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MicroRNAs in Myeloid Hematological Malignancies

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Abstract: MicroRNAs are 19-24 nucleotides noncoding RNAs which silence modulate the expression of target genes by binding to the messenger RNAs. Myeloid malignancies include a broad spectrum of acute and chronic disorders originating from from the clonal transformation of a hematopoietic stem cell. Specific genetic abnormalities may define myeloid malignancies, such as translocation t(9;22) that represent the hallmark of chronic myeloid leukemia. Although next-generation sequencing provided new insights in the genetic characterization and pathogenesis of myeloid neoplasms, the molecular mechanisms underlying myeloid neoplasms are lacking in most cases. Recently, several studies have demonstrated that the expression levels of specific miRNAs may vary among patients with myeloid malignancies compared with healthy individuals and partially unveiled how miRNAs participate in the leukemic transformation process. Finally, *in vitro* experiments and pre-clinical model provided preliminary data of the safety and efficacy of miRNA inhibitory molecules, opening new avenue in the treatment of myeloid hematological malignancies.

Keywords: miRNAs, Acute myeloid leukemia, Myelodysplastic syndrome, Myeloproliferative neoplasms.

1. INTRODUCTION

MicroRNAs (miRNA) are 19-24 nucleotides noncoding RNAs (ncRNA) which regulate the expression of target messenger RNAs (mRNAs) [1, 2]. Mature miRNA are processed from long, capped and polyadenylated precursors which are cleaved in the nucleus, exported to the cytoplasm where duplex miRNA are transformed into single strand miRNA and associated with RISC (RNA-induced silencing complex) [1, 2]. By binding with the seed sequence, mainly at the 3'-untraslated region (3'-UTR) of the target messenger, miRNAs inhibit the translation or favor the destabilization of mRNAs depending on the degree of nucleotide pairing [3, 4].

Recently, several studies have elucidated the physiological roles of miRNAs as key regulators of hematopoiesis [5-7]. The discovery that miRNAs are able to finely tune cell machinery at crucial points suggested the possibility that genetic aberrations at miRNAs gene sequences may contribute to cancer development [8]. Interestingly, miRNAs function, as oncogenes or tumor-suppressors respectively, is strictly dependent on the on the target genes but also may associate with the specific context of normal and tumor cells. The identification of specific miRNA expression profile among normal and tumor tissues has several diagnostic, prognostic and therapeutic implications. Although preliminary, recent findings support the possibility of using miRNAs expression to predict response to specific treatment or outcome among patients with hematological malignancies [9, 10]. Finally, the inhibition of miRNAs by means of antagonist inhibitory molecules in pre-clinical models provided new insights in the understanding of the intricate miRNAs network in the pathogenesis of hematological malignancies and opened a new avenue in their treatment [11].

2. ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) represents a heterogeneous group of malignancies including subtypes with different biological features and outcomes [12, 13]. The revised World Health Organization (WHO) classification of myeloid malignancies emphasized the crucial role of specific genetic abnormalities in the definition of AML subtypes [13]. However, the availability of more accurate genetic assessment tests allowed us to better understand the molecular mechanisms underlying the most common genetic abnormalities and to provide evidence of the role of new molecular markers that were not unveiled by conventional cytogenetic analysis [12]. Moreover, gene expression profiling (GEP) of patients with normal cytogenetics AML (NC-AML) have shown the presence of recurrent gene mutations explaining the unexpected clinical heterogeneity among patients with NC-AML [14].

In AML the aberrant expression of miRNAs, the association of miRNA expression profiles with cytogenetic characteristics and their impact on treatment response and outcome has been proven by different groups [9, 15-19]. Nevertheless, the pathogenetic mechanisms, i.e. the pathways that miRNAs interfere with in leukemic cells, are in some cases lacking.

In this review we will explore the association between AML and specific miRNAs or miRNA profiles following the main categorization of AML into favorable (core binding

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factor, *CBF* leukemias; acute promyelocitic leukemia, APL; AML with mutations of CCAT/enhancer binding protein-alpha, *CEBPA* or C/EBPα; AML with mutations of nucleophosmin, *NPM*) and unfavorable AML (AML with fms-like tyrosine kinase receptor 3 internal tandem duplications, *FLT3-ITD*; AML with abnormalities of the myeloid and lymphoid lineage, MLL gene; AML with deletion 7q or monosomy 7; AML with KIT or *BAALC,* brain and acute leukemia cytoplasmic, overexpression) [12, 13]. Then we will analyze the role of miRNAs among patients with myelodysplastic syndrome (MDS) and myeloproliferative neoplasms (MPN).

Importantly, the integration of prognostication system with miRNA profile might contribute to better distinguish between low and high risk subtypes addressing clinicians in the choice of therapy strategy among several treatment options including standard chemotherapy, bone marrow transplantation and new biological agents. Furthermore, preclinical models and *in vitro* experiments suggest that targeting miRNAs can increase leukemia cells susceptibility to chemotherapy.

2.1. AML with Favorable Risk

CBF-AMLs include cases harboring either translocation (t)(8;21) (q22;q22) or inversion (inv)(16) (p13q22)/t(16;16) which lead to the formation of *AML1/ETO* or *RUNX1/RUNX1T1* and CBFB-MYH11, fusion gene, respectively [20, 21].

It has been reported the *miR-126* is upregulated in both t(8;21) and inv(16) leukemias [18, 19, 22]. Following large-scale miRNA expression profiling, including 47 *de novo* AML, significantly higher levels of *miR-126* were observed in CBF leukemias without evidence of amplification at the *miR-126* locus (9q34.3). Likely, a lower degree of DNA methylation at the *miR-126* locus may explain its upregulation in leukemic blasts from CBF leukemias [22].

