Chapter 12
MicroRNAs in Leukemia

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Abstract Deregulated gene expression plays a crucial role in leukemia and recent evidence suggests that in hematologic malignancies deregulated microRNAs (miRNAs) have the ability to function as both tumor suppressor and oncogene. Microarray-based miRNA expression profiling has been performed for different leukemias and can identify leukemia subtypes and prognostic classification. Specific expression patterns also suggest that miRNAs contribute to leukemogenesis. Indeed, in chronic lymphocytic leukemia (CLL), the putative tumor suppressor *miR-15* and *miR-16* are down-regulated and recent data from animal models suggest that the deregulation of *miR-15* and *miR-16* can cause CLL/lymphoma. Similarly, a growing number of particular miRNAs are associated with clinical course or specific genetic aberrations. In this chapter, we will summarize current knowledge of the role of miRNAs in acute and chronic leukemia.

12.1 Introduction

Myeloid and lymphoid leukemias are hematopoietic stem cell disorders characterized by the clonal expansion of malignant myeloid or lymphoid cells. Over past years our knowledge of these hematologic malignancies has increased tremendously resulting in a classification based on morphology, immunophenotype, genetic and clinical features. Current classifications thus attempt to define biologically and clinically relevant entities. Nevertheless, in many cases the pathogenic event(s) are still unknown, and even well-defined leukemia subgroups (e.g. based on cytogenetics) exhibit considerable heterogeneity. Therefore, an improved understanding of the underlying tumor biology represents a prerequisite for refined molecular taxonomy of myeloid and lymphoid malignancies.

Chronic myeloid leukemia (CML) was the first malignancy associated with a recurrent chromosomal abnormality, the Philadelphia chromosome, which turned

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out to be also the first aberration that could be shown to result from a translocation of genetic material among chromosomes. By forming a fusion gene (*BCR-ABL*) the Philadelphia chromosome translocation t(9;22) is responsible for the pathogenesis of the disease (Chen et al. 2010). With the invention of chromosomal banding techniques, recurrent cytogenetic aberrations were also discovered in acute myeloid leukemia (AML), the most common acute leukemia in adults. Here, the translocation t(15;17) associated acute promyelocytic leukemia (APL) was the first malignancy to be successfully treated with a targeted approach that specifically overcomes the transforming potential of the molecular defect by adding all-*trans* retinoic acid to leukemia therapy (Huang et al. 1988). Likewise, CML has become the first disorder in which a small molecule inhibitor has been designed to specifically target the aberrant tyrosine kinase function responsible for the malignant transformation (Druker et al. 1996).

In addition, during the last decade genomics technologies have provided further "high resolution" insights into the molecular variation underlying the biological and clinical heterogeneity of malignancies. In many ways leukemias proved to be valuable models to demonstrate the utility and promise of the use of DNA microarray technology to study human tumors. For example, based on genome-wide gene expression profiling studies of acute lymphoblastic leukemia (ALL) and AML samples it was first demonstrated that distinct tumor subclasses exhibit characteristic gene expression changes that can be used for tumor class prediction (Golub et al. 1999).

In chronic lymphocytic leukemia (CLL) genomics approaches comparing gene expression profiles of CLL samples with unmutated and mutated *IGHV* led to the discovery of the differentially expressed gene *ZAP70* (Rosenwald et al. 2001; Rosenwald and Staudt 2002), which became the first microarray analysis derived marker that was clinically implemented.

As deregulated gene expression plays a crucial role in leukemias, recent evidence has been provided that in hematologic malignancies deregulated microRNAs (miRNAs) that have the ability to function as both tumor suppressors and oncogenes might also play a crucial role (He et al. 2005). Here, the observations that the putative tumor suppressors *miR-15* and *miR-16* are down-regulated in patients with CLL (Calin et al. 2004; 2005), and that microarray based miRNA expression profiling can identify leukemia subtype specific expression patterns in ALL (Lu et al. 2005) further suggested miRNAs to be important players in leukemogenesis. Similarly, in AML the expression of miRNAs could be correlated with morphology and genomic aberrations (Chen et al. 2010), and in CML the deregulation of the polycistronic *miR-17-92* cluster seems to also be of pathogenic relevance (Venturini et al. 2007).

