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The role of micro-ribonucleic acids in normal hematopoiesis and leukemic T-lymphogenesis

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Abstract

Micro-ribonucleic acids (microRNAs) are small molecules containing 20-23 nucleotides. Despite their small size, it is likely that almost every cellular process is regulated by them. Moreover, aberrant microRNA expression has been involved in the development of various diseases, including cancer. Although many data are available about the role of microRNAs in various lymphoproliferative disorders, their impact on the development of acute lymphoblastic leukemia of T-cell progenitors is largely unknown. In this review, we present recent information about how specific microRNAs are expressed and regulated during malignant T-lymphopoiesis and about their role during normal hematopoiesis.

Key words: microRNAs; Gene expression; Leukemia; Acute lymphoblastic leukemia; T cells

Introduction

For more than 50 years the term “gene” has been synonymous of genomic regions encoding messenger RNAs (mRNAs). However, recent studies have demonstrated that the entire genome gives rise not only to mRNAs but also to thousands of small regulatory RNAs, the so-called non-coding micro RNAs (microRNAs) (1). The first microRNA was described by the group of V. Ambros in the nematode *Caenorhabditis elegans* and was named *lin-4* (2). Five years later, in 1998, the process of RNA interference (silencing of specific genes) carried out by microRNAs was described in plants (3,4). Subsequently, it was assumed that microRNAs regulate gene expression at the post-transcriptional level in presumably every multicellular organism (5). At present, it is clear that more than 3% of human genes encode microRNAs, regulating approximately 90% of the protein-coding genes (6).

microRNAs consist of small molecules containing 20-23 nucleotides (nt) and function most frequently as negative regulators of gene expression. They exert their effects post-transcriptionally by inhibiting mRNA translation or inducing mRNA degradation, and thus participate in a wide variety of physiological and pathological cellular functions (7). microRNAs are now among the most studied non-coding RNAs and it is not an exaggeration to say that their identification opened a new era in cancer research. Over the past few years, molecular oncology research has revealed that abnormalities in both protein-coding genes and microRNAs can be identified in tumors and that the interplay between proteins and microRNAs is involved in the initiation, progression, and metastasis of various types of human cancer (8). Such malignancies include brain, liver, prostate, ovarian, and oral cancer, as well as lymphomas, but this list is probably much longer (9-14). Aberrant microRNA expression could also play a vital role in the pathogenesis of leukemia and thus microRNAs have rapidly emerged as potential targets for anti-leukemia therapy (10).

This review focuses on recent information about the important roles of microRNAs during the normal hematopoietic development as well as their implications during the progress of leukemia and more specifically of T-cell malignancies.

Biogenesis and cellular functions of microRNAs

microRNAs are encoded within different genomic regions including protein-coding genes and non-coding transcription units. Approximately 50% of all synthesized microRNAs are derived from non-coding RNA transcripts,

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while an additional ~40% are located at introns of the protein-coding genes. The majority of microRNAs are transcribed by RNA polymerase II (RNA pol II) and bear a 7-methyl guanidine cap at the 5'-end of the molecule and a poly-adenine (poly-A) tail at its 3' end (15).

The mechanism giving rise to mature microRNAs involves two processing cleavages by the RNAse III enzymes *Drosha* and *Dicer*. Following transcription by RNA pol II, *Drosha* processes the primary microRNA transcript (pri-microRNA) into ~60-100-nt hairpin structure designated as precursor RNA (pre-microRNA). Through interaction with *exportin*-5 and *Ran-GTP*, the pre-microRNA is transported into the cytoplasm, where it is processed *de novo* by the catalytic activity of *Dicer*. This ultimate cleavage gives rise to a double-stranded ~20-23-nt product, which consists of a mature microRNA guide strand and a passenger strand (microRNA*). The mature microRNA is then loaded into effector complexes known as RNA-induced silencing complex, while the passenger strand is degraded (16).

microRNAs inhibit gene expression by binding to the complementary 3' untranslated region (3'UTR) of the target messenger RNA. Every organism synthesizes hundreds of unique microRNAs, each predicted to regulate specific target genes. Moreover, nearly all microRNAs identified in humans, plants and animals are evolutionary conserved (17).

