Review Article

Diagnostic & prognostic role of microRNAs in paediatric acute myeloid leukaemia

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Dysregulation in microRNAs (miRNAs) expression has been observed in distinct acute myeloid leukaemia (AML) subtypes, and their potential as an effective diagnostic and prognostic biomarker is slowly being realized. Certain miRNAs have been found to be associated with various cytogenetic and molecular abnormalities of prognostic significance in AML. Experimental evidences have indicated the potential of modulating miRNA expression as an effective antileukaemic strategy. This has opened a new window for miRNAs-based targeted therapies. In this review, we present results of some studies analyzing the dysregulation in miRNAs expression pattern in paediatric AML and also discuss their use as diagnostic and prognostic markers.

Key words Biomarker - cytogenetics - diagnostic - leukaemia - microRNA - prognosis

Introduction

Paediatric acute myeloid leukaemia (AML) is a heterogeneous disease in terms of diverse cytogenetic and molecular abnormalities, all leading to malignant transformation of haematopoietic progenitors[1,2]. It accounts for almost 15-20 per cent of all paediatric leukaemia. Although there has been a significant improvement in the overall survival rate of paediatric AML patients from 30 to 73 per cent, still nearly half of them relapse[3]. Therefore, diagnostic and prognostic biomarkers for classifying different risk groups as well as more effective molecular targeted therapies are urgently needed for better management of paediatric AML patients.

MicroRNAs (miRNAs) are a group of non-coding RNAs, which mainly function through complementary base pairing to the 3' untranslated region of target messenger RNA (mRNA), followed by degradation of mRNA and/or translational inhibition[4]. These miRNAs, now recognized as epigenetic biomarkers, play vital functions in numerous cellular events ranging from organogenesis to immunity[5,6]. Deregulation of miRNAs affects normal cell growth and development including multiple cellular events including cell cycle regulation, differentiation and cell death, leading to various diseases including cancer[7]. The expression pattern of miRNA has been studied in adult AML patients, where abnormal expression of different miRNAs has

been detected in distinct adult AML subtypes leading to activation or inhibition of essential pathways in leukaemogenesis[8]. Though information on diagnostic, prognostic and functional importance of miRNAs expression is available in adult AML[8], in paediatric AML, information about miRNA expression has been gathered only in a limited series of patients so far[9-18]. Hence, in the present review, efforts have been made to summarize the findings of studies published so far in the area of miRNAs in paediatric AML.

MicroRNA expression profile in paediatric acute myeloid leukaemia: A diagnostic and prognostic tool

Table I summarizes studies which have explored the efficacy of miRNA expression as a diagnostic and prognostic biomarker in paediatric AML patients[9-18]. Most of these studies have tried to explore the possibilities of identifying specific miRNAs expression or miRNAs expression signature from bone marrow which can distinguish paediatric AML from normal controls. Except two studies in which miRNA microarray platform was used for profiling of human mature miRNAs[9,14], in all other studies quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) using miRNA-specific stem-loop primers and probes was used for the analysis of miRNA expression (Table I). The miRNA expression was detected using bone marrow samples derived from paediatric AML patients ranging from a minimum of 68 patients[13] to a maximum of 169 patients[10]. The numbers of normal controls were comparatively less, which could be due to ethical considerations involved in bone marrow aspiration from this group of subjects. Despite having variations in methodology and patients recruited and miRNA assayed amongst all the studies, a common feature was dysregulation in miRNA expression in paediatric AML patients when compared with normal controls. Specific miRNAs such as miR-125b[9,10], miR-100[9,11], miR-99a[9,13], miR-375[15] and miR-335[9,17] were highly expressed, while miR-29a[16] and miR-663[18] were underexpressed in paediatric AML patients when compared to normal controls. Using miRNA microarray platform, Zhang et al[9] found upregulation of 17 miRNAs and downregulation of 18 miRNAs in paediatric AML patients as compared to normal controls. The expression of a few of these miRNAs was further validated by qRT-PCR, which confirmed the upregulation of miR-99a, miR-100, miR-125b, miR-146a and miR-335 in paediatric AML patients[9]. Table II lists those miRNAs, which were differentially expressed in the bone marrow of paediatric AML patients as compared to controls.