The expression levels of *miR-193a* were found downregulated in cells isolated from patients with t(8;21) AML and associated with event-free (EFS) and overall survival (OS) [23]. In leukemic blasts, AML1/ETO repressed *miR-193a* and *PTEN* by binding at the AML1-binding sites and recruiting chromatin-remodelling enzymes. The silenced *miR-193a* resulted in the loss of the inhibition over the oncoprotein AML1/ETO that *miR-193a* usually exerts [23]. Furthermore, *miR-193a* may directly repress not only *AML1/ETO* but also *DNMT3a* (DNA methytransferase), *HDAC* (histone deacetylase), *KIT (*CD117*)*, the cyclin *CCND1* and *MDM2* (murine double model 2) genes whereas the tumor suppressor *PTEN*, a protein involved in the PTEN/PI3K pathway, is upregulated by *miR-193a* [23]. The *miR-193a* circuitry provides explanation for the observation that KIT is usually overexpressed and predicts poor outcome in patients with t(8;21) AML [24] and suggests that the enhancement of *miR-193a* in leukemic blasts might reverse the overexpression of its target genes and restore *PTEN* activity, apoptosis and cell differentiation [23].

Likewise, either t(8;21) or inv(16) AML overexpressing KIT antigen display downregulation of *miR-221/222* and *miR-223* compared with normal bone marrow precursors [25]. *miR-221/222* binds at the 3' UTR of the *KIT* messenger and, thereby, low *miR-221/222* levels result in *KIT* upregulation. Similarly, AML1/ETO binds at the AML1-binding site of the myelopoiesis regulator *pre-miR-223* gene silencing its trascription [7]. Interestingly, the ectopic expression of *miR-223* in HL60 and SKNO-1 cell lines along with demethylating treatment or RNA inhibitory molecules against AML1/ETO, reprogrammed myeloid differentiation overcoming maturation block [7].

In blasts from patients with overt t(8;21) AML following myelodysplastic syndrome, *RUNX1* (or AML1) may delocalize subnuclearly [26]. The same subnuclear localization was observed in murine myeloid progenitor 32D cell lines following single amino acid substitution. Subnuclear RUNX1 associated with deregulation of *miR-24-23-27* cluster and *miR-181,* enhancement of cell proliferation and blockade of myeloid differentiation via *miR-24*/MAP kinase pathway. Furthermore, 32D cells transfected with *miR-24* and *miR-23* and cultured in growth factors enriched medium (IL-3 and G-CSF) showed morphological hallmarks of myeloid differentiation and, limited to *miR-24* transfected cells, increased expression of myeloid lineage markers of differentiation (granzyme B, CD11b and myeloperoxidase) suggesting that *miR-24/miR-23* may play a crucial role in leukemogenesis of t(8;21) AML [26].

Recently, a dutch group investigated the miRNA profiles from 90 pediatric patients with *de novo* AML including cases with well characterized cytogenetic abnormalities, that is t(8;21), inv(16), t(15;17), t(7;12) and MLL-rearrangements AML [27]. Despite the selection criteria could have biased the results, *miR-9* has been shown downregulated in t(8;21) AML cases (p=0.001). By gain-of-function experiments in leukemic blasts and in xenograft models the authors proved that *miR-9* is a tumor suppressor, which can inhibit tumor growth and colony-forming capability, and induce monocytic differentiation [27].

Acute promyelocitic leukemia (APL) is defined by the t(15;17) translocation resulting in the juxtaposition of the PML and RARA genes, [28]. The backbone of treatment for patients with APL includes all-trans retinoic acid (ATRA) in combination with trioxide arsenic (ATO) or antracycline-based chemotherapy. The ATRA based regimens have significant prolongated the OS with manageable toxicity [29].

Numerous miRNAs are predicted target of PML-RARA response elements by seed sequence pairment [30]. By using a quantitative real time PCR (qRT-PCR) approach, a restricted signature of miRNAs distinguished promyelocitic blasts from normal promyelocytes. Out of 12 granulocyte differentiation signature miRNAs, nine miRNAs resulted overexpressed whereas *miR-107*, *miR-342* and *let-7c* were downregulated [31]. Additionally, by SNP array study in blasts from 93 patients with APL, the most common copy number alteration (CNAs) was the deletion of 1q31.3 encompassing the coding regions for *miR-181a1/b1*. Although, mir181a1 and mir181b1 precursors were not differentially expressed in samples with or without del1q31.3, the 1q31.3 deletion was associated with higher number of copy number alterations (CNAs) and increased risk of relapse (HR=28.9, p=0.0031) [32].

Interestingly, downregulation of *miR-181b* and upregulation of *miR-15a, miR-15b*, *miR-16*, *miR-107*, *miR-223*, *miR-342* and *let-7* family were noted following successful treatment with ATRA and chemotherapy [30, 31, 33]. Several mechanisms have been proposed to explain how ATRA acts on miRNAs network in APL blasts [30, 31, 34]. Precisely, ATRA treatment is associated with reduction and/or disappearance of PML/RARA gene-product that, in turn directly regulates the expression of *miR-342* and *let-7c* [31]. Alternatively, following ATRA treatment PU.1 and IRF9 binding at the promoter region restored *miR-342* levels and myeloid differentiation [34]. Finally, homeobox-family genes seemed to be modulated by PML/RARA through miRNAs [11, 30].