Functions of microRNA in normal hematopoiesis

Hematopoiesis is sustained by continuous differentiation of multipotent hematopoietic stem cells (HSCs) into hematopoietic progenitor cells (HPCs), giving rise to different lineages and mature blood cells. Regulation of hematopoiesis consists of two aspects: self-renewal of HSCs (progressive restriction of lineage potential) and differentiation of HSCs/HPCs (development into differentiated precursors up to mature cells). HSC differentiation leads to the formation of multipotent cells, which have largely lost their capacity for self-renewal, but retain the ability to differentiate into many types of blood cells. These HPCs generate common lymphoid progenitors, giving rise to B- and T-lymphocytes, and common myeloid progenitors, generating erythrocytes, megakaryocytes, granulocytes, and monocytes/macrophages (Figure 1) (18).

microRNAs are differentially expressed during normal hematopoiesis (Figure 1). Chen et al. (19) were the first to report that microRNAs are specifically expressed and dynamically regulated during murine hematopoietic cell development (19). Since that report, several groups have used different techniques to isolate progenitors, to induce uni- or multilineage differentiation, and to describe the microRNA expression profile and/or function during murine and human hematopoiesis.

During human erythropoiesis, microRNA-221 and microRNA-222 are highly expressed in $CD34^+$ HPCs and are gradually down-modulated during unilineage erythroid differentiation. The decline of microRNA-221 and microRNA-222 expression during the exponential erythroid growth unblocks *c-kit* protein translation. Since *c-kit* is a receptor of the kit ligand or stem cell factor, this can lead to expansion of early erythroblasts (20). Two additional pathways essential for erythropoiesis are also modulated by microRNAs: ALK4 and GATA-1. MicroRNA-24 is a negative regulator that inhibits erythroid differentiation by targeting active type I receptor ALK4 (21). The second pathway is a globin transcription factor 1 (GATA-1-regulated microRNA locus, consisting of microRNA-144 and microRNA-451, which regulates erythroid development in zebrafish embryos (22).

With respect to the common erythrocyte-megakaryocyte precursors, microRNA-150 was found to drive *in vitro* and *in vivo* megakaryocytic differentiation at the expense of the erythroid lineage (23). Furthermore, a regulatory pathway in megakaryocytopoiesis involving the promyelocytic leukemia zinc finger (PLZF) transcription factor, microRNA-146a and the stromal-cell-derived factor 1 receptor CXCR4 has been identified. microRNA-146a was initially reported to be an endotoxin-responsive gene in human monocytes. PLZF protein is a transcription factor that is involved in the regulation of limb and skeleton formation, hematopoietic proliferation and differentiation, leukemogenesis, and tumorigenesis. In this recently identified pathway, PLZF transcriptionally inhibits microRNA-146a, which in turn regulates the CXCR4 co-receptor. In $CD34^+$ HPC megakaryopoietic cultures, PLZF was up-regulated, a fact leading to a decrease of microRNA-146a expression and to increased levels of CXCR4 protein. MicroRNA-146a overexpression as well as PLZF or CXCR4 silencing impaired megakaryocyte proliferation, differentiation, maturation, and colony formation (24-26).