The impact of aberrant miRNA expression has been studied on clinical and therapeutic outcome in adult AML[8]. In line with this, a few studies also explored the utility of miRNA expression as a potential prognostic indicator in paediatric AML[9-13,15-17] (Table II). These studies tried to establish a correlation between miRNA expression pattern and clinical outcome in terms of therapeutic response, overall survival and relapse in paediatric AML patients. Zhang et al[9] concluded that miR-125b and miR-126a can predict favourable prognosis for M3 and M2 AML patients, respectively. However, the study failed to establish any correlation between miRNA expression and central nervous system (CNS) relapse. In another study by Zhang et al[10], expression of miR-125b decreased in paediatric AML patients who achieved complete remission post-therapy, but the levels remained high in patients with relapse. Similar results were also observed in another study by Zhang et al[13] using miR-99a. The increased expression of miR-100 has been shown to be associated with poor relapse-free and overall survival as well as unfavourable day 7 response to induction chemotherapy in paediatric AML patients[11]. However, another group failed to establish any correlation between miR-196a/b expression and overall survival[12]. Further, increased expression of miR-375[15] and miR-335[17] and low expression of miR-29a[16] in paediatric AML patients have been shown to be associated with poor relapse-free survival and short overall survival in both univariate and multivariate analyses.

The above-mentioned data suggest that quantification of miRNA expression may have clinical utility for risk assessment in paediatric AML. However, as the literature on the expression of miRNAs in paediatric AML is very limited, it is not feasible to evaluate their true potential as an effective diagnostic and prognostic biomarker. What is clear from these studies is that miRNA signatures or expression of specific miRNA is not consistent among different studies. This lack of homogeneity in the findings could be attributed to a number of factors, such as (*i*) variability in the recruitment of number of patients and controls, (*ii*) variability in the frequency of distribution of paediatric AML patients in various cytogenetic or genetic groups, (*iii*) use of unselected mononuclear cells from the bone marrow of healthy donors instead of purified CD34^{+} cells, and (*iv*) different methods for profiling of miRNA.

Table I. A summary of studies on miRNAs expression profiling in paediatric acute myeloid leukaemia (AML)						
Reference	Number of patients/ controls	Ethnicity	Sample	Method for miRNA profiling	miRNA analyzed	Key findings
Zhang et al[9]	99[¶]/12	China	Bone marrow	miRNA microarray and qRT-PCR with molecular beacon probe	576 human mature miRs and validation of differentially expressed miRs such as miR-100, -34a, -146a, -335, -126,-125b	Highly variable expression of miRNAs in primary AML with 17 miRNAs upregulated and 18 downregulated. Upregulation of miR-100, -125b, -335, -146a and -99a in paediatric AML. miR-125b and miR-126 were correlated with favourable prognosis of M3 and M2 patients, respectively. miRNA expression not correlated with CNS relapse in paediatric AML group.
Zhang et al[10]	169[*]/13	China	Bone marrow	qRT-PCR with molecular beacon probe	miR-125b	miR-125b was highly expressed in paediatric APL patients. Post-therapy, expression of miR-125b reduced in patients achieving complete remission. miR-125b expression remained higher in patients who relapsed.
Bai et al[11]	106[†]/20	China	Bone marrow	qRT-PCR with TaqMan probe	miR-100	miR-100 was highly expressed in paediatric AML patients. High expression of miR-100 correlated with unfavourable day 7 response to induction chemotherapy, and poorer relapse-free and overall survival in multivariate analysis.
Danen-van Oorschot et al[12]	82[φ]/2	Europe	Bone marrow or peripheral blood	qRT-PCR with TaqMan probes	miR-29a, miR-155, miR-196a, miR-196b	miR-196a/b was highly expressed in AML patients positive for *MLL* gene rearrangements, *NPM1* mutations or *FLT3*-ITD in a cytogenetically normal background. Low miR-196a/b expression in CEBPA mutated cases. Direct correlation between the expression of miR-196a/b and *HOXA* and *HOXB* genes. miR-155 was upregulated in AML patients carrying *FLT3*-ITD and *NPM1* mutations. Downregulation of miR-29a expression in MLL-rearranged cases. No correlation between miR-196a/b expression and overall survival.

