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The roles of microRNAs in the pathogenesis and drug resistance of chronic myelogenous leukemia (Review)

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Abstract. Chronic myeloid leukemia (CML) is characterized by the accumulation of Philadelphia chromosome-positive (Ph^+) myeloid cells. Ph^+ cells occur via a reciprocal translocation between the long arms of chromosomes 9 and 22 resulting in constitutively active BCR-ABL fusion protein. Tyrosine kinase inhibitors (TKIs) are used against the kinase activity of BCR-ABL protein for the effective treatment of CML. However, the development of drug resistance, caused by different genetic mechanisms, is the major issue in the clinical application of TKIs. These mechanisms include changes in expression levels of microRNAs (miRNAs). miRNAs are short non-coding regulatory RNAs that control gene expression and play an important role in cancer development and progression. In the present review, we highlight the roles of miRNAs both in the progression and chemotherapy-resistance of CML. Our understanding of these mechanisms may lead to the use of this knowledge not only in the treatment of patients with CML, but also in other type of cancers.

Contents

1. Introduction
2. microRNAs (miRNAs)
3. The roles of microRNAs in the pathogenesis and drug resistance of CML
4. Conclusion

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1. Introduction

Chronic myeloid leukemia (CML) has a yearly incidence of approximately 1 in 50,000 individuals and accounts for 15% of all adult leukemias. The onset of this disease is ~45-55 years, with the majority of patients being asymptomatic at diagnosis which is commonly made after routine blood tests. The evolution of CML occurs via a biphasic or triphasic course. The majority of the cases (~85%) are diagnosed during the asymptomatic chronic phase (CP) where the cells are mainly differentiated, minimally invasive and maintain their functionality (1). If left untreated, the disease inevitably progresses after three to five years to an intermediate accelerated phase (AP) and to blast crisis (BC) (2). Nevertheless, up to a quarter of patients progress directly to the BC phase (3). In 1960, Rudkin *et al* detected a consistent chromosomal abnormality characteristic of CML, which later was named the Philadelphia (Ph) chromosome (4). The Ph chromosome results from a reciprocal translocation, which involves the Abelson (ABL) proto-oncogene on chromosome 9 and breakpoint cluster region (BCR) on chromosome 22, t(9;22)(q34;q11) (5). This translocation creates the BCR-ABL fusion gene, which is believed to be the principal cause of CML and is considered as a hallmark of this disease. Depending on the breakpoint in the BCR gene, three main types of fusion proteins are formed: p210BCR/ABL (M-bcr breakpoint), which is the most common in CML, p230BCR/ABL (μ-bcr breakpoint) and p190BCR/ABL (m-bcr breakpoint) (5). The BCR-ABL protein is exclusively localized to the cytoplasm and it is able to trigger multiple downstream pathways leading to enhanced cell proliferation and transformation, reduced growth factor dependence, resistance to apoptosis, perturbed adhesion to bone marrow and stroma, and genetic instability (6). This results in the expansion of the leukemic cell population, initially characterized by overproduction of mature myeloid cells with normal morphology (7). Many BCR-ABL substrates and binding partners have been identified, and current efforts are directed at linking these pathways to specific defects for CML (5,8,9). Selective therapies are aimed for the treatment of CML since its target is well defined in contrast to other cancers (10). Hundreds of protein kinases are known in the human genome and a drug is required that targets a single adenosine triphosphate (ATP) binding site of protein

kinase. By blocking the binding of ATP, phosphorylation is prevented and BCR-ABL-expressing cells either have a growth disadvantage or they undergo apoptosis (10). Imatinib (IM) (STI571) is the first BCR-ABL tyrosine kinase inhibitor (TKI) that prevents ATP from binding by itself to the ABL domain via interaction with six hydrogen bonds (10). Hydrogen bonds involve the pyridine-N and backbone-NH of Met-318, the aminopyrimidine and side chain hydroxyl of Thr-315, the amide-NH and side chain carboxylate of Glu-285, the carbonyl and backbone-NH of Asp-381, the protonated methylpiperazine with the backbone-carbonyl atoms of Ile-360 and His-361. Additionally, a number of van der Waals interactions contribute to binding (11-13). Resistance faced by imatinib can be subdivided into BCR-ABL-independent and dependant mechanisms (14). The dependent mechanism is associated with the duplication of the BCR-ABL tyrosine kinase gene in the DNA sequence leading to higher expression of the protein (10). A point mutation in the kinase domain of BCR-ABL leads to disruption in the binding site of imatinib on the tyrosine kinase, resulting in the loss of sensitivity of the drug (14). T315I is a unique mutation due to its resistance to all approved BCR-ABL inhibitors, prior to ponatinib (15). It may be due to the displacement of cytosine to thiamine (C->T) base pair at 944 of the ABL gene. It causes the elimination of critical O_2 molecules needed for hydrogen bonding between imatinib and BCR-ABL kinases (10). The most common mutation occurs in ATP binding and the activation loop. It causes the derangement of loops as a result of which the kinase domain cannot assume inactive conformation required for imatinib binding (14). BCR-ABL-independent resistance occurs either due to overexpression of the P-glycoprotein efflux pump, activation of rous sarcoma oncogene cellular homolog (Src) family kinase or may be due to low expression, activity or polymorphism of organic cation transporter 1 (OCT1) (10-16).

