than Imatinib in managing CML, and reduce the frequency of CP to BP transition [Demarquet et al., 2011].

TKI resistance can also occur independently of *BCR-ABL*, which can make new generations of TKIs ineffective. Approximately 20% of CML patients develop either primary (i.e., lack of responsiveness) or secondary (i.e., loss of response after initial treatment) TKI resistance [Cortes et al., 2012]. These patients ultimately progress to BP or BC with high mortality. Several mechanisms have been identified that mediate TKI resistance [Bixby and Talpaz, 2009]: (i) alterations in drug importers and exporters; (ii) CML stem cell disorder where quiescent stem cells are insensitive to TKIs; (iii) activating alternative signaling pathways to

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rescue cells from inactivation of BCR-ABL including WNT, JAK-STAT, autophagy, and Hedgehog signaling; (iv) defective DNA-damage repair systems leading to enhanced genome instability; and (v) the inflammatory microenvironment. Conceivably, tackling these pathways in combination with TKIs can improve disease outcome. However, various subtypes of TKI resistance require sensitive biomarkers for classification and selection for appropriate inhibitors in combination therapy.

Attempts to identify clinically relevant CML biomarkers have revealed several candidates, but their actual clinical utility remains to be determined. A landmark microarray study 10 years ago examined the molecular signatures and early biomarkers for CML by comparing the transcriptomes of CML patients with those of healthy individuals [Nowicki et al., 2003]. They identified genes differentially expressed between these samples, most of which were directly or indirectly activated by the BCR-ABL fusion protein [Nowicki et al., 2003]. For example, mutation of specific tumor suppressor genes like *INK4a/ARF* and *p53* were correlated with disease progression in some patients [Nagy et al., 2003]. Many other studies also utilizing microarray approaches on CML patient tissues and cell lines have identified hundreds of genes that are differentially expressed in each stage [Ohmine et al., 2001; Radich et al., 2006; Zheng et al., 2006; Oehler et al., 2009]. A study performed in 2009 used a probabilistic model to identify a group of six regulatory and structural genes (*NOB1, DDX47, IGSF2, LTB4R, SCARB1*, and *SLC25A3*) as potential biomarker candidates [Oehler et al., 2009]. These gene biomarkers were able to differentiate between patients that are responsive and nonresponsive to TKI treatment. While these results are encouraging, a subset of patients in these studies did not exhibit gene expression patterns consistent with these biomarkers. This suggests that there is a critical need to develop more precise biomarkers for identifying and staging CML, and predicting patient response to TKI treatments.

Several groups have found a class of regulatory genes, the microRNAs (miRNAs) that are misexpressed in CML [Godley, 2007; Venturini et al., 2007; Agirre et al., 2008; Bueno et al., 2008; San Jose-Eneriz et al., 2009; Eiring et al., 2010; Flamant et al., 2010; Zimmerman et al., 2010; Chim et al., 2011a,b; Lopotova et al., 2011; Machova Polakova et al., 2011; Suresh et al., 2011; Rokah et al., 2012; Scholl et al., 2012; Yu et al., 2012; Li et al., 2013a,b; Gebauer et al., 2014; Joshi et al., 2014; Kaymaz et al., 2014; Taverna et al., 2014; Xiong et al., 2014; Xishan et al., 2014; Xu et al., 2014a,b; Fallah et al., 2015; Hershkovitz-Rokah et al., 2015a,b], opening new avenues into CML biomarker research.

miRNAs IN CML

miRNAs were first discovered in the roundworm *Caenorhabditis elegans*, where they were found to regulate key development processes [Lee et al., 1993]. They have since emerged as essential regulators in nearly all biological processes throughout the metazoan kingdom [Lee et al., 1993; Pasquinelli et al., 2000]. miRNAs are short, noncoding RNAs responsible for post-transcriptional regulation of messenger RNAs (mRNAs). The human genome contains ~2,000 distinct mature miRNAs [Friedlander et al., 2014]. Following transcription, miRNAs undergo several processing steps to form the mature miRNA, which are ~22 nucleotides in length. Mature miRNAs are incorporated into the RNA induced silencing complex (RISC)

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[Hammond et al., 2001], where they guide the RISC to complementary sites on target mRNAs via standard Watson–Crick base pairing. This pairing has been shown to require as little as six consecutive nucleotides in the 5′ end, or seed region, of the mature miRNA. Perfect complementarity within the seed region is considered the canonical indicator of miRNA targeting. However, many recent studies indicate that miRNAs are capable of targeting noncanonical elements in target mRNAs [Lal et al., 2009; Cevec et al., 2010; Azzouzi et al., 2011; Liu et al., 2011; Chi et al., 2012; Wolter et al., 2014]. These interactions are rarely driven by perfect complementarity, and typically contain loops and non-Watson–Crick base pairing. Because of the small and degenerate nature of these elements, miRNA targets are generally difficult to identify. As such, the vast majority of miRNA targets remain unknown.