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Reference	Number of patients/ controls	Ethnicity	Sample	Method for miRNA profiling	miRNA analyzed	Key findings
Zhang et al[13]	68[Ψ]/12	China	Bone marrow	qRT-PCR with molecular beacon probe	miR-99a	miR-99a was upregulated in M1-M5 paediatric AML. Its expression decreased in patients achieving complete remission. In relapsed patients (with M2), expression of miR-99a increased.
Daschkey et al[14]	102[#]/2	Germany	Bone marrow or peripheral blood	miRNA microarray followed by validation using qRT-PCR with TaqMan probe	Validation of miR-126, miR-146a, miR-223, miR-100, miR125b, miR-181a, miR-181b	miR-27a, -126, -150 and miR-223 were upregulated while miR-21 was downregulated in t(8;21)-positive paediatric AML samples than in t(15;17)-positive samples. miR-100 and miR-125b were highly expressed in t(15;17)-positive leukaemia. A miRNA signature consisting of 22 miRNAs could correctly classify almost 87 per cent of patient samples belonging to various cytogenetic risk groups such as t(8;21), t(15;17) and MLL-rearranged AML. miR-146a and miR-181a/b were highly expressed in t(15;17), while miR-146a was highly expressed in t(8;21).
Wang et al[15]	106[†]/20	China	Bone marrow	qRT-PCR with TaqMan probe	miR-375	miR-375 was highly expressed in paediatric AML subtype M7 and also in patients with unfavourable karyotype when compared with other risk groups. In multivariate analysis, high expression of miR-375 predicted poor relapse-free survival and shorter overall survival.
Zhu et al[16]	106[†]/20	China	Bone marrow	qRT-PCR with TaqMan probe	miR-29a	Downregulation of miR-29a expression in paediatric AML patients (subtype M7) than controls. miR-29a was highly expressed in AML patients with favourable karyotypes. In multivariate analysis, low expression of miR-29a predicted poor relapse-free survival and shorter overall survival.
Lin et al[17]	106[†]/20	China	Bone marrow and serum	qRT-PCR with TaqMan probe	miR-335	High miR-335 expression in the bone marrow and serum of paediatric AML patients than in controls. AML patients with subtype M7 and those with unfavourable Karyotype had significant upregulation in the serum levels of miR-335. Increased expression of miR-335 in serum predicted shorter relapse-free and overall survivals.

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Reference	Number of patients/controls	Ethnicity	Sample	Method for miRNA profiling	miRNA analyzed	Key findings
Yan-Fang et al[18]	70/30	China	Bone marrow	qRT-PCR	miR-663	miR-663 expression was lower in paediatric AML patients compared to controls. Gene encoding miR-663 was highly methylated in paediatric AML patients (41.4%) compared to controls (10.0%).

