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REVIEW ARTICLE

miRNA: AN INDISPENSABLE BIOMARKER FOR LEUKEMIA

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Abstract

MicroRNAs are small non–coding RNA molecules of 18-22 nucleotides which are well - conserved in eukaryotes. They are single stranded RNA which plays an important role in gene regulation. The aberrant expression of miRNA leads to interruption in various cellular functions which is a hallmark of different disease like leukemia. miRNA studies with the help of high throughput technologies help us to know different types of cytogenetic abnormalities related to leukemia. This review emphasizes mainly on miRNA biogenesis and leukemia pathology, role of miRNA as Oncomirs, latest techniques used for getting genetic signature of leukemia and examples of deregulated miRNAs expression in different types of leukemia. Thorough study of miRNA expression can lead to identification of new biomarkers which may be useful for detection of patterned signatures in subtypes of leukemia and diagnosis of disease will be more specific.

Introduction:

It was sixteen years ago when Ambros, Ruvkun, and their colleagues reported that the developmental timing of the nematode *Caenorhabditis elegans* by modulating the expression of the protein-coding gene lin-14 is controlled by small RNA encoded by the lin-4 (lineage-deficient-4)locus [1]. Few years later it was found that lin-4 like small RNA is not present in nematodes only but is present in bilateria also that includes *Homo sapiens*. miRNAs participates in very important biological processes such as- haematopoiesis, cell development, cell proliferation, cell differentiation and programmed cell death[2].

MicroRNAs are 20-25 molecules RNA that are endogenous and abundant in nature, and regulate gene expression by increasing the speed of mRNA decay or decreasing the speed of translation. Nearly 1000 miRNA genes are expected to be present in the human genome, based on the data available till now. They are highly conserved in both animals and plants which varies from species to species[3]. These evolutionary conserved sequences are highly used in bioinformatics approach for the identification of miRNA[4]. It includes small nuclear RNA (snRNA) engaged in mRNA splicing, short interfering RNA(siRNA) involved in regulation of gene expression and small nucleolar RNA (snoRNA) involved in modification of ribosomal RNA[5].

The very first step of miRNA biogenesis starts in nucleus where the transcription of long primary (pri)-miRNA transcripts by RNA polymerase II (Pol II) is done[6]. Due to difference in size of pri-mRNA sequences they are further cleaved by RNase III enzyme Drosha to a form of hairpin precursor called pre-miRNA[7]. The folding of pre-miRNA is done to give imperfect stem loop structure which gets transferred from the nucleus to the cytoplasm by the

involvement of exportin-5 and further processed by another RNase III endonuclease known as Dicer[8,7]. The stem loop structure is changed by dicer to give a duplex of unstable nature which consists of mature miRNA and miRNA coming from the opposing arm of pre-miRNA. The loading of miRNA strand of the duplex on the RNA-induced silencing complex (RISC) is followed by the separation of miRNA from the duplex and its degradation[9].
The altered expression of miRNA is always a typical sign of leukemia. Leukemia is a disorder of the bone marrow in which increased proliferation of immature blood cells takes place. Altered chromosomal abnormalities and oncogene activation can be easily found in large number of people suffering from leukemia[10]. Pathologically, it is divided into acute leukemia and chronic leukemia. The main feature of acute leukemia is storage of hematopoietic precursor cells and blockage of their differentiation. This type of leukemia is very fast in appearance and needs urgent solution for uninterrupted production of healthy leukocytes from the bone marrow. Oppositely Chronic Leukemia takes more time for the onset of disease and mostly involves more proliferation of extra mature hematopoietic cells phenotype, but never causes faulty production of leukocytes and replacing of normal bone marrow[11]. Moreover, it is divided according to hematopoietic lineage into myeloid or lymphoblastic type but it has already been established that all leukemia types are mainly originated from leukemic stem cell(LSC)[12].
Study of miRNA in leukemia is always related to distinct signatures which can be used for detection of different types of leukemia and can be used as biomarker for leukemia. The up regulation or down regulation of miRNA expression in disease can be exploited for defining leukemia pathogenesis and categorizing it into different subtypes. The emergence of unknown miRNAs can still be helpful for developing new biomarkers for leukemia and decreasing the mortality rate of this mortal disease.