Nilotinib (AMN107) and dasatinib (BMS-345825) are second generation drugs that were aimed to have less resistance and intolerance than imatinib (10). Nilotinib is a selective inhibitor and binds to the inactive conformation of the ABL kinase domain, largely through lipophilic interactions and thus blocks its catalytic activity, being 10- to 30-fold more potent than imatinib (17-19). It is effective against all type of resistance except the T315I mutation. Its failure against T315I is due to the loss of an H-bond interaction between threonine-*O* and aniline-NH on nilotinib and a steric clash between the isoleucine-methyl and 2-methylphenyl phenyl groups of nilotinib (17,18). Dasatinib is a multi-targeted inhibitor of wild-type BCR-ABL and Src family kinases having additional inhibitory activity against downstream kinases (19). Contrary to most TKIs, dasatinib binds to active conformation of ABL kinase (13). First and second generation inhibitors have provided promising results, but new mutations are continuously being encountered that requires discovery of more drugs.

Bosutinib is based on a quinolone scaffold and it also has the ability to inhibit the mutation of T3151 (18). One of the most promising TKIs able to inhibit the T315I mutation is ponatinib (AP24534). In addition, it inhibits Src, vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR) and platelet-derived growth factor receptor (PDGFR) family kinases (20). Clinical trials with ponatinib are ongoing and initial results from a phase II trial, called the Ponatinib P-positive Acute Lymphocytic Leukemia (ALL) and CML Evaluation (PACE), involves CML and Ph-positive ALL patients with dasatinib and/or nilotinib intolerance or resistance (including T315I). However, this drug was correlated with a nearly 12% incidence of cardiovascular events such as serious arterial thrombotic events in adults, resulting in withdrawal from the market (21,22).

Bafetinib (INNO-406), an oral dual ABL/LYN tyrosine kinase inhibitor, demonstrates specific LYN kinase activity with no or limited activity against other Src-family member kinases. Several BCR-ABL kinase domain mutations are sensitive to INNO-406 *in vitro*, including the F317L and F317V mutations. Kantarjian *et al* found that INNO-406 can be used in Ph-positive CML or ALL post-imatinib resistance or intolerance. Such a drug with efficacy against various point mutations in the BCR-ABL kinase, with few adverse effects and with narrower kinase spectra, is also in phase II clinical trials (23). Befitinib and imatinib have structural and binding similarities, the notable difference being hydrophobic interaction between the trifluoromethyl group and the hydrophobic pocket created by Ile-293, Leu-298, Leu-354 and Val-379 (24).

Although more potent BCR TKIs are available, imatinib still remains the frontline TKI. Nilotinib, dasatinib, bosutinib and ponatinib are approved for the treatment of imatinib resistance or intolerant CML. The availability of highly potent TKIs, such as nilotinib, has broadened the treatment armamentarium in CML. Nilotinib appears to overcome imatinib resistance in patients with chronic, accelerated and blastic phase CML, producing sustained cytogenetic and hematological responses (25). The first line data for these drugs are encouraging and suggest that some or all of them may replace imatinib as a frontline standard BCR-ABL tyrosine kinase inhibitor in the near future.

In recent years, miRNAs have received wide attention as important regulators of gene expression in leukemogenesis and they are associated with resistance to BCR-ABL TKIs.

2. MicroRNAs (miRNAs)

miRNAs are conserved non-coding RNAs with a short sequence (~20-23 nucleotides) that participate in the traslational regulation of many genes involved in the control of important biological processes by selectively binding to their messenger RNAs (mRNAs) and generally blocking their protein expression (26,27). Most miRNA genes are coded in the intergenic regions of the genome that are distant from other genes and they have their regulatory elements derived from independent transcription units (28). Nevertheless, genomic studies have revealed that 30% of miRNAs are included in the introns of the pre-mRNA of protein-coding genes and frequently in the same orientation, implying that they are transcribed in the same mRNA primary transcript (although they could also be independently transcribed through an alternative promoter) (28). The majority of human miRNA genes are isolated from each other within the genome, but others can be clustered together and transcribed as a polycistronic primary transcript (28). Since they were discovered and characterized on 1993 by Lee *et al* (29), the number of miRNA sequences deposited in miRBase databases is continuously growing, and

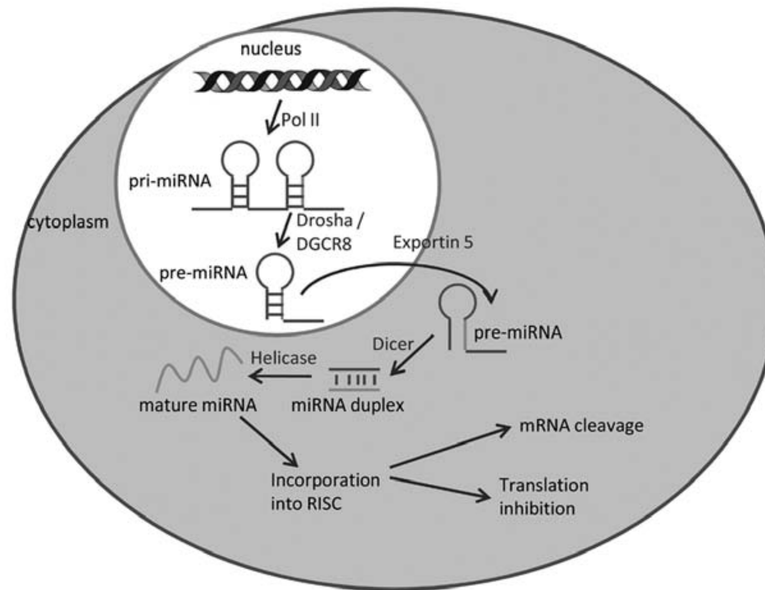

Figure 1. miRNA biogenesis.