The key role of microRNAs was also demonstrated in human monocytopoiesis by two separate studies (27). The first examined the role of microRNAs of the 17-5p-92 and 106a-92 clusters in monocytic differentiation (28). The microRNA-17-5p-92 cluster consists of seven microRNAs located on chromosome 13 and transcribed as a polycistronic unit (15). This cluster is highly homologous to the microRNA-106a-92 and microRNA-106b-25 clusters located on chromosomes Y and 7, respectively (29). During monocytic differentiation, low levels of microRNA-17-5p/20a/106a allow translation of acute myeloid leukemia 1 (AML1) transcription factor that inhibits microRNA-17-5p-92 and microRNA-106a-92 clusters. It also *trans*-activates the expression of monocyte colony-stimulating factor (M-CSF), thus inducing monocytic cell differentiation. This study indicates that monocytopoiesis is controlled by a circuitry sequentially involving microRNA-17-5p/20a/106a, AML1 and M-CSF receptor, whereby microRNA-17-5p/20a/106a functions as a gene complex interlinked with AML1 in a mutual negative-feedback loop (27). Interestingly, the microRNA-17-92 cluster is also essential for B-lymphocyte development *in vivo*. Its targeted

deletion in mice resulted in lung hypoplasia and inhibition of the pro-B to pre-B transition (30). On the other hand, transgenic expression of microRNA-17-92 in mice caused the development of lymphoproliferative diseases and autoimmunity disorders (31).

Additional microRNAs are also involved in the PU.1-dependent regulatory pathway, which is required for normal human monocytic differentiation (28). It was found that the master transcription factor PU.1 directly activates microRNA-424 transcription during monocytic differentiation of promyelocytic blasts as well as human HPCs in unilineage monocyte cultures. microRNA-424 was found to be a potent inducer of monocyte differentiation, particularly by targeting the transcription nuclear factor I-A (NFI-A), whose down-regulation is critical for the progression to monocytic differentiation (28).

microRNAs have also been linked to macrophage functions during inflammation and development of innate immunity. microRNA-155 was found to be up-regulated in primary murine macrophages after exposure to interferon β and poly(I:C) (32). Sustained expression of microRNA-155 in HSCs causes abnormal proliferation of myeloid cells (33).

During myelopoiesis, microRNA-223 levels rise progressively and their suppression blocks granulocytic maturation. The levels of microRNA-233 are modulated by competitive binding to its upstream part of two *CCAAT*-box-binding proteins: NFI-A and C/EPBα. These two proteins bind to the *CAAT* elements located on the pre-microRNA promoter. NFI-A maintains microRNA-223 at low levels, while its replacement by C/EPBα activation results in microRNA-223 up-regulation and granulocytic differentiation (34). The transcriptional induction of the myeloid-specific microRNA-223 is responsible for granulocyte-monocyte precursor progenitor proliferation and granulocyte function *in vivo* (35,36).