Consistent with the role of miRNAs in various developmental processes, their misregulation can broadly contribute to the phenotypic characteristics of all cancer subtypes investigated to date [Croce, 2009]. In chronic lymphocytic leukemia, the loss of the *miR-15/16* cluster is the most frequently observed genetic aberration, occurring in approximately 70% of cases [Calin et al., 2002]. In CML, increased expression of *miR-150* and *miR-146a*, and reduced expression of *miR-142-3p* and *miR-199b-5p* was observed after 2 weeks of TKI treatment [Flamant et al., 2010], suggesting that this drug has the ability to rearrange the miRNA profiles of tumor cells. Using a TaqMan Low-Density Array system, miRNA levels in blood samples were found to change significantly in newly diagnosed CML patients before and within the first 2 weeks of Imatinib treatment [Flamant et al., 2010], potentially identifying easily measurable biomarkers to monitor the TKI response.

Taken together, these results suggest that miRNA signatures could represent novel biomarkers in CML research, to allow staging of CML and are predictive of patient response to TKI treatment. In this review, we discuss the most promising biomarker candidates that have recently emerged.

miR-150 as a Biomarker in CML Diagnosis and Treatment Response

Early diagnosis of CML prior to BP has a significant impact on patient survival rates. *miR-150* has consistently been observed to be down-regulated across multiple studies making it a promising candidate for early CML diagnosis (Table 1). Multiple reports indicate that lowered expression of *miR-150* represents poor prognosis and a more advanced state of CML, while reintroduction of *miR-150* is found to alleviate symptoms in cell lines [Agirre et al., 2008]. Down-regulation of *miR-150* was observed in $CD34^+$ cells derived from six CP CML patient samples [Agirre et al., 2008], suggesting that the down-regulation of this miRNA plays a role in disease initiation.

Further, evidence for down-regulation of *miR-150* as a diagnostic biomarker of CML was shown in a study that used a reverse transcription polymerase chain reaction approach on 50 newly diagnosed CP CML patient samples, and found significant down-regulation of *miR-150* [Fallah et al., 2015]. A study in 2010 performed a microarray analysis on 10 CP CML patient samples and further validated the potential of *miR-150* as a biomarker for CML diagnosis [Flamant et al., 2010]. Importantly, this report uncovered that *miR-150*, which was only known to be down-regulated in CP, is also down-regulated in BP [Flamant et al., 2010],

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suggesting that while this miRNA may have potential for the diagnosis of CML, it is not an effective biomarker to distinguish between CP and BP.

miR-150 expression levels can also be used as a biomarker for treatment response. The down-regulation of *miR-150* was reported in the BCR-ABL transformed leukemia cell line Mo7e-p210 (megakaryoblast), and could be restored in response to Imatinib treatment [Agirre et al., 2008]. Another study used patient samples at different phases of CML and provided evidence that *miR-150* could act as a biomarker for diagnosis and treatment response [Machova Polakova et al., 2011]. The study profiled miRNA expression levels using microarrays and Q-RT PCR, and the results reinforced that *miR-150* is down-regulated in both CP and BP. Most importantly, these results also showed that *miR-150* expression levels were not restored in patients developing resistance to Imatinib treatment. Together, this result suggests that lower expression levels of *miR-150* are indicative of poor prognosis for patients receiving TKI treatment, and strengthen the use of *miR-150* as a potential biomarker for drug response.

miR-203 as a Biomarker for Diagnosis

The methylation patterns of the *miR-203* gene locus have been suggested as a potential biomarker for CML diagnosis (Table 1). Using methylation specific PCR (MS-PCR) and microarray expression profiles, one study reported methylation of the *miR-203* upstream promoter, and the subsequent reduction of *miR-203* levels in both murine and human T-cell cell lines [Bueno et al., 2008]. The authors then compared several leukemia cell lines and detected significant down-regulation and hypermethylation of *miR-203* in CML cell lines expressing BCR-ABL fusion protein, but not in other cell lines of related myeloproliferative diseases [Bueno et al., 2008].

A mechanism of action for *miR-203* in CML was proposed in 2008. In this study, the reintroduction of *miR-203*, to CML cell lines deficient in *miR-203* expression via transfection, resulted in a marked decrease of BCR-ABL expression and a consequential drop in the rate of proliferation [Bueno et al., 2008]. The study also confirmed the predicted interaction of *miR-203* with the 3′UTR of *BCR-ABL* by utilizing a luciferase reporter system. These results indicate that the deregulation of this miRNA, perhaps in conjunction with other miRNAs, could be used as a biomarker for the diagnosis of CML.