MicroRNA as Oncomirs:

It has been suggested through clinical experiments that miRNAs may act as oncogenes and their raised expression level can be easily seen in tumours. These are also known as Oncomirs which causes tumour progression[3].miR17-92 cluster placed on chromosomal location X,13 and 7 plays a role of oncogene[13]. The cluster of genes positioned on chromosome 13 gets transcribed to single primary miRNA and further processing is done to give seven single mature miRNA molecules: miR-17-3p,miR-17-5p,miR-18,miR-19a,mir-19b-1,miR-20 and miR-92-1[14].With the help of frequent microarray studies it has been concluded that almost all family members of miR17-92 cluster are over-expressed in important types of acute myeloid leukemia[15].

The relation between the expression of miR-17-92 and c-Myc gene was found which maintains the expression of E2F1, a cell cycle transcription factor gene. Researchers showed that c-Myc enhances the transcription process of E2F1 and miR-17-92 but two miRNAs from mir-17-92 gene cluster ,mir-17-5p and miR-20a slows down the translation process[16].

miR-126 was newly identified oncomir whose increased expression in normal hematopoietic progenitor cells causes large number of colony formation[17]. Polo -like kinase(PLK)-2 acts as a target of miR-126 which confirms it as a oncomir. Increased programmed cell death in hematological malignancies shows the expression of PLK-2[18]. miR-372 and mir-373 are the two new examples of oncomirs which promotes development of tumour and increases cell proliferation by balancing p53-mediated CDK inhibition[19].

The role of miR17-92 gene cluster as oncomirs is confirmed in which all family members over express but mir-17-5p and miR-20a are involved in repressing the translation process. The emergence of miR126, miR-372 and mir-373 as oncomirs clearly demonstrates the possibility of new findings of miRNA which acts as oncogenes.

Markers used for getting genetic signature of Leukemia

Up-regulated/Down-regulated miRNA expression:
It is used for detailed study of miRNAs function involving knockdown or over expression of particular miRNA in leukemia regulation. It involves mainly antisense inhibitors, particular promoters, transgenics and point mutants[3].

Northern Blotting
It is a trusted method for the detection of gene expression at mRNA level [3]. The use of northern blotting is conducive due to easy availability, less use of technology, and no need of advanced instruments. The main lacunae of this method are that it is time consuming and less sensitive. The alternative for getting higher sensitivity and less time consuming includes the use of Locked Nucleic Acid (LNA) oligonucleotides probes[20].

Real Time PCR

It can be used for the quantification of miRNA expression profiles and to study the role of miRNA in leukemia pathogenesis. It simply includes first strand cDNA synthesis, designing of primers, detection of PCR products and normalization of data[21]. As the use of this technique is at its mature stage, the proper implementation of fluorescent reporters and endogenous controls can lead the technology to become user friendly, cost effective and faster in time.[22]

miR-Q RT- PCR

A new method developed by Sharbati- Tehrani et al. is based on extension of primers and includes innovative qPCR approach which uses three DNA oligonucleotides. It does not require use of any fluorochromic probes or Locked Nucleic Acid and demonstrates higher sensitivity in detection of miRNAs. It shows high specificity and does not allow any type of cross reaction in closely related miRNA families. Cost effectiveness, as well as simplified and higher linearity, shows the future demand of this method[23].

Gene Expression Profiling

It is a technology used to measure the level of mRNA transcripts using oligonucleotides or cDNA probes[24]. The up regulation of members of HOXA and HOXB gene families in leukemia is found after the study of Gene Expression Profiling for normal karyotype[25].