microRNA expression and significance during normal and malignant T-lymphopoiesis

The earliest progenitors of T-lymphocytes are derived

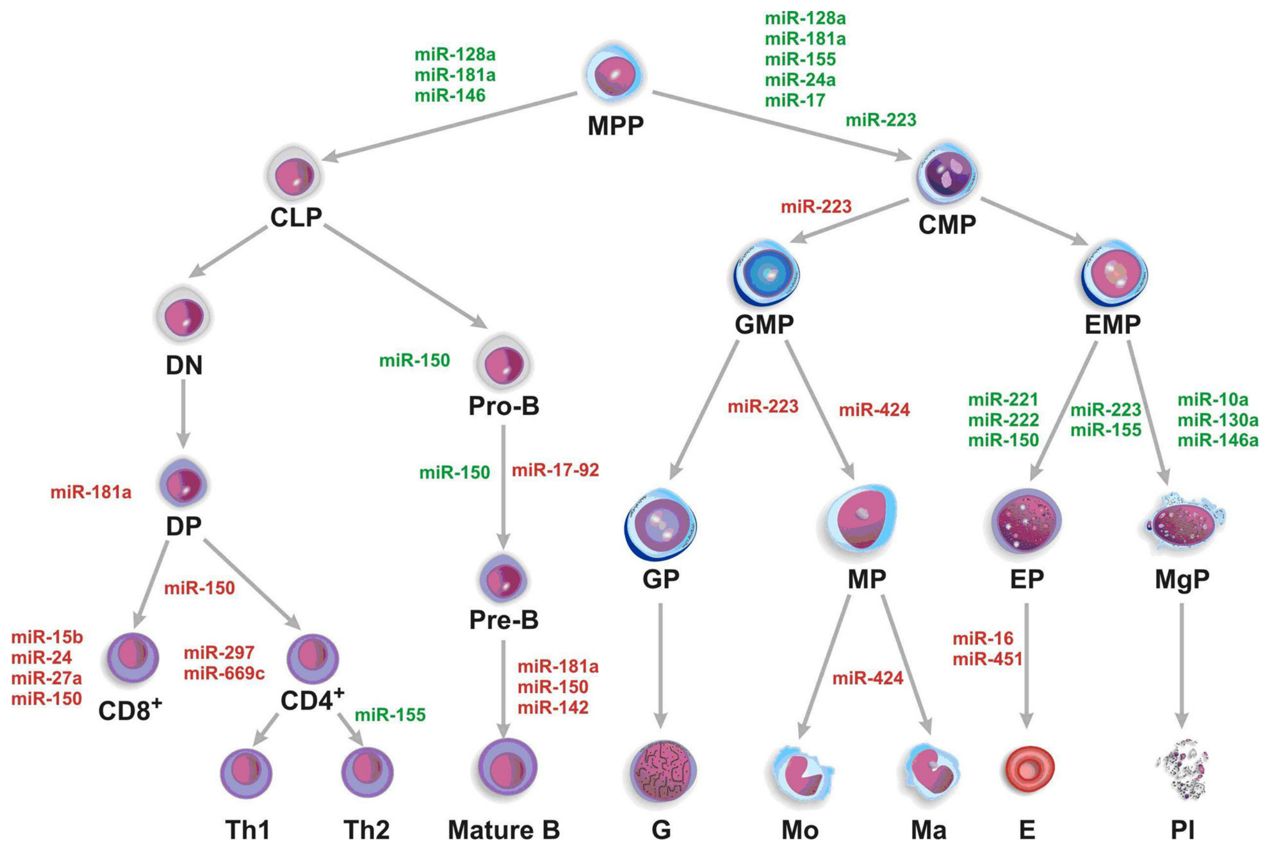

Figure 1. Role of microRNAs in normal hematopoiesis. The figure shows the microRNAs involved in, and their expression during the hematopoiesis and differentiation of MPP cells into different lineages. Red and green color indicates microRNAs that are overexpressed and underexpressed, respectively. MPP = multipotent progenitor cell; CLP = common lymphoid progenitor; DN = double negative; DP = double positive; Th1 = T-helper 1; Th2 = T-helper 2; CMP = common myeloid progenitor; GMP = granulocyte-monocyte precursor; GP = granulocyte precursor; G = granulocyte; MP = monocyte-macrophage precursor; Mo = monocyte; Ma = macrophage; EMP = erythrocyte-megakaryocyte precursor; EP = erythrocyte precursor; E = erythrocyte; MgP = megakaryocyte precursor; Pl = platelets.

from the bone marrow. They are extremely rare and typically are present within complex populations of hematopoietic stem cells (37). Although they are generated within the bone marrow, most of T-cell development occurs in a specialized organ, i.e., the thymus. These distinct differentiation compartments underscore the unique capacity of the thymic microenvironment to support T-cell lineage restriction and differentiation. The development of T-lymphocytes is supported by many thymus-derived signals such as Notch, morphogenetic, and protein kinase signals (38). The development of T cells in the thymus as well as their activation at the periphery, are controlled by complex protein signaling pathways that are regulated by the microRNAs. Expression profiles of these microRNAs vary during the different stages of T cell and cellular subset development. Two specific microRNAs, microRNA-17-92 and microRNA-155, have been implicated in T-cell development and probably account for some of the phenotype of *Dicer* deficiency in T cells. Recent data also indicate a role of microRNAs in the differentiation of T cells into different effector T-helper cell subsets. For example, in microRNA-155-deficient mice, T cells develop directly into Th2 cells, which indicate that microRNA-155 regulates the differentiation into Th type 1 cells. Also, mice with conditional deletion of *Dicer* or *Drosha* in regulatory T cells (T_{reg}) show requirement for the microRNA pathway in forkhead boxP3^{+} T_{reg} cells. These animals develop a lethal autoimmune inflammatory disease accompanied by impaired development and function of T_{reg} cells (39). Moreover, mice lacking microRNA-155 were immunodeficient and displayed increased lung airway remodeling due to the impaired functions of B- and T-lymphocytes and dendritic cells (40). The development of T cells and the regulatory role of microRNAs is described in Figure 2 (41).