12.2 Deregulated MiRNA Expression in AML

Initial studies revealed lineage specific expression of miRNAs in hematopoietic differentiation (Chen et al. 2004) (Fig. 12.1), and *miR-223* became one of the best investigated miRNAs in myelopoiesis (and AML) due to its specific expression

in differentiated myeloid cells. Profiling miRNA expression in hematopoietic subpopulations as well as in a human APL cell line (NB4) upon differentiation with ATRA revealed *miR-223* to be expressed at low levels in the stem cell compartment with increasing expression throughout myeloid differentiation (Chen et al. 2004). Lentiviral over-expression of *miR-223* in NB4 cells induces myeloid differentiation (Fazi et al. 2005), and in primary AML cells minimal over-expression of *miR-223* (less than 2-fold expression levels) led to similar results (Fazi et al. 2007). This was the first study demonstrating that changes in miRNA expression levels can promote reprogramming of AML cells.

Interestingly, over-expression of *miR-223* and loss of *miR-223* might have distinct effects, as genetic depletion of *miR-223* led to a significant increase of myeloid progenitor cells as well as the number of circulating and bone marrow neutrophils (Johnnidis et al. 2008). Despite its important role in granulopoiesis, broader profiling studies did not connect *miR-223* expression to a particular AML subtype (Isken et al. 2008; Garzon et al. 2008a, 2008b; Jongen-Lavrencic et al. 2008; Debernardi et al. 2007; Marcucci et al. 2008). However, Fazi and colleagues demonstrated that the AML1-ETO oncoprotein, the product of the gene fusion resulting from the t(8;21), induces heterochromatic silencing of *miR-223* expression by recruiting chromatin remodeling enzymes at an AML1-binding site on the *miR-223* gene (Fazi et al. 2007), thereby showing for the first time that epigenetic silencing of a miRNA locus can be associated with the pathogenesis of AML.

12.2.1 Profiling Deregulated MiRNA Expression in AML – Association of MiRNA Profiles with Distinct AML Subtypes

Based on the initial observations and considering the steadily increasing number of newly discovered miRNAs, it was reasonable to perform broader miRNA expression profiling studies in AML. Indeed, the miRNA expression profiles of AML patient samples have been recently addressed by several studies that used different methodological approaches and screened slightly different patient subgroups (Isken et al. 2008; Garzon et al. 2008a, 2008b; Jongen-Lavrencic et al. 2008; Debernardi et al. 2007; Marcucci et al. 2008; Cammarata et al. 2010).

For example, Garzon and colleagues profiled miRNA expression in 240 AML patient samples with predominantly intermediate and poor cytogenetics using DNA microarrays. With this approach, the authors could identify molecular signatures associated with balanced 11q23 translocations, isolated trisomy 8 and *FLT3* mutations (*FLT3*-ITD), implying that miRNA profiles are at least partially driven by cytogenetics (Garzon et al. 2008b). Based on these findings the role of miRNAs in AML carrying *NPM1* and *FLT3-ITD* mutations, the two most frequent molecular aberrations in AML, was further investigated (Garzon et al. 2008). A signature distinguishing mutated *NPM1* from wildtype cases included the up-regulation of *miR-10a*, *miR-10b* as well as *let-7* and *miR-29* family members. Interestingly, an independent study confirmed a correlation of *FLT3-ITD* samples and *miR-155*

up-regulation (Garzon et al. 2008a; Jongen-Lavrencic et al. 2008), though FLT3 inhibitor studies showed that the up-regulation of *miR-155* was independent from FLT3 signaling in these cases (Garzon et al. 2008b).

In accordance, a large study exploring the expression of miRNAs by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in a large AML patient cohort could also associate miRNA expression patterns with cytogenetic and molecular subtypes (Jongen-Lavrencic et al. 2008). Interestingly, for a miRNA based prediction of leukemia classes the necessary number of miRNAs varied drastically between AML subtypes. For example, a class predictor of only 10 miRNAs could reliably predict AML with t(8;21) and a set of 7 miRNAs AML with t(15;17). In contrast, a predictor comprising 72 miRNAs was necessary for AML with inv(16), thereby suggesting that not all cytogenetic aberrations might have a quite unique miRNA expression pattern and indeed some of the heterogeneity may stem from the fact that current subgroups may need to be further subdivided. In addition, miRNA expression levels might mainly be influenced by the differentiation stage of the leukemic cells, and thus e.g. inv(16) might be hard to distinguish form other inv(16)-negative AML cases with an identical morphology.