Few studies have been conducted to validate miRNAs as prognostic markers within large randomized multi-institutional clinical studies (Table **1**). The absence of *NPM1* mutations and the occurrence of *FLT3-ITD* are clearly have been clearly associated with worse prognosis among patients with CN-AML [35]. ***NPM1*** **mutations** that result in cytoplasmic localization of nucleophosmin (NPMc+ AML) have been observed in one third of AML among adults and, in absence of *FLT3-ITD*, are associated with favorable outcome [14, 35]. Among 85 patients with *de novo* AML, upregulation of *miR-10a*, *miR10b*, *let-7* and *miR-29* family was found to clearly distinguish patients with *NPMc+* mutated from wild type (wt) *NPM1* cases [16]. Notably, among patients with *NPMc+* AML *miR-10a* positively correlated with homeobox-family gene *HOXB4* that is located at the same locus of *miR-10a* in chromosome 17q21 suggesting that both genes may be regulated by the same cis elements [16].

Recently, it has been reported that high baseline *miR-10* family members expression levels predict the probability of complete remission among 54 previously untreated patients with AML at a single center (p=0.002) [36]. At the multivariable analysis including *miR-10*-5p, NPM1 mutational status, age, unfavorable prognostic group, normal cytogenetics) *miR-10-5p* expression levels and NPM1 mutational status were significantly associated with survival (p=0.019 and p=0.005, respectively). Similar results were noted among a validation cohort of 183 patients of 60 yrs or older with *de novo* CN-AML treated on Cancer and Leukemia Group B (CALGB)/ALLIANCE protocol [36]. Despite the previous demonstration of a strong positive correlation between HOX-related genes and *miR-10* and *miR-20a* [37], the authors failed to prove *miR-10-5p* affecting apoptosis and cell proliferation at the baseline and after chemotherapy in cell lines and primary blasts by functional experiments [36].

On the contrary, *miR-204* is downregulated in patients with NPMc + AML. By performing western blotting in cell lines with high expression of HOXA9, HOXA10 and MEIS1 (NPM1+ OCI-AML3 cell line and MEG-01 cell line), transfection with *miR-204* lentivirus has been shown to robustly downregulate *HOXA10* and *MEIS1* but not *HOXA9* protein. Accordingly, HOXA10 and MEIS1 protein levels were reversed after an antisense oligonucleotide against *miR-204* was introduced [14].

MiR-181 family members have been found overexpressed among patients with *CN-AML* and *CEBPA* mutations and associated with event-free (p<0.001), disease-free (p=0.004) and overall survival (p=0.009) independently from other molecular and clinical features [38]. Indeed, the truncated C/EBPα-p30 isoform, that derives from *CEBPA* mutated gene, has been demonstrated to bind *miR-181a-1* promoter and upregulate its transcription [39].

2.2. AML with Unfavorable Risk

Several studies observed that *miR-155* expression levels are independently associated with high risk AML and poor outcome. (Table **2**) [9, 15] The role that *miR-155* plays in hematopoiesis and hematological malignancies was described by Mallardo *et al.* [85]

By using miRNA expression profiling, 12 (*miR-181* family, *miR-124*, *miR-128-1*, *miR-194*, *miR-219-5p*, *miR-220a*, *miR-320*) out of 305 miRNAs were differently associated with event-free survival (EFS) among patients younger than 60 yrs and with normal karyotype, wild type *NPM1* and *FLT3-ITD* [17]. The miRNA signature that was derived from 64 patients treated in the CALGB 19808 multicenter study, was confirmed by the analysis of a validation cohort including 55 patients from the CALGB 9621 study who received similar treatment but significantly differed from training group in the white-cell count, the grade of blast bone marrow infiltration, the percentage of circulating blasts, and finally the proportion of patients with high levels of *ERG* gene expression in leukemic cells. In the confirmatory group, the miRNA summary value was confirmed to correlate with EFS (p=0.03) and the percentage of circulating blasts (p=0.004) [33]. The CALGB group confirmed *miR-181* as prognostic markers in a larger population of patients with CN-AML, especially within poor molecular risk patients with *FLT3-ITD* and/or wt*NPM1* [40]. *Mir-181* was measured at the baseline in bone marrow cells from 187 younger adults (< 60 yrs) treated with intensive induction therapy and consolidation autologous bone-marrow transplantation according with CALGB9621 and 19808 and in 122 older patients (≥ 60 yrs) (validation cohort). Higher *miR-181* expression levels at the baseline were significantly associated with better CR rate (p=0.04) and longer OS (p=0.01). In both reports, the multivariable analysis including the most reliable molecular markers, *miR-181* retained its prognostic power [17, 40]. Interestingly, the expression levels of *miR-181* family were inversely associated with the risk of event [17] and were associated with the expression levels of predicted target genes encoding proteins that mediate the intracellular transduction following the activation by toll-like receptors and interleukin-1β [17, 18, 40].

The CALGB group analyzed the gene expression signature of 72 patients aged ≥ 60 yrs with primary CN-AML harboring *FLT3-ITD* and treated frontline with intensive chemotherapy [41]. Although the treatment included in some cases investigational drugs, patients with *FLT3-ITD* had significantly shorter DFS and OS compared with *FLT3-WT* (p 0.007 and < 0.001, respectively). However, the difference disappeared among patients aged ≥ 70 yrs. As expected, *FLT3-ITD* samples correlated with increased expression of *miR-155* and *miR-125b-2*. Additionally, m*iR-144*, *miR-451*, *miR-488*, *miR-486-5p* were encountered among the most downregulated miRNAs in the same group of patients [41].