Recent data suggest that microRNA genes are new epigenetic targets in cancer development. Their altered regulation by oncogenic proteins and more specifically by fusion proteins expressed during hematological malignancies is potentially relevant to the pathogenesis of leukemic diseases. Numerous oncogenes and tumor suppressor genes are potentially regulated by microRNAs (42). microRNAs may play a role in oncogenesis as oncogenes, probably by variety of mechanisms involving the elimination

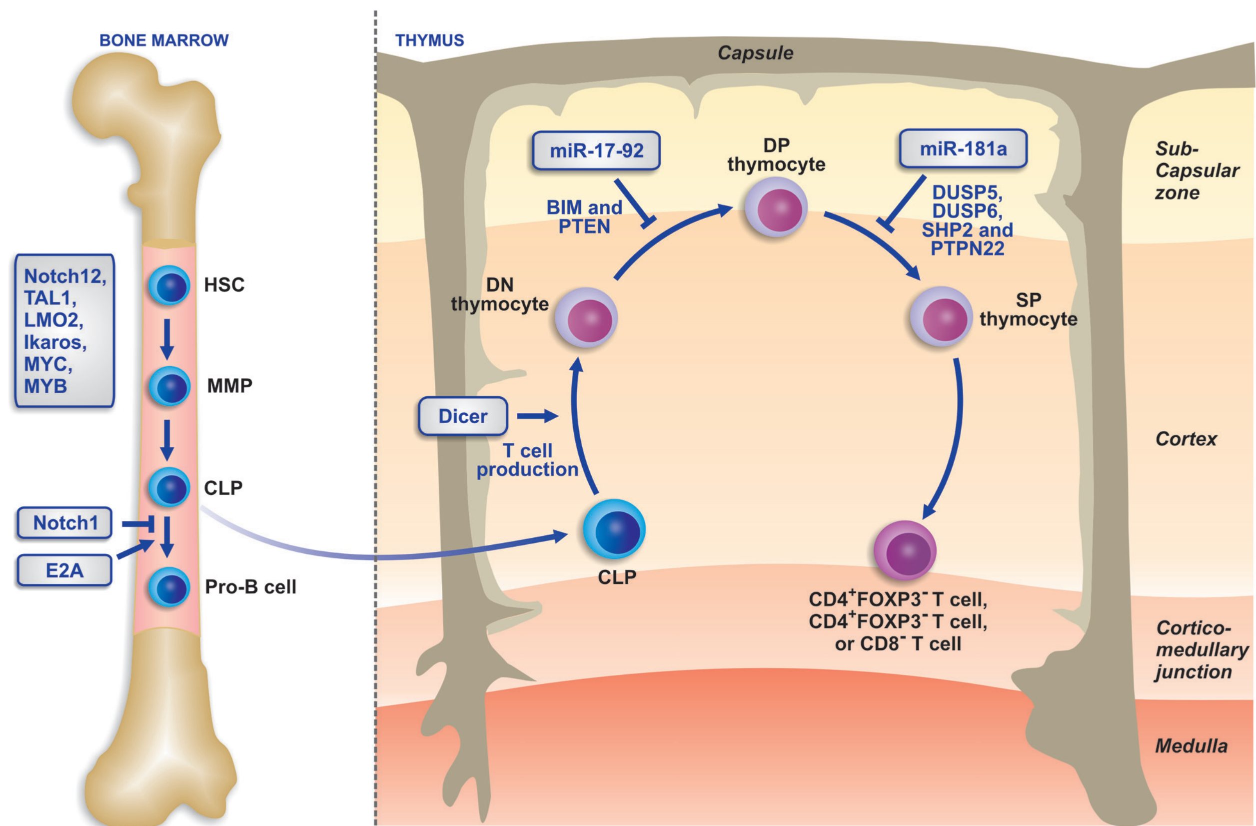

Figure 2. Schematic diagram of T-cell development and regulation and the participation of microRNAs in its regulation. HSC = hematopoietic stem cell; MMP = multipotent progenitor cell; CLP = common lymphoid precursors; DN thymocyte = double-negative thymocyte; DP thymocyte = double-positive thymocyte; SP thymocyte = single-positive thymocyte. (Reproduced from Ref. 41, with permission from Macmillian Publishers Ltd.).

of tumor suppressor proteins or suppression of genes by targeting oncogenic mRNAs (43).

microRNA-mediated tumorigenesis may result from either down-regulation of tumor suppressor genes or up-regulation of oncogenes. Usually microRNA genes are located at fragile sites of the genome as well as in minimal regions with loss of heterozygosity, regions of amplification (minimal amplicons) or common breakpoint regions (42). The aberrant expression at such sites has been associated with solid tumors and hematopoietic malignancies, as suggested by the following examples: frequent deletion of microRNA-15a and microRNA-16-1 in chronic lymphocytic leukemia, increased levels of microRNA-155 in diffuse large B-cell lymphomas, and increased levels of microRNA-181 in acute myeloid leukemia (AML) types M1 and M2 (43). Besides, aberrancies of the normal microRNome have been well documented in almost all hematological malignancies, suggesting in most cases the so-called specific microRNA-signatures. Also of importance is the fact that some of the microRNA-expression abnormalities described in leukemias and lymphomas can be interpreted on the basis of the microRNome variations during normal hematologic ontogenesis. This suggests that their origin is from a particular differentiation stage in which the hematopoietic differentiation is “frozen” in the malignancy (44).