Similarly, smaller genome-wide miRNA expression studies using bead-based miRNA profiling approaches, microarrays and qRT-PCR confirmed miRNA expression patterns characteristic of cytogenetic subgroups such as t(15;17), t(11q23), t(8;21) and inv(16) (Cammarata et al. 2010; Li et al. 2008), as well as molecular subtypes like AML with *CEBPA* and *NPM1* mutations or deregulated *MN1* expression (Langer et al. 2008). Interestingly, all studies pointed towards the deregulation of miRNAs located in the *HOX* gene cluster, such as *miR-10a/b* and *miR-196a/b*, as well as *miR-221*, *let-7* family members, *miR-155*, *miR-29*, *miR-125b*, members of the *miR-17-92* cluster in AML. This suggests that a defined group of miRNAs might be involved in leukemogenic processes such as impaired differentiation and increased self-renewal.

12.2.2 Prognostic Impact of Altered MiRNA Expression in AML

Being correlated with altered gene expression and cytogenetic and molecular genetics aberrations, miRNA expression signatures have been shown to also confer prognostic information. While the study of Garzon and colleagues could identified two miRNAs, *miR-191* and *miR-199a*, to be significantly correlated with overall and disease-free survival (Garzon et al. 2008b), a large AML cohort by the Rotterdam group (based on qRT-PCR) could not identify any miRNAs that were correlated with overall or event-free survival independent of known factors such as cytogenetics (Jongen-Lavrencic et al. 2008). On the other hand, Marcucci and colleagues could identify a miRNA signature associated with event-free survival studying cytogenetically normal AML samples from adults under the age of 60 years who had high-risk molecular features such as *FLT3*–ITD, a wildtype *NPM1*, or both (Marcucci et al. 2008). However, in the future additional studies are needed to determine the impact of miRNAs as reliable biomarkers for diagnosis as well as prognosis in AML.

12.2.3 MiRNAs of Pathogenic and Functional Relevance in AML

Today, no study has been published yet investigating miRNA signatures in leukemic stem cells. So far, only few in vivo studies demonstrated the potential of a single miRNA to contribute to the development of myeloid neoplasia. Recently, Han and colleagues showed that retroviral over-expression of *miR-29a* can induce AML in mice (Han et al. 2010), and O'Connell and colleagues demonstrated similar findings through over-expression of *miR-155* in primitive hematopoietic cells that led to a myelo-proliferative syndrome (O'Connell et al. 2008). In contrast to miRNAs that can function as proto-oncogenes, there also has been evidence that distinct miRNAs can function as tumor-suppressor in AML. For example, recently Garzon and colleagues highlighted the potential of *miR-29b* as tumor suppressor by inducing apoptosis and reducing tumorgenicity in a xenograft AML model (Garzon et al. 2009a). Furthermore, miRNAs have also been shown to represent both targets and effectors of the epigenetic machinery. Similar to other genes, miRNA expression can be affected by DNA promoter methylation and histone modifications. As mentioned above AML1-ETO can lead to heterochromatic silencing of the *miR-223* genomic region and demethylation can restore *miR-223* expression followed by differentiation of leukemic blasts (Fazi et al. 2007). On the other hand, miRNA expression can impact the epigenetic modifications as e.g. *miR-29b* targets DNA methyltransferases in AML (Garzon et al. 2009b).

With regard to novel biological insights, the list of miRNA-target gene interactions likely involved in leukemogenesis is rapidly growing. Here, further in-depth analyses of the miRNA transcriptome using next generation sequencing will also provide novel insights by being potentially less error-prone than hybridization-based technologies (Kuchenbauer et al. 2008). These novel approaches might significantly add to the understanding how miRNAs contribute to the development of cancer, by revealing novel miRNAs, miRNA isoforms, mutations and absolute sequence counts, thereby highlighting additional miRNAs that might serve as future therapeutic targets in AML.