RUNX-1 mutations in elderly with wt*NPM1* are associated with poor outcome [42, 43]. Recently, a study has

Table 1. Results of miRNA profiles within clinical trials including patients with AML.

References	Cell source	Training cohort (n°)	Validation cohort (n°)	Dysregulated miRNAs	Prognostic or Risk-group correlation	Multivariable analysis	Parameters included at the multivariable analysis
Havelange *et al*, 2014	Pre-treatment BM	54 adult patients with *de novo* AML	183 elderly patients (≥60 yrs)	*miR-10* family	Higher *miR-10a* and *miR-10b* levels associated with the odds of achieving CR	Yes	miR-10a and -10b levels, NPM1 mutations, age, unfavorable cytogenetic, CN-AML (training cohort). miR-10a and -10b, NPM1 mutations, BAALC expression (validation cohort)
Li *et al*, 2013	pre-treatment BM and PB	81 patients AML-M2 (AML/ETO+ and AML/ETO-)	89 patients with(8;21) AML	*miR-193a*	overexpression of *miR-193a*: better EFS and OS	No	NA
Marcucci *et al*, 2013	pre-treatment BM and PB	363 patients with *de novo* NC-AML	Not included	*miR-155*	High *miR-155*levels were associated with WBC counts, percentage of blasts, *FLT3-ITD*, *RUNX1* mutated, *WT1* mutated, high *ERG* and *BAALC* High miR-155 had lower odds of achieving CR, shorter DFS and OS	Yes	Age, race, WBC count, *miR-155, NPM1*status*, FLT3-ITD*, *BAALC* and ERG expression
Blum *et al*, 2010	pre-treatment BM	53 previously untreated AML patients ≥60 yrs	Not included	*miR-29b*	High pre-treatment *miR-29b* had better response to decitabine	No	NA
Schwind *et al*, 2010	pre-treatment BM	187 adult patients with CN-AML < 60 yrs	122 patients with CN-AML, ≥60 yrs	*miR-181a*	Higher *miR-181a* associated with better CR, longer OS and a trend in longer DFS	Yes	Age, WBC count, *miR-181a, ERG and BAALC* expressione, *CEBPA and NPM1* mutation*, WT1, FLT3-ITD*.
Whitman *et al*, 2010	pre-treatment BM or PB	243 adult patients with CN-AML	not included	*miR-155, miR-144 miR-451*	Overexpression of *miR-155* and underexpression of *miR-144* and *miR-451* associated with *FLT3-ITD*		

(Table 1) contd….

References	Cell source	Training cohort (n°)	Validation cohort (n°)	Dysregulated miRNAs	Prognostic or Risk-group correlation	Multivariable analysis	Parameters included at the multivariable analysis
Garzon *et al*, 2008	pre-treatment BM	85 adult patients with AML	not included	*miR-10a, miR-10b, miR-204, miR-155*	*miR-10a*, *miR-10b*, *let-7*, *miR-29* family were upregulated in NPMc+ AML *miR-155* was upregulated in *FLT3-ITD* AML	No	NA
Marcucci *et al*, 2008	pre-treatment BM	64 patients with high molecular risk CN-AML < 60 yrs	55 patients with high molecular risk CN-AML	*miR-181 family, miR-124, miR-128, miR-194, miR-219, miR-220a, miR-320*	*miR-181* family was inversely associated with EFS (death, lack of CR, or relapse) *miR-124*, *miR-128*, *miR 194, miR-219, miR-220a, miR-320* were positively with EFS	Yes	MicroRNA summary value, ratio of *FLT3-ITD* to *FLT3* (high vs low) and WBC count (log), age, sex, race, Hb level, platelet count, percentage of blasts, the presence or absence of extramedullary involvement, *NPM1* mutation, and *BAALC* and *ERG* expression

Annotations: CN-AML, cytogenetics normal, acute myeloid leukemia; yrs, years; EFS, event free survival; OS, overall survival, WBC, white blood cell; FLT3-ITD, fms-like tyrosine receptor internal tandem duplications; WT1, Wilm's tumor gene; BAALC, brain and acute leukemia cytoplasmic gene; CR, complete response; DFS, disease free survival; NPM1, nuclephosmin; CEBPA, CCAAT/enhancer binding protein-alpha gene; NPMc+, nucleophosmin cytoplasmic positive.

Table 2. miRNA profiles associated with specific molecular or cytogenetic abnormalities in acute myeloid leukemia and their targets.

Disease and Specific Molecular Abnormalities	Dysregulated miRNAs	Predicted Target	Reference
CBF [t(8;21) and inv(16)]	*miR-126/miR-126** (upregulated)	PLK2, SPRED1	Li *et al*, 2008
	miR-193 (downregulated)	AMl1/ETO, DNMT3a, HDAC, KIT, CCND1, MDM2	Li *et al*, 2013
	miR221/222 (downregulated)	KIT	Brioschi *et al*, 2010
	miR-9 (downregulated)	LIN28B/HMGA2	Emmrich *et al*, 2014
	miR-223 (downregulated)		Fazi *et al*, 2007
t(15;17)	*miR-224, miR-368, miR-382* (upregulated)	Notevaluated	Li *et al*, 2008
	miR-181 (downregulated)	Notevaluated	Nowak *et al*, 2012

(Table 2) contd….