Intriguingly the study of methylation in the *miR-203* genomic locus by MS-PCR, found no significant hypermethylation in 11 CML primary patient samples but significant hypermethylation in other leukemia subtypes [Chim et al., 2011b]. Methylation of *miR-203* was also observed in Ph negative leukemia patients [Chim et al., 2011a]. These results suggest that the regulation of *miR-203* in CML patients may be more complex than is currently understood, warranting further investigation.

miR-17/92 Cluster as Biomarker to Distinguish Between CML and AML

The *miR-17/92* cluster has significant potential for use as a biomarker as it is one of the best-characterized miRNA clusters in leukemia (Table 1). This cluster consists of six miRNAs: *miR-17*, *miR-18a*, *miR-19a*, *miR-19b-1*, *miR-20a*, and *miR-92a-1*. The overexpression of

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miR-17/92 cluster was first reported to CML in K562 cells [Venturini et al., 2007]. The *miR-17/92* cluster is a promising biomarker for disease progression as the overexpression of the cluster was reported in $CD34^{+}$ cells from patients in CP, but not in BP [Venturini et al., 2007]. Similar findings were also reported in 50 CP CML patients using reverse transcription polymerase chain reaction analysis, showing increased expression of *miR-17* and *miR-20a* [Fallah et al., 2015]. Although these findings are promising, they conflict with previously published microarray expression results showing increased expression of *miR-17*, *miR-19a*, *miR-19b*, and *miR-20a* during the BP of CML [Machova Polakova et al., 2011]. This suggests that the expression patterns of some members of the cluster require further investigation.

The *miR-17/92* cluster also shows promise as a biomarker to differentiate between late stage CML and AML. Comparative transcriptome sequencing of the CML cell line K562 versus the AML-specific cell lines HL-60 and THP-1, show increased expression of *miR-20a* only in K562 cells, suggesting that higher *miR-20a* expression levels are a potential biomarker for CML [Xiong et al., 2014].

miR-10a as Candidate for Diagnosis and Drug Response

miR-10a is an emerging candidate for CML diagnosis (Table 1). Using a Q-RT PCR approach on 85 newly diagnosed CP CML patients, down-regulation of *miR-10a* was observed in 71% of patients, displaying its clinical relevance as a biomarker for diagnosis [Agirre et al., 2008]. In concordance with its down-regulation, the authors provide evidence that *miR-10a* targets the well-characterized cell proliferative transcription factor upstream stimulatory factor (USF1), which is up-regulated in 60% of CP CML patients [Agirre et al., 2008].

Interestingly, a great deal of variability is seen in the expression of *miR-10a* among cell lines derived from CML patients in the BP. The BP derived cell lines, Mo7e-p210, TCC-S, and K562, show increased *miR-10a* levels, as opposed to BV173 and KU812, which show decreased *miR-10a* levels [Agirre et al., 2008; Xiong et al., 2014]. These findings suggest that while emerging from similar sources, there may be significant differences in the molecular mechanisms driving CML in these cell lines, and that *miR-10a* may provide an effective biomarker for distinguishing among them.

Other studies have also explored the relevance of *miR-10a* as a biomarker for drug response. *miR-10a* levels in the Mo7e-p210 cell line were unaffected by Imatinib treatment [Flamant et al., 2010], however, a significant increase of *miR-10a* was observed by microarray analysis of patient samples 2 weeks post Imatinib treatment [Flamant et al., 2010]. Rescue of *miR-10a* levels by transfection into KU812 cells resulted in a significant decrease of proliferation, suggesting that *miR-10a* may play a role in CML pathogenesis [Agirre et al., 2008]. Taken together, the results suggest that down-regulation of *miR-10a* has potential to be a biomarker for the CP of CML. However, the lack of consensus about *miR-10a* regulation among cell lines in response to Imatinib requires further investigation.

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miR-29a/b as Biomarker of Drug Resistance

MiR-29a/b is consistently down-regulated in CML, and its expression level correlates to clinical resistance to Imatinib, making it a promising biomarker candidate for drug response (Table 1). While studying miRNA expression profiles of patients receiving Imatinib treatment over 12 months, *miR-29a* was found to be down-regulated in drug resistant patients [San Jose-Eneriz et al., 2009]. *miR-29b* expression levels correlate strongly with poor prognosis as its down-regulation is seen during the diagnosis and BP of CML [Machova Polakova et al., 2011], displaying some potential use as a biomarker for diagnosis. However, other studies have shown that the down-regulation of *miR-29a/b* occurs in both CML and AML patients, suggesting that these two miRNAs play a strong underlying regulatory role in myeloid cells.