MicroRNA Profiling

The main principle behind microRNA profiling is Watson-Crick base pairing of nucleic acids. It involves mainly profiles of bead -based arrays, microarrays and qRT-PCR. Microarray allows detection of hundred of miRNAs at one time. A sample of RNA is hybridised with the capture probes which are spotted on glass slides. The sensitivity of the array is compromised due to short length of miRNA which causes difficulty to normalize the melting temperature of the probes[26]. The lacunae was sorted out by the emergence of Locked Nucleic Acids (LNAs).It contains minimum one LNA monomer and locked by connecting methylene bridge at 4'carbon atom and 2' oxygen atom of ribose moiety[27].

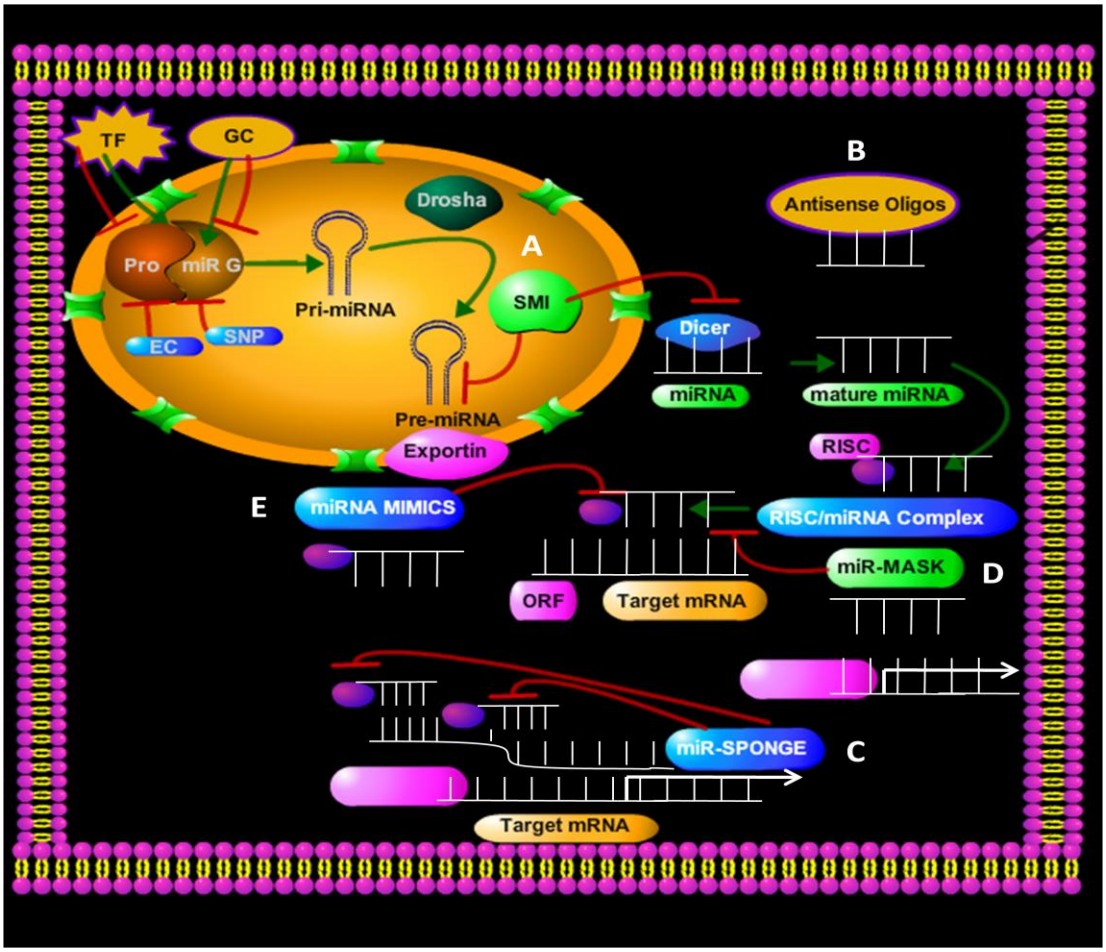

Fig 1. Biogenesis of miRNA showing causes of cancer such as defect in chromosomes, mutations, SNPs and changes due to epigenesis and latest anti-cancer therapeutic methods exploiting miRNA. (A) Use of small molecule inhibitors in transcription,(B)Involving antisense oligonucleotides which are single strands of RNA which arrests protein translation of mRNA by attaching to them,(C)Utilising miRNA-sponges which are oligonucleotide constructs having random miRNA binding sites that can be inserted into cell via mammalian expression vectors. They are used to soak up the complementary endogenous miRNA and suppressing the mechanism of specific oncomirs, (D) Introducing miRNA-masks which use antisense oligonucleotide technology attaching them to specific miRNA sites in the 3'-UTR of target mRNA and covers the miRNA target, thus slowing down the specific oncomir activity and (E) miRNA mimics are used which are small chemically modified double stranded RNAs that are similar to endogenous miRNAs and capable of bringing back the endogenous action of miRNA and heal the lost tumour suppressor miRNA expression. ORF: open reading frame. Pre-miRNA: Precursor microRNA. Pri-miRNA: Primary microRNA. RISC: RNA-induced Silencing Complex**. [(Source: Micro RNAs as a new therapeutic target towards leukemia signaling, M.Y. Murray et al. ,Cellular Signalling 24 (2012) 363–368, MicroRNAs in Cancer: Small Molecules With a Huge Impact, Marilena V. Iorio et al., J Clin Oncol 27(2009):5848-5856]**