they have been proven to play an important role in cellular regulatory pathways (30).

miRNA biological relevance: Involvement in gene expression control. Considering that a single miRNA can target several mRNAs, a single mRNA may contain in the 3'-untranslated region (UTR) sequence several signals for miRNA recognition and it has been determined that at least 10-40% of human mRNAs are a target for miRNAs (31). In fact, a single miRNA may bind to as many as 200 gene targets and these targets can be diverse in their function; they include transcription and secreted factors, receptors and transporters. Thus, miRNAs potentially control the expression of about one-third of human mRNAs (32). Therefore, great interest is concentrated on the identification of validated targets of miRNAs. This specific field of miRNA research has confirmed that the complex networks constituted by miRNAs and RNA targets coding for structural and regulatory proteins lead to the control of highly regulated biological functions, such as differentiation (33), cell cycle (34) and apoptosis (35). Since a single 3'UTR of a given mRNA contains signal sequences for several miRNAs, which miRNA should be targeted in order to achieve alteration of the expression of the gene should be experimentally evaluated. With respect to the possible effects of the expression of other mRNA targets, it should be clearly stated that alteration of a single miRNA may retain multiple effects. In contrast, miRNAs usually bind to their targets with incomplete complementarity; for this reason, the identification of gene targets with only a simple BLAST search is impossible. However, current bioinformatic approaches have taken advantage of the fact that miRNAs within families have highest homology at the 5'-end of the mature miRNA, which is crucial for the stability and proper loading of the miRNA into the miRNA-mediated silencing complex (miRISC) (36).

Biogenesis of miRNAs. Various miRNAs are encoded by unique genes (intergenic miRNAs) (37) and others are embedded into the intronic regions of protein-coding genes (intragenic miRNAs) (38). The transcription by RNA polymerase II of these miRNA genes gives rise to long primary miRNAs (pri-miRNAs) with typical stem-loop structures. These are rapidly processed by the nuclear RNase endonuclease-III Drosha, which, removing the branches, gives rise to precursor miRNAs of ~60-100 nucleotides in length. In both cases of intergenic miRNAs and intragenic miRNAs, the pre-miRNAs are transported from the nucleus to the cytoplasm by exportin-5. In the cytoplasm, pre-miRNAs are further processed by another RNase endonuclease-III (Dicer) to generate mature miRNAs ~22-nt long, which generate the RNA-induced silencing complex (RISC) (Fig. 1).

miRNA and gene regulation. Structurally, miRNAs are small non-coding regulatory RNAs; these small RNAs post-transcriptionally repress gene expression by recognizing complementary target sites most often in the 3'UTR of target mRNAs (39). However, animal miRNAs may also target 5'UTR and coding regions of mRNAs, as documented by experiments involving both artificial and natural mRNAs and also by bioinformatic predictions (40). miRNAs silence the expression of target mRNAs, either by mRNA cleavage or by translational repression. Nevertheless, it has been described that miRNAs can also increase the expression of a target mRNA (41). Perfect miRNA:mRNA complementarity leads to cleavage of the mRNA by Argonaute protein (AGO2); this is the small interfering RNA (siRNA) pathway, which while important experimentally, is not thought to occur with endogenous mammalian miRNAs. Instead, the imperfect pairing with mRNA causes a downregulation of translation, even if the mechanism by which this occurs is still not clear. Whatever the precise nature of the mechanism, affected mRNAs accumulate in granular cytoplasmic ‘P-bodies’ along with RISC proteins (42). Generally, mRNA abundance is ultimately also reduced (43). This is important, as it means that the impact of miRNA activity can be assessed (at least