¶36 patients (18 each of ALL and AML) and 7 normal samples were used as a test cohort for the evaluation of miRNA profiling; 63 paediatric patients (31 ALL and 32 AML) and 5 normal donors were used as a validation cohort. In test cohort, 18 AML patients constituted by 7 t(8;21), 4 t(15;17) and 7 negative for both. In validation cohort, 32 AML patients constituted by 5 t(8;21), 5 t(15;17) and 22 negative for both; *131 AML samples before therapy (including 76 PML-RARα-positive APL samples), 38 APL samples (PML-RARα-positive) after therapy; †Includes 35 patients with favourable prognosis, 52 patients with intermediate prognosis and 19 patients with unfavourable prognosis; φIncludes 9 patients of MLL t(9;11), 8 patients of MLL t(10;11), 9 patients with t (8;21), 10 patients with inv(16), 11 patients with t(15;17), 26 cytogenetically normal patients and 9 patients with miscellaneous cytogenetic aberrations; ψ68 paediatric AML patients include 41 samples before therapy, 23 samples with complete remission and 4 samples with relapse; #Includes 1 patient with t(4;11), 2 patients with t(6;11), 16 patients with t(9;11), 6 patients with t(10;11), 5 patients with t(11;19), 3 patients with t(11q23), 14 patients with t(15;17), 13 patients with inv(16), 24 patients with t(8;21), 4 patients with normal karyotype and 14 patients with miscellaneous cytogenetic aberrations. AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; PML-RARα, promyelocytic leukaemia-retinoic acid α receptor; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; MLL, myeloid/lymphoid leukaemia; *CEBPA*, CCAAT/enhancer-binding protein alpha; CNS, central nervous system; APL, acute promyelocytic leukaemia; *NPM1*, nucleophosmin 1; miRNA, microRNA

Correlation of miRNA expression with cytogenetics in paediatric acute myeloid leukaemia

The heterogeneity and complexity of AML in terms of various cytogenetic abnormalities is well established[2]. Although many of these abnormalities are rare, some of these occur frequently and are clinically very important as these have been found to be associated with therapeutic outcome and survival[2]. The commonly detected cytogenetic abnormalities have been classified according to the prognostic information they carry. For example, t(8;21)(q22;q22) and inv(16)(p13.1q22) or t(16;16)(p13.1;q22) and t(15;17)(q22;q12-q21) confer a relatively favourable outcome. However, patients with balanced translocations involving band 11q23 and the myeloid/lymphoid leukaemia (MLL) gene [t(v;11) (v;q23)/MLL] other than t(9;11)(p22;q23), inv(3) (q21q26.2) or t(3;3)(q21;q26.2), t(6;9)(p23;q34), deletion or loss of 5q, monosomy 7, structural alterations of 17p or a complex karyotype [defined as more than or equal to three chromosome aberrations in the absence of t(8;21), inv(16) or t(16;16), t(15;17), t(9;11), t(v;11), t(6;9) and inv(3) or t(3;3)] have a very poor prognosis[2,8]. Patients with other chromosome aberrations such as t(9;11) (p22;q23); those not classified as favourable or unfavourable or those with cytogenetically normal AML (CN-AML) are classified as having an intermediate prognosis[2].

Using miRNA expression profiling, only a couple of studies have looked at the correlation between specific miRNA signatures and cytogenetic subtypes

Table II. List of microRNAs (miRNAs) found to be dysregulated in paediatric acute myeloid leukaemia (AML)

Highly expressed miRNA in paediatric AML patients as compared to controls for diagnostic purpose	Less expressed miRNA in paediatric AML patients as compared to controls for diagnostic purpose	miRNA with prognostic information in paediatric AML
miR-375, miR-335, miR-100, miR-125b, miR-146a, miR-99a, miR-34a, miR-210, miR-213, miR-181c, miR-146b, miR-126, miR-181a, miR-181d, miR-130a, miR-195, miR-181b, miR-222, miR-99a	miR-29a, miR-663, miR-331, miR-505, miR-150, miR-30e-3p, miR-142-3p, miR-197, miR-138, miR-191, miR-590, miR-339, miR-144, miR-363, miR-148a, miR-338, miR-143, miR-145, miR-142-5p, miR-582	miR-125b, miR-126, miR-99a, miR-100, miR-375, miR-335, miR-29a
Source: Refs 9-18		

Table III. List of microRNAs associated with various cytogenetics and molecular alterations in paediatric acute myeloid leukaemia (AML)