An in-depth study of *miR-29b* has proposed a potential mechanism of action for this miRNA [Li et al., 2013a]. *MiR-29b* is predicted to target BCR-ABL transcripts and is commonly present at lower levels in patient samples at diagnosis. A direct interaction between *miR-29b* and the 3′UTR of *Abl-1* was observed using a luciferase assay [Li et al., 2013a]. The overexpression of *miR-29b* in K562 cells lowers ABL-1 protein levels, and induces G1 phase cell arrest by activating p21 and p27. In K562 cells, exogenous *miR-29b* also leads to an increase in apoptosis and induces Caspase 3 activity, leading to cleavage of PARP and increased expression of BAX proapoptotic factor [Li et al., 2013a].

The well-characterized mechanism of action in CML cell lines and its consistent down-regulation in the CP and BP patient samples, indicate that *miR-29b* is a good candidate as a miRNA biomarker for diagnosis, and shows some promise as an indicator for TKI treatment response.

CONCLUSION

CML presents a challenge for biomarker research, where early detection strategies have a significant impact on patient prognosis. Current techniques are adequate for the diagnosis of CML, but they are insufficient to monitor disease progression and the development of drug resistance. MiRNAs are dynamic regulatory molecules found to be sensitive to the molecular development of CML, and exhibit great potential as potential drug targets and biomarkers. Unfortunately, they do not address all aspects of the biomarker spectrum. Down-regulation of specific miRNAs, for example, *miR-150* and the *miR-29a/b* cluster, has been well characterized in the context of CML, with specific molecular mechanisms. If confirmed using larger datasets, these miRNAs are promising biomarker candidates for patient prognosis and the development of drug resistance. However, the precise contribution of other miRNAs to disease progression remains controversial. Conflicting reports on *miR-203* and *miR-17/92* clusters indicate that the deregulation of miRNAs can be inconsistent in CML patients [Bueno et al., 2008; Chim et al., 2011a,b; Machova Polakova et al., 2011; Fallah et al., 2015]. High-throughput studies have displayed that many miRNAs are differentially expressed in response to disease progression, but there is a distinct lack of understanding of the molecular mechanisms governing miRNA function. In conclusion, these findings suggest that while the use of single miRNAs may be ineffective,

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combinatorial miRNA expression profiles could be used in principle as effective biomarkers for diagnosis, to monitor disease progression, and drug response.

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TABLE 1

miRNA Expression Patterns Related to CML. ↑, Up-Regulated and ↓, Down-Regulated miRNA Levels Based on Different Detection Techniques and in Different Phases of the Disease

miRNA	chr. locus	Sample type	Origin	Time point/phase	Expression level	Detection technique	Citation
miR-10a	17q21.32	Primary tumor	CD34+ cells	Diagnosis	↓	Q-RT PCR	Agirre et al. [2008]
		Cell line	Mo7e-p210, TCC-S BV173, KU812	–	↑ ↓		
		Primary tumor	Patient	Chronic, post treatment	↑	Microarray	Flamant et al. [2010]
		Cell line	K562, HL-60, THP-1	–	↑	NextGen Sequencing	Xiong et al. [2014]
miR-17-92	13q31.3	Primary tumor	Patient Patient	Chronic Blast	↑ Unchanged	Q-RT PCR	Venturini et al. [2007]
		Cell line	K562	Post treatment	↓	Microarray	
		Primary tumor	Patient	Blast	↑	Microarray	Machova Polakova et al. [2011]
		Cell line	K562, HL-60, THP-1	Chronic, post treatment	↑	NextGen Sequencing	Xiong et al. [2014]
		Primary tumor	Patient	Chronic	↑	Q-RT PCR	Fallah et al. [2015]
miR-29a/b	7q32.3	Primary tumor	Patient	Chronic, pre and post treatment	↓	Microarray	San Jose-Eneriz et al. [2009]
			Patient	Blast	↓	Microarray	Machova et al. [2011]
			Patient	Diagnosis	↓	Q-RT PCR	Li et al. [2013]
			Patient	Chronic	↓	Q-RT PCR	Xu et al. [2014]
miR-150	19q13.33	Primary tumor	CD34+ cells	Diagnosis	↓	Q-RT PCR	Agirre et al. [2008]
			Patient	Chronic Blast Post treatment	↓ ↓ ↑	Microarray	Flamant et al. [2010]
			Patient	Diagnosis Blast Relapse post treatment	↓ ↓ ↓	Microarray and Q-RT CPR	Machova et al. [2011]
			Patient	Diagnosis	↓	Q-RT PCR	Fallah et al. [2015]
miR-203	14q.32.33	Primary tumor	KARPAS-45, PEER, JURKAT, MOLT-4	Chronic	↓	Q-RT PCR and MS-PCR	Bueno et al. [2008]
			Patient		Unchanged	MS-PCR	Chim, et al. [2011]