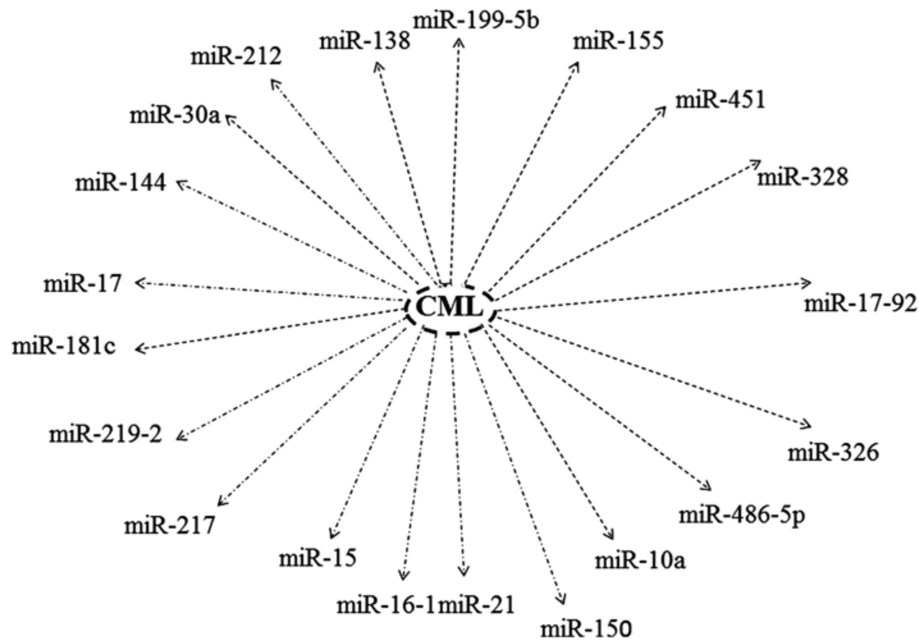

Figure 2. miRNAs involved in CML.

to some extent) by measures of mRNA abundance such as expression profiling.

The basic mechanism leading to alteration of gene expression is based on the recruitment of mature miRNAs at the level of the RISC silencing complex. This process occurs in the cytoplasm, where the pre-miRNA hairpin is cleaved by the RNase III enzyme Dicer, which interacts with the 3'-end of the hairpin and cuts away the loop joining the 3' and 5' arms, yielding an imperfect miRNA/miRNA duplex. One of the strands is incorporated into the RISC, where it binds to the target mRNA sequence. Perfect or near perfect base pairing with the target RNA promotes cleavage of the RNA (44). It is proposed that in the case of partial complementarity, miRNAs, in order to recognize their targets, nucleotides 2-7 of the miRNA (the '*seed sequence*') are important (45). This is the key process permitting mature miRNAs to exert their effects in gene regulation. The final effect of miRNA activity is the inhibition of the synthesis of the protein(s) encoded by the target mRNA(s). This has of course important biological implications depending on the role of the protein in the cellular network. Since a single 3'UTR of a given mRNA contains signal sequences for several miRNAs, applied biological studies are needed to determine which miRNA should be targeted to achieve alteration of gene expression. Possible effects on the expression of other mRNA targets should be considered. An alteration of a single miRNA may exhibit multiple effects, possibly in combination with the targeting activity of other miRNAs, enabling the achievement of a strong biological effect (46,47).

3. The roles of microRNAs in the pathogenesis and drug resistance of CML

Aberrant expression of miRNAs has been observed in hematological cancers, exhibiting unique expression signatures in comparison to normal counterparts. Furthermore, numerous studies have solely identified a loose association between certain hematological cancers and aberrant miRNA expression, while others have been able to illustrate their role in clinical diagnosis, prognosis and cancer therapy (Fig. 2) (32,48-56). The first investigation discovered that miR-15 and miR-16-1 have a deleted region, 13q14, in chronic lymphocytic leukemia (CLL) (57) and they act as tumor suppressors, and their expression is inversely correlated with anti-apoptotic B-cell CLL/lymphoma 2 (Bcl-2) expression (58). Additionally, Bcl-2 and myeloid cell leukemia sequence 1 (Mcl-1) have been predicted as potential target genes of the miR-29 family and both belong to the Bcl-2 family which plays a central role in cell death regulation (26). A recent study found that a significant low expression of miR-29a/29b is related to a high expression level of both Bcl-2 and Mcl-1 in peripheral blood mononuclear cells (PBMCs) derived from acute myeloid leukemia (AML) and CML patients compared with healthy subjects (59). Overexpression of miR-21 has also been implicated in directly modulating the expression of several apoptotic-related proteins such as Bcl-2. Seca *et al* observed that its downregulation caused a decrease in the expression levels of Bcl-2 protein in leukemic cells. In addition, treatment with anti-miR-21 caused both an increase in the autophagy-related proteins (Beclin-1 and LC3-II) and a cellular sensitivity to etoposide or doxorubicin (K562 and KYO-1 cells) (60). Another previous study found that over 50% of miRNA genes are located within regions of loss of heterozygosity, amplification, fragile sites and viral integration sites, and other cancer-related genomic regions (61). Using quantitative reverse transcription-polymerase chain reaction (qRT-PCR), upregulation of miR-96 and downregulation of miR-151, miR-150, miR-125a and miR-10a were detected in $CD34^+$ cells derived from CML patients when compared with the same cells derived from healthy donors. Additionally, transcription factor upstream stimulatory factor 2 (USF2) has been