Chromosome aberration	MicroRNAs upregulated	MicroRNAs downregulated
t(15;17)	miR-29a, miR-100, miR-125b, miR-181a, miR-181b, miR-146a, miR-146b-5p	Let-7b, let-7c, miR-18b, miR-22, miR-24, miR-27a, miR-126-3p, miR-150, miR-223, miR-342-3p, miR-378, miR-196a, miR-196b
t(8;21)	miR-126-3p, miR-146a, miR-146b-5p, miR-181a, miR-181b, miR-155, miR-130, miR-27a, miR-150, miR-223	miR-26a, miR-494, miR-125a, miR-193a, miR-142-3p, miR-196a, miR-196b, miR-21
inv(16)	None reported	miR-196a, miR-196b
MLL rearranged	miR-21, miR-196a, miR-196b	Let-7c, miR-26a, miR-29a, miR-30c, miR-30d, miR-100, miR-125b, miR-126-3p, miR-143, miR-146a, miR-146b-5p, miR-181a, miR-181b, miR-181d, miR-221, miR-222
Molecular aberrations in CN-AML		
NPM1 mutation	miR-29a, miR-155, miR-196a, miR-196b	None reported
FLT3-ITD	miR-155, miR-196a, miR-196b	None reported
CEBPA mutation	None reported	miR-196a, miR-196b

CN-AML, cytogenetically normal acute myeloid leukaemia; *CEBPA*, CCAAT/enhancer-binding protein alpha; *FLT3-ITD*, internal tandem duplication of the Fms-like tyrosine kinase 3 gene; MLL, myeloid/lymphoid leukaemia; miRNA, microRNA; *NPM1*, nucleophosmin 1
Source: Refs 9, 12-17

of paediatric AML[12,14]. Specific miRNAs, which are either upregulated or downregulated in various cytogenetic abnormalities, are listed in Table III. In one of the studies, miR-196a/b was upregulated, whereas miR-29a was downregulated in paediatric AML patients carrying MLL gene rearrangements[12]. In the same study, a relatively low expression of miR-196a/b was observed in patients with t(8;21), inv(16) and t(15;17) as compared to all other patients. However, there was no correlation between miR-155 expression and specific cytogenetic abnormalities[12]. Daschkey *et al*[14] found that miR-27a, miR-126, miR-150 and miR-223 were significantly highly expressed, while miR-21 was significantly underexpressed in t(8;21)-positive paediatric AML samples as compared to t(15;17)-positive samples. Further, miR-100, miR-125b and miR-181a/b were highly expressed in t(15;17)-positive leukaemia, whereas miR-146a was highly expressed in both t(8;21)-positive and t(15;17)-positive leukaemia as compared to other cytogenetic groups[14]. They further concluded that a miRNA signature consisting of 22 miRNAs can correctly classify almost 87 per cent of patient samples belonging to various cytogenetic risk groups such as t(8;21), t(15;17) and MLL-rearranged AML[14]. In other

studies it was found that high expression of miR-375[15] and miR-335[17] was more frequent in paediatric AML patients with unfavourable karyotypes than in with favourable or intermediate karyotypes. In contrast, miR-29a was highly expressed in paediatric AML patients with favourable karyotypes than in with intermediate or unfavourable karyotypes[16].

It is to be noted that the detection of aforementioned cytogenetic alterations involves more uniform and standardized protocols across various laboratories and hence is an accepted method for diagnosis, prognosis and management of AML. This is in contrast to two of the most common methods for miRNA profiling - microarray and qRT-PCR, which have been used in various studies for evaluating the diagnostic and prognostic potential of miRNA in paediatric AML. In miRNA microarray, there is a hybridization between specific miRNA sequences and their respective complementary probes on a slide, thereby producing fluorescent signals, which is measured as distinct spots. As miRNAs are very small and many of these belong to the same family, thereby differing only by a few nucleotides, designing probes for specific miRNAs is a very difficult process and can influence the end result.