Acute lymphoblastic leukemia (ALL) is a genetically heterogeneous lymphoid malignancy, which may involve B or T lineages, and leukemic cells may present immunophenotypic markers characteristic of distinct stages of maturation (45-47). The quantitative and qualitative differences in the expression of these markers are useful for the diagnosis, classification and prognostic stratification of patients with ALL (47). Moreover, there are geographic differences in the distribution of ALL subtypes (48,49). In addition to immunophenotypic markers, ALL is characterized by various underlying genetic abnormalities. Zanette et al. (50) have compared the profile of microRNA expression in $CD19^+$ cells from patients with ALL of B-progenitors with $CD19^+$ cells from the peripheral blood of healthy subjects. The five most highly expressed microRNAs in ALL were microRNA-128b, microRNA-204, microRNA-218, microRNA-331, and microRNA-181b-1. The most represented microRNA in ALL is microRNA-128b with more than a 400-fold difference compared to normal $CD19^+$ B cells. microRNA-128b is a reported homologue of microRNA-128a and has been found in colon, lung, and pancreas solid cancers. On the other hand, the four microRNAs with the lowest expression levels were microRNA-135b, microRNA-132, microRNA-199s, microRNA-139, and microRNA-150 (50).

More recently, Mi et al. (51) performed a large-scale genome-wide microRNA expression profiling assay and identified 27 microRNAs that are differentially expressed between ALL and AML. Using expression signatures of at least two of these microRNAs results in an accuracy rate of >95% in distinguishing and diagnosing AML and ALL. Among these microRNAs differentially expressed between ALL and AML, microRNA-128a, microRNA-128b, let-7b, and microRNA-233 were most significantly distinct. microRNAs 128a and 128b were expressed at higher levels in ALL, whereas microRNAs 233 and let-7b were expressed predominantly in AML. microRNA-233 is also reported to be a “myeloid” gene that plays a critical role in myeloid functions and differentiation (51).