considered as an miR-10a target and it appears to be upregulated in CML patients (62). Wang *et al* observed a miR-486-5p overexpression in CML CD34 cells compared with normal CD34 cells (63). In addition, the investigators showed that miR-486-5p levels were increased after erythroid differentiation in normal CD34 cells and its inhibition reduced such differentiation of both normal CD34 and CML CD34 cells. These results indicate an important role for miR-486-5p in modulating normal and leukemic hematopoietic progenitor growth and erythroid differentiation (63). Furthermore, this miRNA was able both to enhance phosphatidylinositol-3 kinase (PI3K)/AKT (protein kinase B) signaling and to reduce phosphatase and tensin homolog (PTEN) and Forkhead box O1 (FoxO1) levels in hematopoietic cells. Meanwhile, downregulation of miR-326 was a possible mechanism for the unrestricted activation of smoothened (Smo) signal transducer of the oncogenic Hedgehog (Hh) pathway and resulted in elevated cell proliferation and decreased rate of apoptosis in $CD34^+$ CML cells (64). One study suggested an oncogenic role for the miR-17-92 cluster (which encodes seven miRNAs: miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1) in CML. Chromosomal amplification at 13q31-q32 led to overexpression of the miR-17-92 cluster encoded by the chromosome 13 open reading frame 25 (C13orf25) gene in $CD34^+$ CML. Its overexpression was found to be associated with CP-CML but not BC-CML (65). A novel role of miRNAs has been identified during blast crisis. Aberrant activity of RNA binding proteins (RBPs) is related to increased BCR-ABL activity (66). One RBP affected is heterogeneous nuclear ribonucleoprotein E2 (hnRNP E2) which arrests myeloid differentiation through interaction with CCAAT/enhancer-binding protein-α (C/EBP-α) (67,68). miR-328 downregulation has been shown in BC-CML secondary to BCR-ABL activity both *in vitro* and *in vivo* using microarray, northern blotting and qRT-PCR methods (66). Postulating that miRNA and RBPs may interact, Eiring *et al* demonstrated that hnRNP E2 mediated the loss of miR-328 in BC-CML and re-expression of miR-328 was able to restore differentiation and block blast survival by simultaneously interacting with the hnRNP E2 and the mRNA encoding the survival factor provirus integration site for Moloney murine leukemia virus 1 (Pim 1) (66). Another study found that the heterogeneous nuclear RNP (hnRNP) A1 protein is also upregulated in BC-CML and it is linked to pri-miR-17-92 at the same time (68). Another study provided support for the idea that the lack of miR-17-92 expression can play a pivotal role during BC stage in CML (67). Another hallmark of CML is miR-150 whose reduced expression is found in $CD34^+$ cells derived from CML patients at diagnosis (62) and also in total leukocytes of PBMCs derived from CML patients in AP and BC phases (69). Moreover, increased expression of miR-150 and miR-146a and decreased expression of miR-142-3p and miR-199b-5p were observed in PBMCs derived from 140 patients newly diagnosed with CML and treated with imatinib for two weeks. Expression levels of these miRNAs also tended to normalize after imanitib treatment (69). Another recent study, conducted on 17 patients, demonstrated that the miR-451 expression level increased after imanitib therapy and it was inversely correlated with the BCR-ABL transcript in some CML patients. However, this study suggests that expression of miR-451, being heterogeneous among patients, can be regulated by other mediators (70). Recently, Rokah *et al* characterized the miRNA expression profile of CML cell lines and patients compared to a normal counterpart derived from healthy donors, using miRNA microarrays and miRNA real-time PCR. The expression levels of miR-31, miR-155 and miR-564 were decreased in CML and influenced by BCR-ABL activity. After 30 days of BCR-ABL inhibition (by imatinib), upregulation was observed in all three miRNAs indicating that BCR-ABL does not play a role in repressing these miRNAs. Notably, the analysis identified CML disease as possibly associated with major deregulation of these miRNAs (71). In addition, miR-130a expression was also found to be regulated by BCR-ABL in K562 cells. Small interference (si)RNA knockdown of BCR-ABL in K562 cells, decreased miR-130a and miR-130b, and increased the expression of their putative target, the growth negative regulator CCN3 (72). Another study identified miR-96 upregulation, and miR-120, miR-151 and miR-10 downregulation in CML. Conversely, miR-10 downregulation was shown to be independent to BCR-ABL signalling (62). Consistent with these data, a recent study demonstrated that the expression of miR-424 is markedly low in CML cell lines and patient samples at the time of diagnosis. In addition, with the aid of bioinformatic analysis and via luciferase assays, the authors revealed a conserved target site for miR-424 in the 3'UTR of the ABL gene, showing that this miRNA directly targets BCR-ABL. In fact, its overexpression was able to induce apoptosis of K562 cells as well as sensitize these cells to imatinib treatment. These data suggest that miR-424 acts as a tumor suppressor by downregulating BCR-ABL expression (73). Furthermore, Fallah *et al* found differential expression of miRNAs, derived from leukocytes in the peripheral blood of 50 newly diagnosed CML patients in chronic phase using stem-loop reverse transcription-polymerase chain reaction. Some onco-miRNAs were found to be downregulated (miR-155 and miR-106), and some tumor-suppressor miRs (miR-16-1, miR-15a, miR-101 and miR-568) were upregulated. These results showed that few miRNAs alone are good candidates for CML diagnosis independently of conflicting results, but together could be an additional tool for CML diagnosis (74).

CML treatment has been revolutionized by TKIs most notably imatinib, which acts by inhibiting BCR-ABL. A significant number of patients, however, suffer from imatinib resistance (75). For example, miR-146 upregulation targets members of the nuclear-factor-κ-B (NF-κB) pathway [interleukin-1-associated kinase 1 (IRAK1)/tumor-necrosis-factor -receptor-associated factor 6 (TRAF6)] that are found to be constitutively activated in CML by BCR-ABL (76). Furthermore, inhibition of the NF-κB pathway in CML results in apoptosis, suggesting that the upregulation of miR-146 post-imatinib treatment makes CML cells more sensitive to apoptotic signaling (77). miR-199-5b was found to be a regulator of the Notch pathway through its targeting of the transcription factor Hes1 and it may promote cancer growth in CML, inhibiting Hes1 (70). Xu *et al* showed the feedback regulation among BCR-ABL, GATA1 transcription factor and miR-138. In fact, these authors demonstrated that overexpression of miR-138 represses BCR-ABL and cyclin D3 (CCND3) by binding to the coding and 3'UTR regions, respectively (78).