12.3 MiRNAs in CML

Important contributions to the understanding of CML pathogenesis have been shown for several miRNAs. GEP-studies identified specific miR expression profiles for CML compared to other tumor types as well as malignant hematopoietic cell lines and purified normal human blood cells (Lu et al. 2005; Ramkissoon et al. 2006).

BCR-ABL and c-MYC dependent regulation of miRNAs encoded within the polycistronic *miR-17-92* cluster were identified in CML cell lines (Venturini, 2007). Previous reports had already shown the involvement of this cluster in malignancies of B cell origin (Ota et al. 2004) and a direct regulation by c-MYC has been reported for the *miR-17-92* cluster (He et al. 2005; O'Donnell et al. 2005). In the recent study the authors linked BCR-ABL-driven leukemogenesis with c-Myc dependent regulation of the *miR-17-92* cluster. The link was identified through

inhibition of BCR/ABL after treatment with STI-571 (Imatinib) and by using RNAi. Moreover, the authors suggested a BCR/ABL-c-MYC-*miR-17-92* pathway that mediates enhanced miRNA expression in early chronic phase $CD34^+$cells in CML (Venturini et al. 2007).

Agirre and colleagues reported a specific miR profile after analysing expression levels in mononuclear cells from healthy persons and from patients with newly diagnosed CML (Agirre et al. 2008). *MiR-10a*, *miR-150*, and *miR-151* were down-regulated, whereas *miR-96* was up-regulated in CML cells. Down-regulation of *miR-10a* was not dependent on BCR-ABL activity and contributed to the increased cell growth of CML cells. The group identified the upstream stimulatory factor 2 (USF2) as a potential target of *miR-10a* and showed that over-expression of USF2 increases cell growth. There were some indications in the study for clinical relevance: In a group of 85 newly diagnosed patients with CML expression of *miR-10a* was down-regulated in 71% of the patients. Conversely expression of USF2 was up-regulated in 60% of the CML patients and there was an inverse correlation with over-expression of USF2 being significantly associated with decreased expression of *miR-10a.* While direct evidence is lacking, the results indicate that down-regulation of *miR-10a* may increase USF2 and contribute to the increase in cell proliferation of CML (Agirre et al. 2008).

A subsequent study identified miRNAs differentially expressed between resistant and responding samples that might, if confirmed, predict resistance to imatinib in patients with newly diagnosed CML. Potential targets for these miRNAs included membrane transporters which have been implicated in resistance to chemotherapy in general and specific imatinib resistance as well (San Jose-Eneriz et al. 2009).

Soon after the report on the role of the *miR-17-92* cluster, a second microRNA, *miR-203*, involved in the regulation of the ABL/BCR-ABL pathway was identified by analysing a fragile region on the mouse chromosome 12. This region was found to be frequently lost in γ-radiation-induced T cell lymphomas and contained approximately 12% of the known miRNAs.

Down-regulation of this miRNA turned out to be caused not only by deletion but also by silencing through promoter hypermethylation of the upstream region of *miR-203* in a majority of Ph-positive tumors, including B-ALL, primary CML, and cultured CML cell lines. Further analysis using computational prediction, mRNA expression analysis and functional validation identified *miR-203* as a regulator of ABL and BCR-ABL fusion protein levels. These data suggest that miR cannot only be regulated by BCR/ABL but may control oncogene levels as e.g. ABL or BCR/ABL. Reexpression of *miR-203* resulted in inhibition of tumor cell proliferation (Bueno et al. 2008).

CML blast crisis progenitors have been shown to loose their ability for differentiation by suppressed expression of CEBPα, a transcription factor controlling myeloid differentiation. Altered post-transcriptional gene regulation by the translational regulator poly(rC)-binding protein hnRNP E2 has recently been shown to be centrally involved in the pathogenesis of blast crisis in CML. High expression of hnRNPE2 leads to extensive down-regulation of CEBPα protein levels through interaction with

the mRNAs 5′ untranslated region (Melo and Barnes 2007; Perrotti et al. 2002; Perrotti and Neviani 2007; Chang et al. 2007).