[TF=Transcription factors, GC=Genomic changes: deletion, amplification, translocation, Pro= Promoter, miR G=miRNA Gene, EC=Epigenetic changes and SMI=Small Molecule Inhibitors]

Expression of MicroRNAs in different type of Leukemia
Acute Lymphocytic Leukemia:
It is one of the prominent pediatric malignancy caused due to clonal proliferation of premature B and T –lymphocyte progenitors which causes accumulated leukemic lymphoblasts in different extramedullary sites and bone marrow[28]. Some cytogenetic abnormalities like: t(12;21)TEL AML1; the t(8;14), t(2;8) and t(8;22) MYC-related translocations; TAL1; t(1;19) E2A-PBX; 11q23 translocations (MLL); t(9;22) translocations (BCR-ABL) etc characterizes ALL[29]. The first report of the relation of miRNA with ALL was shown in B-cell ALL patient when an inserted human homologue of lin-4 ,miR-125-1 was found in a rearranged heavy – chain immunoglobulin gene locus[30].

27 miRNAs are partially expressed between AML and ALL which includes miR-128a and miR-128b up regulation and down regulation of let-7b and miR-223 in ALL in comparison with AML. The over expression of miR-128b in ALL was found when compared to normal CD19+ cells[31]. The top five over expressed miRNAs in ALL are miR-128b,miR-204,miR-218.miR-331 and miR-181b-1.miR-17-92 cluster up regulation in ALL was also seen in ALL[32].

During the comparison of ALL with normal CD34+ cells ,up regulation of 14 miRNAs (miR-128a, miR-142-3p, miR-142-5p, miR-150, miR-181a, miR-181b, miR-181c, miR-193a, miR-196b, miR-30e-5p, miR-34b, miR-365, miR-582, miR-708) and down regulation of 5 miRNAs (miR-100, miR-125b, miR-151-5p, miR-99a, let-7e) was seen[33]. There was an attempt to differentiate ALL based on T-cell lineage and B-cell lineage, The cluster analysis that was done revealed that over expression of miR-148,miR-151 and miR-424 characterizes T-ALL and over expression of miR-425-5p,miR-191,miR-146,miR-128,miR-629 and miR-126 characterizes B-ALL[34].