Notably, miR-138 expression is increased by GATA1, and repressed by BCR-ABL in addition to imatinib resistance in CML (78). Turrini *et al* found that miR-212 increased the ATP-binding cassette subfamily G member 2 (ABCG2) expression upon treatment with imatinib in CML (79). Another recent study showed that imatinib markedly inhibits expression of miR-30a in human CML cells. This miRNA is a potent inhibitor of autophagy by downregulating Beclin-1 and autophagy protein 5 (ATG5) expression and miR-30a mimic enhances imatinib-induced cytotoxicity and promotes mitochondrial-dependent intrinsic apoptosis. In contrast, knockdown of miR-30a by antagomir-30a increases the expression of Beclin-1 and ATG5, and inhibits imatinib-induced cytotoxicity. These findings indicate that dysregulation of miR-30a may interfere with the effectiveness of imatinib-mediated apoptosis by an autophagy-dependent pathway, thus representing a novel potential therapeutic target in CML (80). Different studies were performed to find a relationship between epigenetic dysregulation of miRNAs and CML. A recent study found that 48 miRNAs of CpG-rich 212 miRNAs were upregulated after imatinib treatment via microarray analysis. In particular, imatinib induced the demethylation of the miR-203 promoter region, resulting in low expression of targeted BCR-ABL gene, and loss of proliferation of leukemic cells (81). Recent evidence indicates that the miRNA expression could be linked to the onset of resistance to TKIs. In this context, a study demonstrated the relationship among expression changes of specific miRNAs and resistance to imatinib or responsiveness to imatinib after treatment in CML patients. Nineteen miRNAs differentially expressed between resistant and responder patients (imatinib) were identified: 18 of them were downregulated (hsa-miR-7, hsa-miR-23a, hsa-miR-26a, hsa-miR-29a, hsa-miR-29c, hsa-miR-30b, hsa-miR-30c, hsa-miR-100, hsa-miR-126#, hsa-miR-134, hsa-miR-141, hsa-miR-183, hsa-miR-196b, hsa-miR-199a, hsa-miR-224, hsa-miR-326, hsa-miR-422b and hsa-miR-520a) and only one was upregulated (hsa-miR-191) in resistant CML patients (82). Lopotová *et al* (83) and Scholl *et al* (84) reported that CML patients with imatinib-resistance showed lower levels of miR-451 compared with responders. In particular, the increased levels of 13 miRNAs (miR-19a, miR-19b, miR-17, miR-20, miR-92a, miR-106a, miR-221, miR-222, miR-126, miR-146a, miR-181a, miR-181b, let7c and miR-55) and decreased levels of 4 miRNAs (miR-103, miR-150, miR-451 and miR-144) in PBMCs from Blast Crisis (BC)-CML patients were used to uncover signaling pathways and their role in CML (83,84). Several miRNAs (e.g. miR-191, miR-29a, miR-422b, miR-100, miR-326 and miR-26a) are promising predictors of imatinib resistance in newly diagnosed CML patients. A study suggested that miR-181b and miR181d are associated with myeloid cell leukemia-1 (Mcl 1) in Lyn-mediated imatinib-resistant CML cells. Incubation of these cells with a Lyn inhibitor (dasatinib) increased miR-181b and miR-181d expression (85). In line with this study, Liu *et al* (86) described a reciprocal regulatory correlation between c-Myc and miRNA-144/451. In particular, Myc is upregulated in imatinib-resistant K562 cells, where it inhibited miRNA-144/451 transcription. Additionally, You *et al* analyzed the circulating miRNA profile in the culture supernatant of imatinib-resistant K562 CML cells (K562-R) by microarray chip analysis. They found that specific miRNAs are associated with nilotinib sensitivity by comparison of the miRNA expression patterns from the culture supernatant of nilotinib-treated K562-R cells with the culture supernatant of untreated K562-R cells (miRNA-221, -379, -548, -603 and -648). The information obtained from this study may have the potential to identify a novel biomarker to predict drug response in the future (87). In another study, the authors examined the expression levels of miR-17 which possesses oncogenic activities through downregulation of cyclin-dependent kinase N1A (CDKN1A), p21 and E2 transcription factor 1 (E2F1) tumor suppressor genes, in imatinib-sensitive and -resistant K562 cells compared to PBMCs derived from healthy donors by stem-loop RT-PCR. A significant decrease was observed in miR-17 levels in response to imatinib, dasatanib and nilotinib in K562 IM-R cells. These data proved that miR-17 may be a crucial target for the treatment of CML (88). Consistent with these data, Mosakhani *et al* performed miRNA microarray (followed by qRT-PCR verification) on available diagnostic bone marrow core biopsies derived from CML patients including 4 imatinib-resistant and 5 imatinib-responder CML patients. They found a significant downregulation of miR-181c in imatinib-resistant vs. imatinib-responders and some miR-181c target genes such as pre-B cell leukemia homeobox 3 (pre-PBX3), heat shock protein 90 kDa β member 1 (HSP90B1), N-myristoyltransferase 2 (NMT2) and RAD21 were associated with the drug response (89). Finally, Ohyashiki *et al* identified downregulation of miR-148b in patients of the STOP-IM group and in a subset of the imatinib group (90). In this context, a recent study found both new and differential expression of miRNAs in $CD34^+$ CML stem/progenitor cells obtained at diagnosis from bone marrow of CML patient IM-responders, IM-non-responders after imatinib therapy, and of healthy donors. Bioconductor Illumina deep sequencing (DESeq) analysis revealed 63 differentially expressed miRNAs in the $CD34^+$ cells from CML and healthy donor samples. Notably, 12 of these were differentially expressed in $CD34^+$ cells from the IM-responders and non-responders. Most of the 63 differentially expressed miRNAs identified were present at reduced levels in the $CD34^+$ CML cells as compared to cells derived from healthy donors, while 17 miRNAs were increased. In addition, 34 novel miRNAs were identified in the $CD34^+$ CML stem/progenitor cells. The authors, next, validated the sequencing data in $CD34^+$ cells from IM-responders and IM-non-responders and normal individuals using a high-throughput quantitative microfluidic device. This study confirmed the differential expression in $CD34^+$ CML cells of 32 of the 63 identified miRNAs, including an increased level of oncomiRs miR-155 and miR-17-92, and a decreased level of the tumor suppressors, miR-145, miR-151 and miR-452. Importantly, the authors detected significant changes in some of these miRNAs in $CD34^+$ cells from CML patients after three months of nilotinib treatment (23 normalized after three months of nilotinib treatment, whereas 10 showed little change). To further correlate miRNA profiles with corresponding mRNA expression changes and to identify potential target genes, RNA-sequencing was performed on the same RNA samples. Differentially expressed mRNAs (1,210) were identified that were predicted targets of the deregulated miRNAs in the comparison of CD34 cells derived from CML patients and normal individuals. Strikingly, only seven differentially