The role of miRNAs in this process has recently been elucidated. D. Perrotti and colleagues (Eiring et al. 2010) identified *miR-328* which was differentially modulated in a BCR-ABL dose and kinase-dependent manner through the MAPK-hnRNP2 pathway. The group showed decreased levels of *miR-328* in blast crisis CML. *MiR-328* was identified to harbour a C-rich element that resembles the negative regulatory hnRNP E2-binding site contained in the CEBPα intercistronic mRNA region. As hypothesised, *miR-328* turned out to competitively target hnRNP E2 for binding and decreases association of hnRNP E2 to CEBPα. In further experiments it was possible to recover maturation in BCR-ABL positive cells that lost the ability for differentiation by reconstitution of *miR-328* expression with consecutively decreased survival of leukemic blasts mediated through the described mechanisms and by targeting PIM1 survival factor (Eiring et al. 2010).

The interaction with hnRNP E2 was independent of the miRNA's seed sequence but lead to the release of *CEBPα* mRNA from hnRNP E2-mediated translational inhibition. Importantly, these data reveal the ability of a miRNA to control cell fate not only through base pairing with mRNA targets but also through a "decoy" activity that interferes with regulatory proteins.

12.4 MiRNA in CLL

CLL is the most prevalent form of adult leukemia in the Western world showing an estimated incidence about 3.9 per 100,000 people per year. Men are affected two times more often than women and the median age at diagnosis peaks at 72 years (Dores et al. 2007).

CLL has turned out to be a disease with multiple facets in its pathogenic mechanisms including genetic aberrations, antigen drive and microenvironmental interactions (Zenz et al. 2010). Different risk groups can be closely linked to the clinical and genetic features. Identification of recurring genomic aberrations, gene mutations such as *TP53* (Döhner et al. 1995) and *ATM* (Schaffner et al. 1999), the detection of somatic mutations in the variable regions of the immunoglobulin (Ig) heavy chain (*IGHV*) genes (Fais et al. 1998; Hamblin et al. 1999) and the evidence of biased *IGHV* usage and stereotyped B cell receptors (BCRs) (Fais et al. 1998; Tobin et al. 2003; Stamatopoulos et al. 2007) have essentially led to better prognostication and insight into CLL-biology (Zenz et al. 2010).

Chromosomal aberrations occur in approximately 80% of all CLL cases. Most often chromosome 13q14 is affected ($13q^-$: 55%) by monoallelic (76%) or biallelic (24%) deletion followed by 11q deletion ($11q^-$: 18%), trisomy of chromosome 12 (16%) and the 17p deletion ($17p^-$: 7%) (Döhner et al. 2000).

Although research succeeded to map the ataxia teleangiectasia-mutated (*ATM*) gene to the affected minimal consensus regions at the chromosome bands 11q22.3-q23.1 and the tumor suppressor gene *TP53* is inactivated in 80% of cases with deletion of 17p13 respectively, no classical tumor suppressor gene could be located

at the minimal deleted region (MDR) at 13q14.3 despite intensive investigations for many years (Bullrich et al. 2001; Corcoran et al. 1998; Migliazza et al. 2001; Stilgenbauer et al. 1998).

Interestingly, the cells of the precursor state of CLL named monoclonal B-lymphocytosis (MBL) also exhibit 13q14 deletions at rates of more than 50% (Rawstron et al. 2008). In addition this characteristic deletion is present in a variety of other cancers with remarkable regularity (DLBCL, mantle cell lymphoma and multiple myeloma as well as mature T cell lymphoma (Rosenwald et al. 1999); Cigudosa et al. 1998; Stilgenbauer et al. 1998) prostate cancer (Dong et al. 2001).

12.4.1 MiR-15a and MiR-16-1

The missing link to the mechanism underlying the tumor suppression at band 13q14 was found in 2002 when it became evident that a cluster of two newly cloned miRNA genes, *miR-15a* and *miR-16-1* (Lagos-Quintana et al. 2001; 2002; Lee et al. 2001; Lau et al. 2001), was exactly located in a 30-kb region within the *DLEU2* gene at the MDR on chromosome 13q14.3 (Calin et al. 2002). Both *miR-15a* and *miR-16-1* were either deleted or down-regulated in most investigated CLL cases (Calin et al. 2002).