Differences in the methods of probe designing, probe labelling, hybridization and use of different microarray platforms can also influence the relative abundance of miRNA. Further, qRT-PCR is also a highly variable method and factors such as (*i*) use of different endogenous miRNA control for normalization, (*ii*) use of different chemistries such as SYBR-Green, TaqMan probe and molecular beacons, and (*iii*) sensitivity of different platforms of real-time PCR machines can influence the end-point measurement of expression of specific miRNAs. Thus, it becomes crucial to optimize various methods for the measurement of miRNA expression before integrating them in routine clinical settings for diagnosis, prognosis or management of paediatric AML.

Correlation of miRNA expression with molecular markers in paediatric acute myeloid leukaemia

Nearly half of the AML patients do not have any cytogenetic abnormalities and are classified as CN-AML. However, in various studies it has been found that CN-AML is actually a heterogeneous group with a number of genetic abnormalities, leading to defects in gene expression[2]. Many of these molecular alterations also carry prognostic information, which makes their analysis clinically very important and viable[19,20]. Molecular alterations such as an internal tandem duplication of the Fms-like tyrosine kinase 3 gene (*FLT3*-ITD), partial tandem duplication of the MLL gene, mutations of the Wilms tumour 1 (*WT1*) and high expression of the brain and acute leukaemia, cytoplasmic, erythroblast transformation-specific-related gene and meningioma (MN) (disrupted in balanced translocation) 1 (*MN1*) genes confer adverse prognosis, whereas mutations in the nucleophosmin (*NPM1*) and CCAAT/enhancer-binding protein alpha (*CEBPA*) genes confer favourable prognosis[2,8]. Further, numerous combinations of these markers have also provided useful information for predicting clinical outcome of CN-AML patients. In one such example, AML patients positive for *NPM1* mutations, but negative for *FLT3*-ITD, showed a better outcome than patients who were positive for *FLT3*-ITD, regardless of *NPM1* mutations, or have wild-type *FLT3* and *NPM1* alleles[2].

As many of these molecular alterations carry important prognostic information in paediatric AML, it is imperative to establish their correlation with the expression of specific miRNAs (Table III). MiR-196a/b was found to be highly expressed in paediatric AML patients positive for *NPM1* mutation or *FLT3*-ITD, whereas patients carrying *CEBPA* mutations were having low expression of miR-196a/b[12]. The authors also observed high miR-155 expression in *FLT3*-ITD and *NPM1*-mutated cases[12]. No correlation could be established between miR-29a expression and FLT3-ITD or neuroblastoma rat sarcoma viral oncogene homolog (*N-RAS*) and V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*K-RAS*) mutations, miR-155 expression and *N/K-RAS* mutations and miR-196a/b expression and presence of *WT1* or *N/K-RAS* mutations[12]. Further studies are needed to establish a significant association between miRNA expression signatures and specific molecular alteration in paediatric AML.

Conclusions and future directions

The development of sophisticated, high-throughput technologies for profiling miRNAs, such as miRNA microarray platforms, array/card-based qRT-PCR and next-generation sequencing methods for small RNA sequencing, have made it possible not only to study the dysregulated miRNA expression in AML but also to attain a global, multidimensional view of gene regulation by combining it with various DNA, RNA or protein-based high-throughput approaches. However, before being universally accepted, the role of miRNAs in paediatric AML needs to be investigated further and should also be validated in different laboratories working on different cohorts across the world. In addition, use of an adequate number of patients and appropriately matched controls to get significant results, standardization of methods for the collection, storage and processing of biological samples, uniformity in assay platforms and interpretation of huge amount of data, are some of the areas which need to be explored. To get specific miRNAs involved in AML, another challenge would be to get reproducible data sets with highly specific and sensitive statistical numbers. Once validated, various *in vivo* studies, with appropriate preclinical knock-in and knock-out animal models, may deliver the functional role of various miRNAs and their involvement in leukaemia development. After functional characterization, specific miRNAs can further be used for designing miRNA-based therapeutic strategies. Available preliminary data suggest that miRNA-based therapeutic approach can be a viable option for disease management.

***Conflicts of Interest*:** None.

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