Interestingly, a special microRNA expression profile was recently identified in children experiencing ALL relapse, which was characterized by high levels of expression of microRNA-7, microRNA-198 and microRNA-663 and low levels of microRNA-126, microRNA-222, microRNA-551a, microRNA-345. Nevertheless, the mechanisms involved in the regulation of these microRNAs have not been identified. This “cascade” of microRNAs was also identified in samples of ALL with subsequent central nervous system relapse, suggesting they may serve as biomarkers in the detection of early central nervous system relapse of pediatric ALL. Interestingly, target prediction of the microRNA pattern revealed that some abnormally expressed microRNAs may putatively target neuron function- and neurotransmitter-related genes (52).

The prognostic value of microRNAs in ALL is further demonstrated by the significance of microRNA-16. In general, low levels of microRNA-16 expression are associated with better outcome, while higher expression levels of this microRNA are associated with the poorest type of prognosis. This microRNA functions by inhibiting cell cycle progression by targeting several cyclins (E, D1), CDK6 and by inducing apoptosis by inhibiting BCL-2 translation. Considering these main functions of microRNA-16, the poor prognosis associated with its higher expression levels in ALL is astonishing. In this respect, researchers have failed to demonstrate a relationship between microRNA-16 expression and malignant lymphocyte proliferation or differentiation. However, investigation of the extent to which microRNA-16 varies within normal peripheral blood leukocyte stimulation, has revealed that its decrease after stimulation is necessary for the induction of lymphocyte proliferation. In contrast, a microRNA-16 increase might act as a restriction point in the entire proliferation process. Thus, microRNA-16 expression levels appear to be of prognostic significance in ALL, which may be controlled in independent and larger series (53).

We have recently studied the microRNA expression profile in a subgroup of patients with T-ALL, associated with a poor prognosis: T-ALL expressing NK markers (CD56 or N-CAM) (54,55). By multiple comparisons, we determined the microRNA expression profile of normal $CD3^+/CD56^-$ and $CD3^-/CD56^+$ cells and of T-ALL/$CD56^+$ and T-ALL/$CD56^-$ leukemic samples. Hierarchical cluster analysis revealed that normal and leukemic samples were grouped into two distinct clusters. The expression of 95 of 157 (60.5%) microRNAs did not differ from that of endogenous RNAs. Most of the differentially expressed microRNAs were less

expressed in leukemic cells than in normal cells. microRNA-29b was significantly more expressed in leukemic cells than in normal cells. By contrast, microRNA-223 was less expressed in these comparisons. Known targets include the *TCL1* and *MCL1* genes for the former and *NFIA-1* for the latter. Interestingly, the relevance of microRNA-223 to hematopoietic differentiation has been demonstrated in myelopoiesis, where there is a direct correlation between the transcriptional and epigenetic regulation of microRNA-223 (34-36).

In our study (54), microRNA-152 was less expressed in leukemic blasts compared to normal cells; however, within the latter, microRNA-152 was more expressed in $CD3^-$/$CD56^+$ than in $CD3^+$/$CD56^-$ cells. microRNA-181a and microRNA-181b were more expressed in T-ALL/$CD56^-$ cells than in $CD3^+$/$CD56^-$ cells, whereas microRNA-374 was more expressed in T-ALL/$CD56^+$ cells than in $CD3^+$/$CD56^-$ and $CD3^-$/$CD56^+$ cells. Comparison between T-ALL/$CD56^+$ and T-ALL/$CD56^-$ cells demonstrated a 270-fold increase in microRNA-221 expression in the former. Among the microRNA-221 targets are $p27^{/kip1}$, an important cell cycle regulator, and the *c-kit* receptor from the tyrosine kinase family, which have constitutively augmented activity in other hematological malignancies.