Disease and Specific Molecular Abnormalities	Dysregulated miRNAs	Predicted Target	Reference
	miR-15a, miR-15b, miR-16, miR-142, miR-181b, miR-223, let-7a, let-7d (upregulated); *miR-107, miR-342, let-7c* (downregulated)	Not evaluated	Careccia *et al*, 2009
MLL-rearrangements	*miR-17-5p, miR17-3p, miR-18a, miR-19a, miR-20a, miR-19b, miR-92 (polycistronic)* (upregulated)	not evaluated	Li *et al*, 2008
	miR-191 (upregulated) *miR-29* (downregulated)	not evaluated	Garzon *et al*, 2008
NPMc+	*miR-10a, miR-10b, let-7, miR-29 family, miR-15a-16-1, miR-17-18a-19a-20a cluster*(upregulated)	HOXAB4 (*miR-10*); HAXA10 MEIS1 (*miR-204*)	Garzon *et al*, 2008
FLT3-ITD	*miR-155* (upregulated)		Garzon *et al*, 2008
	miR-181 family, miR-124, miR-128, miR 194, miR-219, miR-220a, miR-320(upregulated)	Toll-like receptors, IL1-beta, CARD (caspase recruitment domain)	Marcucci *et al*, 2008
*RUNX1*mutated	*let-7, miR-223, miR-99a, miR-100* (downregulated) *miR-211, miR-220, miR-595*(upregulated)	Not evaluated	Mendler *et al*, 2012
*BAALC*overexpressed	*miR-3151*(upregulated)	TP53 pathway	Eisfeld *et al*, 2014

Annotations: CBF, core binding factor; MLL, myeloid and lymphoid gene; NPMc+, nucleophosmin cytoplasmic positive; FLT3-ITD, fms-like tyrosine receptor internal tandem duplications; BAALC, brain and acute leukemia cytoplasmic.

shown that samples with *RUNX1* mutations yielded lower levels of the *let-7* family, *miR-223* (a positive regulator of granulopoiesis) *miR-99a* and *miR-100* whereas *miR-211*, *miR-220* and *miR-595* were upregulated [44].

By gene-expression profiling, 91 pediatric patients with AML exhibited a reciprocal pattern of *HOXA9* (belonging to the homeobox-family) and growth factor independent 1 factor (*GFI1*) profiles with *HOXA9*-like signature clustering 11q23 (*MLL1*) cases and *GFI1*-like signature defining t(15;17), t(8;21) and inv(16) cases [11]. Surprisingly, among the validation cohort including 460 adults with AML and MDS, either MLL translocations leukemias or *NPM1* mutant cases showed *HOXA-9* like signature [11]. HOXA9 interacts with MEIS1 and PBX1 resulting in the formation of a transcriptional factor complex that regulates multiple genes. In mice GFI1 directly interfere with the expression of *HOXA9*, *PBX1* and *MEIS1* during maturation of myeloid precursor [45]. HOXA9 was shown to target *miR-21* and *miR-196b* that were up-regulated in patients with 11q23 abnormalities compared with t(8,21) cases [11].

AML with *MLL* translocations were associated with upregulation of *miR-191* and down regulation of *miR-29* family members [16]. In AML cell lines and primary samples the restoration of nomal levels of *miR-29b* induces apoptosis and shrinkage of tumors. Furthermore, disappearance of tumors were observed in xenograft mouse model that were treated by injection of syntethic *miR-29b* after inoculation of K562 cells in mice flanks [46]. By GEP analysis in K562 transfected with *miR-29b* or scrambled oligonucleotides, several genes related to apoptosis (*MCL-1*, *TRA4*, and *MYBL2*), cell cycle regulation (*CDK4*, *CDK6* and *CCND2*) and cell proliferation (*JAK2* and *IGF1*) were found deregulated according to *miR-29* expression levels. Furthermore, in primary cell lines from patients with AML, *miR-29a* and *miR-29b* correlated with specific signatures and approximately 42% of the *miR-29a*-correlated genes were also associated with the expression levels of *miR-29b*. The genes that were found to be positively or negatively associated with *miR-29* belonged to different pathways including apoptosis, protein metabolism and modification processes [46].

Aberrant hypermethylation and histone modifications may epigenetically alter the expression of genes that participate to normal hematopoiesis [47]. In AML cell lines (Kasumi-1, MV4-11 and K562) *miR-29b* lentivirus infection resulted in a decrease of global DNA methylation [46]. Although a significant reduction of the expression of all three DNA methytransferases *DNMT1*, *DNMT3A* and *DNMT3B* at both RNA and protein levels was observed, *miR-29b* has been shown to directly interact with *DNMT3A* and *DNMT3B* 3'-UTR regions whereas the inhibition of *DNMT1* was mediated by its transactivator Sp1 [46]. In *KIT*-driven AML, the down regulation of *miR-29b* and therefore the increased levels of Sp1, enhance the activity of the Sp1/NFκB/HDAC complex. The Sp1/NFκB complex has been shown to favor the expression of *BAALC* gene and *miR-3151* that are located on chromosome 8q22 [48]. Increased expression of *BAALC* and *miR-3151* is associated with inferior outcome in patients with AML [49, 50]. The *miR-3151* binds at the 3'-UTR of TP53, thereby affecting the TP53-mediated apoptosis pathway in leukemic cells. Furthermore, the expression of *BAALC* is under the control of RUNX1, that is frequently translocated or mutated in AML [48]. The intrigued network involving *miR-29b* and *miR-3151* and their interactions with Sp1/NFκB, KIT, TP53, DNMT and BAALC provide us new elements to better understand the mechanisms of leukemic transformation and suggest that miRNAs are key-regulators of various cellular pathways (Fig. **1**).