Table I. Several miRNAs involved in chronic myeloid leukemia.

miRNAs	Relevance	Putative targets	Authors, year (ref.)
miR-29 family	Bcl-2 family plays a Bcl-2 family central role in cell death regulation		Peláez and Carthew, 2012 (26)
miR-29a/29b	High expression level of both Bcl-2 and Mcl-1 in peripheral blood mononuclear cells (PBMCs) from AML and CML patients	Bcl-2 and Mcl-1	Xu *et al*, 2014 (59)
miR-21	The downregulation of this miRNA caused a decrease in the expression levels of Bcl-2 protein in leukemic cells	Bcl-2 protein	Seca *et al*, 2013 (60)
anti-miR-21	It caused both an increase in both the autophagy-related proteins (Beclin-1 and LC3-II)	Beclin-1, LC3-II	Seca *et al*, 2013 (60)
miR-10a	Transcription factor upstream stimulatory factor 2 (USF2) has been considered as a miR-10a in CML patients	USF2	Aggire *et al*, 2008 (62)
miR-486-5p	miR-486-5 enhances PI3K/AKT signaling in hematopoietic cells in association with reduction in PTEN and FoxO1 levels	PI3K/AKT, PTEN, FoxO1	Wang *et al*, 2015 (63)
miR-326	The downregulation of this miRNA was a possible mechanism for unrestricted activation of Smo signal transducer of the oncogenic Hedgehog (Hh) pathway	Hh receptor components (i.e. Smo and Ptch1)	Babashah *et al*, 2013 (64)
miR-17-92	Chromosomal amplification at 13q31-q32	c-MYC	Venturini *et al*, 2007 (65)
miR-328	HnRNP E2 mediated the loss of miR-328 in the BC-CML pathway	MAPK-hnRNP E2	Eiring *et al*, 2010 (66)
miR-150	Reduced expression of this miRNA is found in CD34$^+$ cells derived from CML patients at diagnosis and also in total leukocytes of PBMCs derived from CML patients in AP and BC phases	CBL E3 ubiquitin-protein ligase (MAPK signaling)	Aggire *et al*, 2008 (62)
miR-150 and miR-146a miR-142-3p and miR-199b-5p	An increased expression of miR-150 and miR-146a and a decreased expression of miR-142-3p and miR-199b-5p in PBMCs derived from 140 patients newly diagnosed with CML and treated with imatinib	The p38 MAPK and JNK signaling pathways	Flamant *et al*, 2010 (70)
miR-31, miR-155 and miR-564	miR-31, miR-155 and miR-564 were decreased in CML and influenced by BCR-ABL activity	MAPK, ErbB, mTOR and VEGF	Rokah *et al*, 2012 (71)
miR-130a and miR-130b	miR-130a expression is also regulated by BCR-ABL in K562 cells siRNA knockdown of BCR-ABL in K562 decreased miR-130a and miR-130b and increased the expression of their putative target, the growth negative regulator CCN3	CCN3	Suresh *et al*, 2011 (72)
miR-155	miR-155 was found to be downregulated in CML	E2F2 (MAPK signaling)	Machová Poláková *et al*, 2011 (69)

Table I. Continued.