Mi et al. (56) have demonstrated that microRNAs-17-92 could play an essential role in the development of mixed lineage leukemia (MLL)-rearranged acute leukemia. This cluster was highly expressed not only in MLL-associated AML but also in ALL. The amplification of 13q31 and up-regulation by MLL fusions possibly contribute to the overexpression of the microRNA-17-92 cluster in MLL-rearranged leukemia. Moreover, microRNA-17-92 significantly increase cell viability, while inhibiting the apoptosis of HeLa (human cervix carcinoma) and 293T (human embryonal kidney) lineage cells and enhance proliferation of mouse normal bone marrow progenitor cells, resulting in the transformation of these cells by microRNA-17-92 and in cooperation with MLL fusions. This suggests that overexpression of the microRNA-17-92 cluster in MLL-rearranged leukemias is attributable to both DNA copy number amplification and direct up-regulation by MLL fusions.

The control of microRNA expression in ALL is still poorly understood. Nonetheless, several experimental data indicate that epigenetic regulation is an important mechanism guiding the regulation of microRNA expression in ALL. Recently, Li et al. (57) observed that histone modifications are involved in microRNA deregulation in human ALL cells. Using NALM-6 cells and primary leukemic cells, this group demonstrated that the silencing of the microRNA-22 gene in all cells is associated with the accumulation of histone modifications in its promoter element, independent of DNA methylation. microRNA-22 is frequently down-regulated in human ALL cells and the enforced expression of the microRNA-22 gene can inhibit the *in vivo* growth of B-cell lymphomas in mice, indicating the anti-tumor effect of microRNA-22. Moreover, histone modifications have been implicated in various cancers and histone deacetylase activity inhibitors possess significant growth inhibitory activity on ALL cells. For further research activities it would be of interest to investigate the functional role of microRNA-22 in histone modification associated with tumor initiation, promotion and progression of leukemia cells (57).

The regulation of microRNA expression is documented in cells infected with human retroviruses. Human T-cell leukemia virus type-1 (HTLV-1) is a causative agent of adult T-cell leukemia, which is an aggressive $CD4^+$ malignancy. Tomita et al. (58) discovered that the expression of selected microRNAs and especially microRNA-146a is altered in HTLV-1-infected T-cell lines compared to uninfected cells. MicroRNA-146a is one of the key molecules in the inflammatory response and its expression is induced by activation by the NF-κB signaling pathway. It is expressed in HTLV-1-infected T-cell lines and is induced directly by the virus-encoded transactivator protein *tax* that plays a central role in viral pathogenecity. Exactly how the *tax*-microRNA-146a relationship regulates cell growth is difficult to determine, but it probably has several targets including IRAK 1, TRAF-6, IL-8, CXCR4, and matrix metalloproteinase 9. Thus, identification of aberrant expression of specific microRNAs in HTLV-1-infected cells may reveal novel retroviral functions in the pathogenesis of latent HTLV-1 infection. In the future, microRNAs could be used in clinical studies to predict the development of HTLV-1 leukemogenesis. Inhibition of microRNA-146a by anti-microRNA inhibitors suppresses the proliferation of HTLV-1-infected T-cell lines but, on the other hand, uninfected cell lines proliferate rapidly. These data suggest that microRNA-146a might be a promising therapeutic target in adult T-cell leukemia (58).

In conclusion, microRNAs are key regulators of cellular differentiation and their aberrant expression has been associated with the characteristic block of T-cell differentiation in T-ALL. Moreover, differential synthesis of some microRNAs has proved to be a prognostic factor for the outcome of ALL and/or could be used for its diagnosis. As a matter of fact, analysis of microRNA expression may prove to be of greater clinical significance than mRNA profiling, in contrast to mRNA expression, for a small number of microRNAs might be sufficient to classify human leukemias and, unlike mRNAs, microRNAs remain intact in routinely collected formalin-fixed or paraffin-embedded clinical tissues. Nevertheless, this assumption still requires extensive independent studies to be conducted before microRNA analysis is introduced into routine clinical exams.

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