Chronic Lymphocytic Leukemia

CLL is featured by storage of clonal CD5-positive B lymphocytes in bone marrow, blood and lymphatic tissue. Moreover, it has been found that more than 90% of leukemic cells are not going under - programmed cell death and they do not divide[35]. When CLL is observed cytogenetically , the chance of deletion of chromosome 13 band q14(13q14) is 10-35% as compared to greater sensitive fluorescence in situ hybridization (probes) which gives a frequency value of 70%[36].

It was found that miR-155, up-regulated in different lymphomas are over expressed in aggressive CLLs but miR-29 family members and miR-181 family members are found to be down-regulated.miR-181 family members are used to regulate TCL1 oncogene found to be up-regulated in aggressive form of CLL[37]. Greater levels of TCL 1 denotes high level of 70-kDa Zeta-Associated Protein(ZAP-70) and deficient of somatic mutation in Immunoglobulin Variable genes(IgVh), molecular markers of aggressive CLL supported by murine models which causes IgV region arrangement[38].

miR-15a and miR-16-1 affects BCL2 expression in CLL mouse models and it was found that expression levels of both miRNA is inversely proportional to the BCL2 protein expression level. Anti-apoptotic protein BCL2 over expression characterizes malignant B cells of CLL[39].

In another study it was found that homeotic gene HOXA11 is repressed due to miR-181a and miR-181b, having the greater role of miR-181a[40]. It is revealed that under expressed levels of itchy E3 ubiquitin protein ligase homolog(Itch) is related to induction of miR-106b and downfall in Itch protein levels is due to processing of caspase-9, non-functional mitochondria and CLL cell apoptosis[41].

Table1. Regulation of different miRNA in leukemia(↓shows down regulation and ↑ shows up regulation of miRNA)

microRNA	Type of leukemia	Reference
miR-17-92 cluster↑	CML	Woods K et al.(2007),O'Donnell KA et al.(2005),Agirre X et al.(2008)
miR-106b↑	CLL	Hammond SM et al.(2006)
miR107↑	APL	Garzon et al.(2007)
miR-125b-1↑	ALL	Zanette et al.(2007)
miR-126↑/126*↑	CBF AML	Langer C et al.(2008)
miR-128↑	ALL	Sonoki T et al. (2005)
miR-155↑	AML with FLT3-ITD and CLL	Garzon R et al.(2006),Garzon R et al.(2008),Burchova H et al.(2007),Georgantas RW et al.(2007),O'Connell RM et al.(2008),sampath D et al.(2009)
miR-191↑/miR-199↑	AML with t(11q23),isolated trisomy 8 and FLT3-ITD	Garzon R et al.(2008)
miR-221↑/miR-222↑	AML	Crazzolara R et al.(2009)
miR-127↑/miR-299↑/miR-323↑/miR-368↑/miR-382↑/miR-17↓/miR-126↓	AML with t(15;17)	Jongen et al.(2008),Li Z et al.(2008),Dixon et al.(2008)
miR-326↑/miR-219↑/miR-17-92↑/miR-196a↑/miR-29s↓/miR-34a↓/miR-16↓	AML with t(11q23)/MLL	Jongen et al.(2008),Garzon et al.(2008), Li Z et al.(2008),Dixon et al.(2008),Popovic R et al.(2009)
miR-124a↑/miR-30d↑	AML with Trisomy 8	Garzon R et al.(2008)
miR-10a↓	CML	Chaubey et al.(2009)
miR-15↓/mir-16-1↓	CLL	Calin et al.(2002), Calin et al.(2005),Linsey et al.(2007),Pfeifer et al.(2007), Scaglione BJ et al.(2007),Raveche et al.(2007),Cimmino et al.(2005)
miR-29↓/miR-181b↓	CLL	Pekarsky et al.(2000),Yan XZ et al.(2006),Fulci V et al.(2007)
miR-10a↑/miR-10b↑/miR-196a↑/miR-196b↑/miR-204↓/miR-128↓/miR-126↓/miR-130a↓/miR-451↓	AML with NPM1 mutation	Jongen et al.(2008),Garzon et al.(2008),Becker H et al.(2010),Coskun E et al.(2010)
miR-181a↑/miR-335↑/miR-34a↓	AML with CEBPA mutation	Jongen et al.(2008),Marcucci et al.(2008),Pullikan JA et al.(2010)
miR-148a↓	CN-AML	Hackanson B et al.(2008)
miR-223↓	AML with t(8;21)	Tanner SM et al.(2001)