High risk AML group includes cases with del(7q) or monosomy 7 [13]. *Mir-29b-1/miR29a* clusters at chromosome 7q32 and in primary AML samples with monosomy 7 *miR-29a* and *miR-29b*, but not *miR-29c,* that resides on chromosome 1q32.2, expression levels were lower than cases with other genetic abnormalities [46]. The observation that the rate of clincial response among patients with del(7q)/monosomy 7 who received decitabine were higher compared with historical population, suggested that hypomethylating agents might counteract the inhibition on DNMT enzymes secondary to low *miR-29* expression levels in this subgroup of patients [51, 52].

3. MYELODYSPLASTIC SYNDROMES

Myelodsyplastic syndromes (MDS) are usually believed pre-leukemic conditions and may precede overt AML. Recently, the availability of next generation sequencing (NGS) allowed to prove the molecular heterogeneity of MDS providing a deeper insight in the pathogenesis of this disease and explanation of different clinical outcomes among affected patients [53]. Studies of miRNA expression in MDS failed in some circumstances to reveal specific miRNA signatures as it has been clearly observed in AML. However, these results may be related to the use of unpurified stem cells as source of tumor cells in studies aiming to analyze miRNA profiles in MDS patients.

Similarly with AML cases, *miR-10*, *miR-181* and *miR-155* were found to be prognosticaly relevant among patients with MDS where they are more likely associated with specific risk categories [54, 55]. However, in contrast with AML, *miR-181* has been correlated to shorter survival in patients with either high risk (International prognostic score system, IPSS, Int-2 or high risk) or low risk MDS [55]. Furthermore, MDS carrying trisomy 8 and del(5q) were more likely to express specific miRNA profiles compared with MDS with normal cytogenetics or with different abnormalities [39, 56].

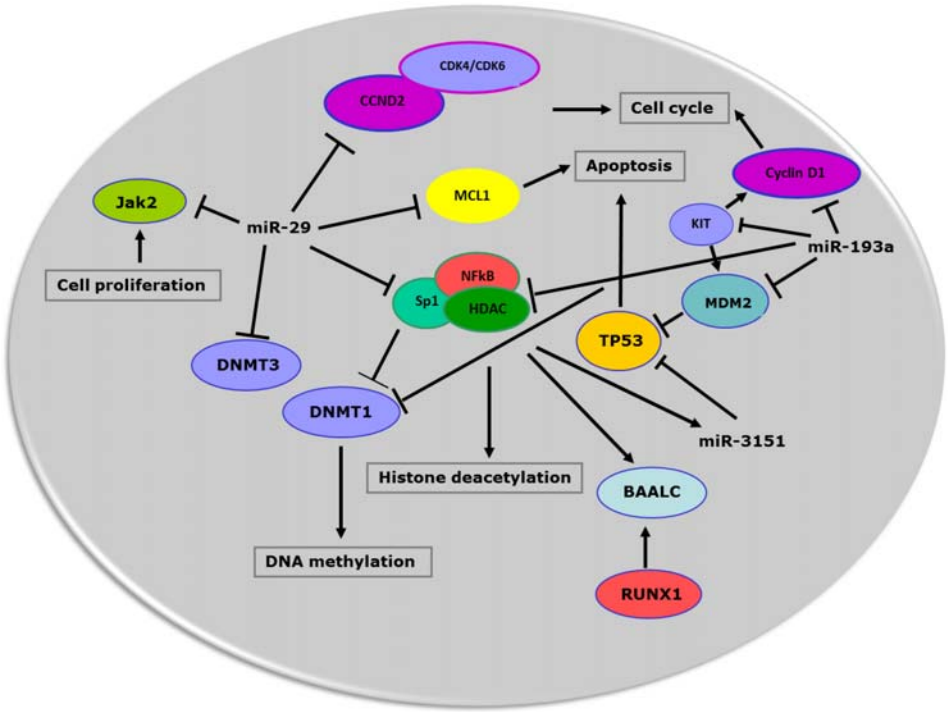

Fig. (1). MiRNAs network in acute myeloid cell. miRNAs interfere with multiple pathways in malignant cell resulting in dysregulation of cell cycle, apoptosis, cell proliferation and epigenetics (DNA methylation and histone deacetylation). Furthermore, different miRNAs may converge on the same cellular pathways suggesting a possible cooperation effect among miRNAs that are aberrantly expressed in leukemic cells.

In mice model with additional sex combs-like 1 (*ASXL1*) mutations displaying myelodysplastic-like features, it has been reported that *miR-125a* and *HOXA9* are derepressed by mutant *ASXL1* through inhibition of polycomb repressive complex 2-mediated (PRC2-mediated) methylation and histone H3K27 [57]. *miR-125* inhibited the expression of C-type lectin domain family 5 (Clec5a), which is involved in myeloid differentiation [57].

Recently, it has been observed that miRNAs expression levels can be modulated in cells as well as in body fluid [58]. Using a high-throughput digital quantification technology to study the miRNA profiles in plasma samples from 77 patients with MDS, a "7 miRNA" signature emerged as an independent prognostic factor of survival within patients with normal cytogenetics (p=0.008) [59].