miRNAs	Relevance	Putative targets	Authors, year (ref.)
miR-150	miR-150 is involved in Myb regulation (a critical oncogene in CML)	MYB	Machová Poláková *et al*, 2011 (69)
miR-146	miR-146 upregulation targets members of the NF-κB pathway (IRAK1/TRAF6) that are found to be constitutively activated in CML by BCR-ABL	NFκB pathway	Taganov *et al*, 2006 (76)
miR-138	Overexpression of miR-138 represses BCR/ABL1 and cyclin D3	GATA-1	Xu *et al*, 2014 (78)
miR-30a	miR-30a is a potent inhibitor of autophagy	Beclin-1 and ATG5,	Yu *et al*, 2012 (80)
miR-203	Imatinib induced the demethylation of the miR-203 promoter region	DNA methyltransferase	Shibuta *et al*, 2013 (81)
miR-181	miR-181 is associated with myeloid cell leukemia-1	Bcl-2 family protein	Zimmerman *et al*, 2010 (85)
miRNA-144/451	miRNA-144/451 transcription is inhibited by c-Myc	c-Myc	Liu *et al*, 2012 (86)
miR-17	miR-17 possesses oncogenic activities through downregulation of CDKN1A, p21 and E2F1 tumor-suppressor genes	CRK and MAPK signaling	Firatligil *et al*, 2013 (88)
miR-181c	miR-181c targets genes such as PBX3, HSP90B1, NMT2 and RAD21	PBX3, HSP90B1 NMT2 and RAD21	Mosakhani *et al*, 2013 (89)
miR-199b and miR-219-2	Downregulation of miR-199b and miR-219-2 in the 9q deleted CML	MAPK signaling and TGF-β signaling pathways	Joshi *et al*, 2014 (92)
miR-217	miR-217 inhibited expression of DNMT3A	AKT, ERK and STAT5 signaling via epigenetic silencing of the PTEN gene expression DNMT3A	Nishioka *et al*, 2014 (93)

expressed mRNAs were identified from comparison of the IM-responders and non-responders. Most of these were predicted to have roles in regulation of the cell cycle, mitogen-activated protein kinase (MAPK) signaling and transforming growth factor-β (TGF-β) signaling pathways. Thus, aberrant, differentially expressed miRNAs and target genes identified in primitive CML stem/progenitor cells may serve as useful biomarkers to predict clinical response of CML patients to TKI therapy (91). Additionally, since the miR-219-2 and miR-199b (centromeric to the ABL1 gene) are frequently lost in CML patients, a study analyzed 150 CML patients in order to identify 9q deletion. Fluorescent *in situ* hybridization (FISH) (BCR-ABL dual color) revealed 9q34.1 deletion in 34 (23%) CML patients. The expression level of miRNA, analyzed by real-time polymerase chain reaction (RT-PCR), showed downregulation of miR-199b and miR-219-2 in the 9q deleted patients (34 CML) as compared to a pool of patients without deletion. However, miR-199b (9q34.11) was significantly downregulated compared to miR-219-2. The follow-up study showed that miR-199b was found to be strongly associated with imatinib resistance, as 44.11% patients showed resistance to imatinib therapy. Hence, the deletion in the 9q34.1 region (ABL) plays an important role in disease pathogenesis (92). Finally, Nishioka *et al* found that long-term exposure of K562 cells to BCR-ABL TKI caused drug-resistance in association with an increase in levels of DNA methyltransferases (DNMTs) and a decrease in levels of miR-217. In addition, an increase in levels of DNMT3A in association with downregulation of miR-217 were observed in leukemia cells isolated from individuals with BCR-ABL TKI-resistant Philadelphia-positive ALL and CML. Further studies with TKI-resistant K562 cells found that forced expression of miR-217 inhibited expression of DNMT3A through a miR-217-binding site within the 3' untranslated region of DNMT3A and sensitized these cells to growth inhibition mediated by the TKI. Notable, long-term exposure of K562 cells to dasatinib in combination with 5-Aza-2'-deoxycytidine (5-Aza-dC) potently inhibited proliferation of these cells in association with upregulation of miR-217 and downregulation of DNMT3A

in vitro. Furthermore, a decrease in levels of DNMT3A and an increase in levels of miR-217 were noted in K562 tumors in immune-deficient mice that were treated with the combination of 5-Aza-dC and dasatinib. Taken together, Ph+ leukemia cells acquired TKI resistance via downregulation of miR-217 and upregulation of DNMT3A (93).

4. Conclusion

A decade after its discovery, miRNAs are rapidly entering the clinic as biomarkers and therapeutic tools. The fact that miRNAs can regulate the expression of multiple genes makes them attractive drug targets, as multiple oncogenes can be inhibited at the same time. However, the fact that miRNAs can regulate multiple genes is also a major drawback, due to the risk of unwanted side-effects. To reduce side-effects, tumor-specific delivery may be necessary. miRNAs, that can act as oncogenes or tumor suppressor genes in CML, contribute to the pathogenesis, disease progression, and response to therapy of CML and resistance to TKIs. The potential of using these small RNAs as therapeutic targets opens up new opportunities for leukemia therapy by either inhibiting or augmenting their activity. In conclusion, with miRNA-based therapeutics entering early phase human clinical trials, hope for a novel class of effective anticancer agents may be realized.

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