Chronic Myeloid Leukemia

CML is a commonly found cytogenetic abnormality in which more than 95% of cases have homeostatic translocation t(9;22)(q34;q11), popularly called Philadelphia chromosome that generates a chimeric BCR-ABL protein having tyrosine – kinase activity. The treatment of this disease mainly emphasizes inhibition of kinase activity of BCR-AML by using Imatinib which causes resistance sometimes. Two protein from Src kinase family Fyn and Lyn were identified in previous studies for effective treatment without causing resistance[42,43].

Over expression of miR17-92 cluster has been reported in CML cell lines due to transactivation caused by breakpoint cluster region-c-abl oncogene(BCR-ABL) and c-MYC,v-myc myelocytomatosis viral oncogene homolog (c-MYC).In general BCR-ABL-MYC pathway can cause expression of pri-miR-17-92 in early chronic phase which is not possible in blast crisis CML[44].

The following 7 miRNAs (miR17-5p,17-3p,18a,19a,20a,19b-1 and 92-1) forms a polycistronic cluster which plays an important role in control of cell cycle by stopping E2F1[16]. The anti-apoptotic effect of miR-17 and miR-20a downregulates E2F1 and stops arrest of cell cycle because of G1 checkpoint due to an accumulated double strand breaks[45].

It was found that miR-10a, miR-150 and miR-151 were down regulated and miR-96 was up regulated when comparison of miRNA profiles of CML patients and healthy controls was done. In this study it has been suggested that miR-10a down regulation is not related to BCR-ABL1 activity whereas it is related to over expressed upstream transcription factor 2(USF2) denoting the usefulness of miR-10a in cell proliferation of CML due to increased USF2 expression[46]. In a separate study it was found that mir-219-2 and miR-199b does not play a significant role in CML pathogenesis[47].

Acute Myeloid Leukemia

It is a disease having a hallmark of accumulated granulocytic or monocytic precursors in peripheral blood and bone marrow. It has been revealed that cytogenetic and molecular subtypes of AML have related miRNA expression profile. Some microRNA having oncogenic and tumour suppressor functions like:miR-155,miR-21 and let-7 are related to specific subtypes. The correlation between heterogeneous pathogenesis of AML and miRNA expression profile is found[48].

In a study it was observed that on the basis of 21 up-regulated (let-7a, let-7b, let-7c, let-7e, miR-21, miR-22, miR-23a, miR-23b, miR-24, miR-26a, miR-27a, miR-27b, miR-125a, miR-130a, miR-199b, miR-221, miR-222, miR-223, miR-335, miR-424 and miR-451) and 6 down –regulated (j-miR-5, miR-128a, miR-128b, miR-130b, miR-151 and miR-210) miRNAs, AML can be separated from ALL[31].

The cytogenetically subtypes of AML i.e., CBF-AML with t(8;21), CBF-AML with inv(16) or t(16;16) and APL with t(15;17) have different miRNA expression profile which helps them to discriminate among others[49]. It has been found that APL with t(15;17) is characterized by up-regulation of 7 miRNAs(miR-127, miR-154, miR-154*, miR-299, miR-323, miR-368 and miR-370) and down regulation of 9 miRNAs(miR-17-3p, miR-185, miR-187, miR-194, miR-200a, miR-200b, miR-200c, miR-330 and miR-339)[48].