4. CHRONIC MYELOID LEUKEMIA (CML) AND MYELOPROLIFERATIVE NEOPLASMA (MPD)

Chronic myeloid leukemia (CML) is a myeloproliferative disorder originating from the hematopoietic stem cell (HSC) and characterized by exuberant cell proliferation and abnormal differentiation of the myeloid precursors. The hallmark of CML is the Philadelphia chromosome (Ph) that results from the translocation of the long arms of cromosomes 9 and 22. The t(9;22) causes the formation of the oncoprotein BCR-ABL1, a constitutively active tyrosine kinase that confers growth factor-independent proliferation and to the hematopoietic progenitors., [60].

Although the vast majority of patients achive hematological, cytogenetic and molecular response by TKIs treatment, a small proportion of patients with CML will eventually fail into because of the occurrence of resistance to TKIs or progression to blast crisis (BC) [61]. The mutations at the drug binding site are responsible of the occurrence of drug resistance in most cases [62]. However, the mechanism(s) of resistance to TKIs are not completely known. In patients with CML harboring the deletion in 9q34, *miR-199b* and *miR-219-2* were downregulated compared with patients without the deletion. However, only *miR-199b* out of the two miRNAs associated with imatinib drug resistance [63]. Hence, the deletion of *miR-199b* located at 9q34.11 may provide explanation of the poor prognosis and suggest the possible mechanism of drug resistance in patients with del(9q) CML [64].

In a model of CML, *miR-328* upregulated the translation of the C/EBPα mRNA through a regulatory RNA-binding protein (hnRNP E2) that represses CEBPA identifying a novel decoy regulatory mechanism [65]. *MiR-328* is markedly reduced in BC-CML CD34+ cells when compared with CP-CML CD34+ cells suggesting that the downregulation of *miR-328* might contribute to disease progression. Accordingly, the re-expression of *miR-328* levels in BC-CML CD34+ cells restored myeloid differentiation in response to stimulation with G-CSF through the upregulation of C/EBPα protein and of PIM1 serine-threonine kinase [65].

In patients with newly diagnosed CML and following two weeks of treatment, *miR-150* and *miR146* were found increased whereas *miR-142-3p* and *miR-199b-5p* were decreased at the same time point compared with pre-treatment levels. Furthermore, *miR-150* resulted downregulated in blasts from patients with overt CB-CML [66]. Despite the paucity of samples, the authors documented positive correlation between miRNAs levels at diagnosis and specific clinical features [66]. Importantly, *miR-142-3p* correlated with Sokal score ($p< 0.05$) and *miR-18* fold change between day 0 and day 14, inversely associated with the time to initial complete hematological response ($r=-0.7198$, $p=0.0125$). The results were confirmed in a validation cohort of 12 patients [66]. Interestingly, imatinib may induce demethylation of *miR-203* which targets *BCR/ABL1*, suggesting that the switch-off exerted by imatinib on constitutively activated ABL kinase might rely on additional mechanisms other than the inhibition at the ATP-binding pocket [67].

In imatinib resistant CML cell lines *miR-181* was significantly repressed compared with drug-sensitive cell lines [68]. Lyn kinase (Lyn) turns off *miR-181* that targets myeloid cell-leukemia 1 (MCL-1), an antiapoptotic protein whose expression may be upregulated in TKIs resistant cells. According to this model, the mechanism of resistance in cells overexpressing Lyn and Mcl-1 is mediated by *miR-181* [68].

Myeloproliferative neoplasms (MPD) include primary myelofibrosis, essential trombocytemia and polycytemia vera [69]. Using NGS, in a training cohort of 22 patients with MPN comparing the genomic DNA of granulocytes with *in vitro* expanded CD3+ T-lymphocytes, as germline control, 141 somatic mutations were identified [70]. Recurrent mutations occurred at 8 gene loci including *miR-662* that was mutated in 7.4% of cases. Similarly, *miR-17*, *miR-19a*, *miR-542* and *miR-663a* were characterized by missense mutations in their stem-loop coding region (*miR-662,-663,-542*) or downstream the seed sequence (*miR-17-5p*) [70].

The thrombopoietin receptor (MPL) mutations have been found in primary myelofibrosis and essential thrombocytemia resulting in constitutive activation of the janus kinase 2 (JAK2) signaling [71-73]. In platelets from patient with MPN, *miR-28* is overexpressed compared with stable levels in platelets from healthy individuals [74]. It has been proposed that *miR-28* exerts its action targeting MPL as well as other proteins essential for megakaryocyte differentiation. Furthermore, the ectopic expression of *miR-28* transfected CD34-derived megakaryocytes inhibited terminal differentiation.

These results shed light on the mechanisms by which miRNAs contribute to the onset in the pathogenesis of myeloproliferative neoplasms [74] and on the mechanism(s) of resistance of TKIs other than mutations of the active binding site.

5. miRNAs AND LEUKEMIC STEM CELLS (LSCs) IN MYELOID HAEMATOLOGICAL MALIGNACIES

Leukemic stem-cells (LSCs) represent the natural reservoir in patients with AML, CML and MDS and are responsible of clinical minimal residual disease (MRD) positivity, relapse and/or chemo-resistance [75, 76].

An interesting finding was that following transfection into CD34+ AML cell line KG1a of *miR-9*, *miR-24* and *miR-125a* oligonucleotides, CD34 protein expression levels

measured by Western blotting were significantly reduced suggesting that low expression of CD34 on the surface of NPMc+ blasts [77] may be due to the up-regulation of specific miRNAs [15]. Recently, distinct miRNA expression profiles were identified in LSCs from adults with *de novo* CN-AML [78]. Among 364 patients with CN-AML, the expression of 44 activated genes in LSCs was associated with high risk molecular features (*FLT3-ITD*, *WT1* and *RUNX1* mutations, wt*CEBPA* and *TET2*, high *ERG*, *miR-155* and *BAALC* gene expression levels) and shorter disease-free and overall survival ($p<0.001$ for both), even at the multivariable analysis including the most common prognostic markers. The observation that miRNAs which are relevant for stem cell function might be upregulated in leukemic blasts suggests the implementation and the inclusion of miRNAs inhibitory molecules in order to eradicate the LSC reservoir, therefore improving the disease-free and overall survival of patients with AML [78].

In CML as in other myeloid malignancies, the persistence of quiescent CML HSCs might contribute to disease progression because leukemic stem cell proliferation and survival are apparently resistant to TKIs inhibition [76]. In patients with CML, the expression of *miR-17~92* polycistronic primRNA transcripts and mature miRNAs (*miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b, miR-92-1*) were found upregulated in chronic phase (CP) but not in BC-CML CD34+ [79, 80]. Accordingly, in 3 *BCR/ABL1* positive cell lines, a downregulation of miRNA encoded within the polycistrionic *miR-17-92* cluster was observed following imatinib treatment or the addition of anti *BCR/ABL1* RNA interference (RNAi). The polycistrionic *miR-17-92* cluster is transcriptionally regulated by c-MYC [81] and c-MYC participates with *BCR-ABL* in the leukemic transformation process [82].

In primary myelofibrosis CD34+ cells, several miRNAs involved in trascriptional control and chromatin remodeling were found deregulated [83]. In particular, overexpression of *miR-155-5p* impaired the activity of JARID2 resulting in increased formation of CD41+ megakariocyte precursors [83].

6. MicroRNAs AS PREDICTIVE MARKERS OF RESPONSE AND THERAPY TARGETS

So far, few studies within clinical trials have identified miRNAs or miRNA signature to predict chemo-sensitivity in AML [9, 38, 44, 51].

In a phase 2 study with frontline single agent decitibine in a single cohort of older patients (≥ 60 yrs) who were not fit or refused other treatments, higher levels of *miR-29* were predictive of clinical response ($p=0.02$) [51]. The observation than high *miR-29* expression levels favor clinical response to standard treatment, suggested to investigate whether the delivery of *miR-29* molecules into leukemic cells has antileukemic effects. One concern when small molecules are introduced into body fluids is that they can be degraded. Transferrin-conjugated nanoparticles containing *miR-29b* have shown significant results in terms of apoptosis induction and cell growth inhibition [84]. More impressive is the fact that mice who were treated with the combination of *miR-29b* nanoparticles followed by decitabine have significantly better outcome compared with mice treated with decitabine as single agent [84]. A similar "priming" effect was noted with the novel HDAC inhibitor AR-42 which sensitized leukemic cells to decitabine by removing the inhibition exerted by HDAC on *miR-29b* expression [52].

MiRNA inhibitory molecules represent a challenging tool for the treatment of patients with hematological malignancies. Experimental data *in vitro* and in pre-clinical models suggest that the interference of miRNAs in combination with standard treatment might contribute to chemo-sensitivity and leukemia control [11]. In a xenograft model of MLL leukemia, the injection of *miR-21* and *miR-196b* antagomirs in combination with daunorubicin and aracytin (that resembles the D3+A7 scheme used as standard induction treatment of human AML) significantly improved the life span of the mice compared with recipients treated with control antagomirs ($p < 0.01$) or AML1/ETO leukemia models [11]. Furthermore, by using lentivirus-delivered CXCR4 shRNA the expression levels of *let-7a* were found significantly increased in OCI-AML3 contributing to sensitize cells to aracytin, a backbone drug in the treatment of AML. The reduction of *let-7a* target genes, such as the antiapoptotic protein *BCL-XL* and *MYC*, would be responsible for inhibition of cell proliferation and chemo-sensitivity [85]. Lenalidomide, an anti-angiogenic drug that has been approved for treatment of myelodysplastic syndrome with del(5q) and of multiple myeloma, may enhance the translation of the C/EBPα-p30 isoform in myeloid blasts from patients with CN-AML and *CEBPA* mutations. The induction of the C/EBPα-p30 isoform results in higher expression of *miR-181a* and inhibition of leukemic growth [39]. Therefore, specific miRNA signature may explain the better chemo sensitivity of certain subtype of AML, such as those with *CEBPA* mutations [9].

Although further studies are warranted to confirm that the RNA inhibitor molecules are effective and not harmful for patient safety, these results suggest that the repression of miRNAs may serve for treatment of certain patients with AML.

7. CONCLUSIONS AND FUTURE DIRECTIONS

Over the last decade, several studies underscored the pivotal role that miRNAs have in myeloid malignancies, including acute myeloid leukemia, myelodysplastic syndrome and myeloproliferative neoplasms. MiRNAs may act as tumor suppressor or oncogene interfering with multiple pathways involved in cell cycle, tumor growth and apoptosis. Interestingly, different leukemic subtypes have distinct miRNAs expression profiles. Furthermore, the identification that miRNA expression profiles may predict outcome in myeloid malignancies or chemo-sensitivity to specific drug suggest to include miRNAs in the prognostication system of these broad spectrum of diseases. Finally, *in vitro* experiments and pre-clinical models provided promising results of the safety and the efficacy of miRNAs inhibitory molecules. Further studies and clinical trials are warranted to confirm the prognostic and predict power of miRNAs and to test the miRNAs inhibitory molecules.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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