In CBF-AML with t (8; 21) and CBF-AML with inv (16) or t (16; 16), the over expression of miR-126 and miR-126* occurs. In AML with chromosomal aberrations of 11q23/MLL 7 up regulated miRNAs (miR-17–5p, miR-17–3p, miR-18a, miR-19a, miR-19b, miR-20a and miR-92) are originated from polycistronic miRNA cluster known as miR-17-92[15]. In AML with FLT3-ITD ,over expression of miR-155 was found which is not dependent on FLT3 signaling[50].

It was observed that expression level of miR-148 a is just opposite to the expression level of BAALC (Brain And Acute Leukemia Cytoplasmic) gene and can be called a negative regulator for BAALC[51]. The up-regulation of miR-181a and miR-335 is related to the mutations in CEBPA genes[52]. The relation between the miR10-a,miR10-b and miR-196a-1 and HOX genes was determined in CN-AML[53]. Havelange et.al describes a positive relation between HOX related genes and miR-10 and miR-20a.In addition, he described the negative correlation between miR-181a, miR-181b, miR-155 and miR-146 expression and IRF7 ,TLR4 genes responsible in inflammation and immunity. He

further showed the positive correlation of BIM and PTEN proapoptotic genes with miR-23a,miR-26a,miR-128a and miR-145[54].

Another study relates the role of miR-10a in NPM1^{mut} AML and its significance in the pathogenesis of this type of AML[55]. The role of ERG in haematopoiesis and leukemia and its regulation by miRNA was studied and it was found that NPM1^{mut} AML patient had higher expression levels of miR-196a and miR-196b in ERG regulation[56]. X-N Geo et al. reported that expression of miR-193a is inversely correlated to C-Kit expression and it is under expressed in leukemia cell lines and primary AML samples[57].

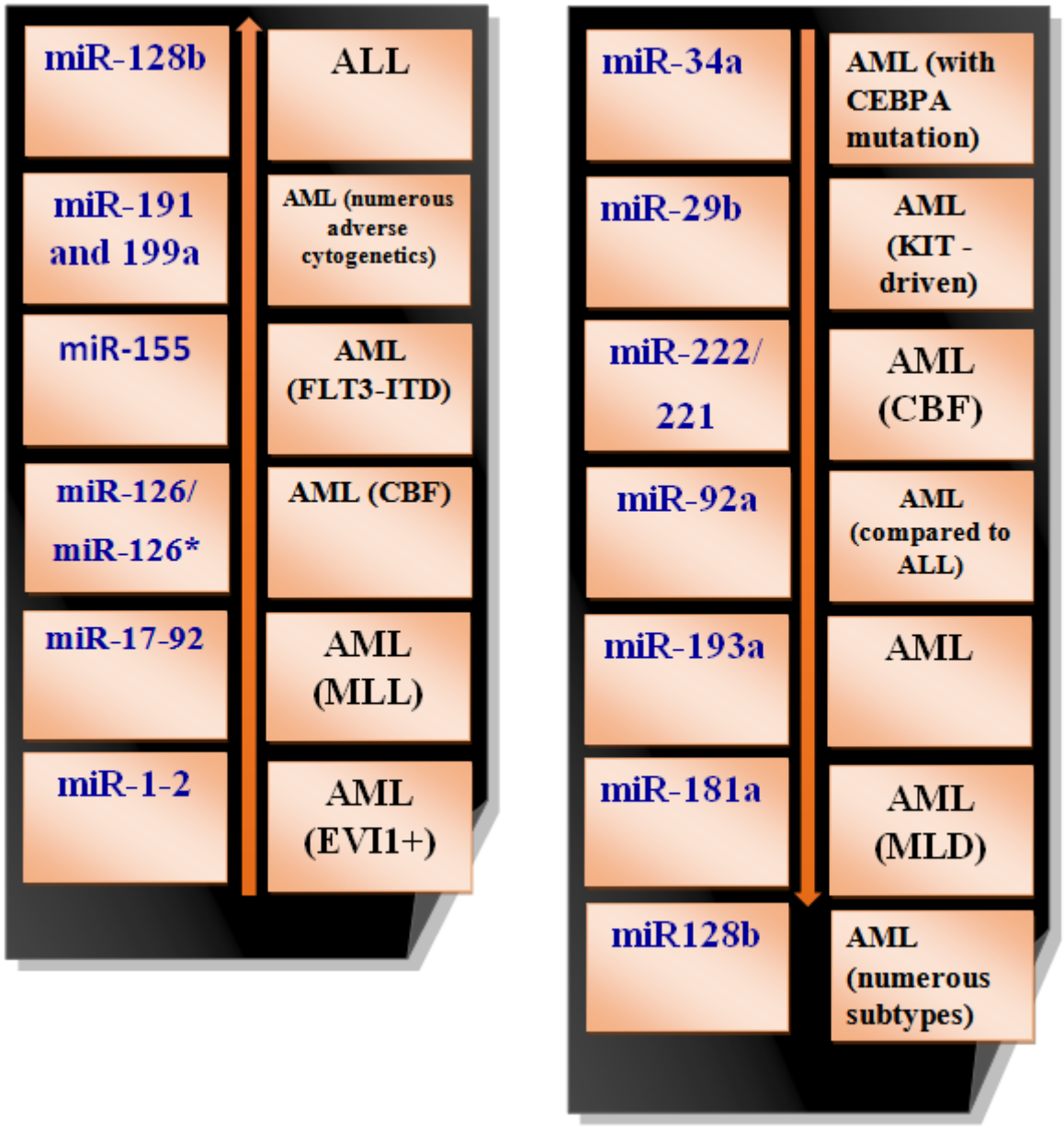

Figure 2: Regulation of miRNA in acute myeloid leukemia. (Source: Micro RNAs as a new therapeutic target towards leukemia signaling, M.Y. Murray et al., Cellular Signalling 24 (2012) 363–368**)**
[Where↑ = Up-regulation and ↓ = Down-regulation]

Conclusion:

The use of miRNA and siRNA as a therapeutic tool started with the discovery of RNAi in 1998 by Fire and colleagues. The study on the role of these miRNAs in various types of leukemia is still not adequate, especially in comparison to siRNA research. Numerous studies of various miRNAs in different types of leukemia are suggested

here and the role of miRNA as oncogenes is also studied. There are many miRNAs present in very small amount in our body whose expression in leukemic person and normal person will always be distinct. Numerous studies has suggested that the up regulation or down regulation of miRNA expression forms a distinct signature of leukemia subtypes which can be used as a biomarker for this disease and can be helpful in diagnosis, prognosis and cure of this disease. With the help of new technologies, the study of miRNA is becoming more specific and fruitful with each passing day. For this task there is a need of developing more new microarray techniques and high throughput technology for better research of this field, e. g. - the use of Locked Nucleic Acid is good for better sensitivity and specificity for research work related to miRNA. Specifically in connection with AML there are many miRNAs of unknown functions and pathogenesis that can be used as biomarker. The new biomarker discovery path is always open for new researchers to fight against leukemia and its deadly mortality rate among human beings. Among them miRNA stands as a major potential biomarker by properly studying and understanding the disregulated expression of them. Moreover, use of miRNA as Antagomirs, synthetic miRNAs, capable of changing expression of particular miRNAs, can be used as potential therapeutic agents to rectify aberrant expression of endogenous miRNAs in AML and can be used as part of a futuristic strategic plan aimed at personalized treatment of different types of leukemia.

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