These findings were further strengthened after investigators reported that a germline mutation in the primary precursor of *miR-15a* and *miR-16-1* seemed to reduce the expression of this cluster in two CLL patients (Calin et al. 2005). Although mutations in the miR locus appear to be very rare, another study identified a point mutation in the 3′-DNA neighbouring the *miR-16-1* region that has been associated with reduced *miR-16-1* expression in the New Zealand black mouse, a model for indolent, late-onset CLL (Raveche et al. 2007).

A characteristic of CLL is that the clonal expansion of $CD5^+$ B cells is nourished only by a small proliferating cell-pool in CLL. Most CLL cells are locked in the G0/G1 phase of the cell cycle and non-dividing. Accumulation of malignant cells results from resistance to apoptosis as a further feature for CLL. CLL cells show consistent over-expression of the B cell lymphoma 2 (Bcl-2) protein (Kitada et al. 1998) which exerts its anti-apoptotic function by blocking the release of cytochrome c from mitochondria (Cory and Adams 2002). Bcl-2 over-expression can be caused by characteristic chromosomal translocations. In follicular lymphoma with the classical translocation t(14;18)(q32;q21), the *BCL2* gene is brought under the control of the Immunoglobulin heavy chain enhancers, resulting in forced transcription of the gene (Horsman et al. 1995). Similar mechanisms were reported for CLL but account only for approximately 5% of cases in which the *BCL2* gene is over-expressed (Adachi et al. 1990). Therefore other mechanisms explaining BCL-2 deregulation still needed to be found.

The search for the targets of *miR-15a* and *miR-16-1* revealed significant complementarity between parts of the *BCL-2* sequence and the seed region of both miRs. Studying the functional aspect of this finding, both miRs turned out to be inversely correlated with BCL-2 protein levels in CLL and were reported to act as direct

Table 12.1 Selected targets identified for *miR-15a/16-1*

Targets (gene symbol)	Tissue/cell lines	References
Bcl-2, WT1, CDC2, ETS1, JUN, MCL1, MSH2, PDCD6IP, RAB9B, WT1	MEG-01 cell lines	Calin et al. (2008); Cimmino et al. (2005)
CCND1, WNT3A	Prostate cancer	Bonci et al. (2008)
CCND1 (by *miR-16-1*)	Mantle cell lymphoma	Chen et al. (2008)
CCND3, CCNE1, CDK6	HeLa, HepG2, and A549 cell lines	(Liu et al. (2008))
c-MYB	K562 myeloid leukemia cells, human $CD34^+$ cells	Zhao et al. (2009)
CCNE1, CHK1, MCM5, CCND2, CDK6, CDK4, CCND3, C-MYB, BCL, CSE1L, ARL2, IGF1Rβ	CLL I83E95 cells	Klein et al. (2010)

post-transcriptional repressors of BCL-2 in transfection experiments on a MEG-01 cell line. Transfection with wild-type *miR-15/16* lead to an increased rate of apoptosis in transfected MEG-01 cells (Cimmino et al. 2005). Moreover, in in vivo studies in immunocompromised nude mice, tumorigenicity of leukemic xenografts using MEG-01 leukemic cells was significantly suppressed by ectopic expression of *miR-15a/16-1* (Calin et al. 2008).

By applying bioinformatic programs for screening and functional experiments for validation, several proteins involved in the regulation of cell cycle, cell growth and apoptosis have been reported to be directly regulated by *miR-15a/16-1* (Table 12.1).

Further and definite evidence for the pathogenic meaning of the 13q14 MDR and *miR-15a/miR-16-1* in B cell malignancies has been shown recently by generating a mouse model deleting the DLEU2/*miR-15a/16-1* cluster (Klein et al. 2010).

Mice presenting deletions of the MDR or with sole *miR-15a/16-1* deletion developed clonal B cell lymphoproliferation at the age of 15–18 months, including $CD5^+$ MBL, CLL/SLL, and NHL in 42% of $MDR^{-/-}$ and 26% of *miR-15a/16-1*$^{-/-}$ cases (Klein et al. 2010). While these data suggest that the deletion of the *miR-15a/16-1* is sufficient for lymphomagenesis, it also suggests that the non-protein coding DLEU2 is also contributing as the phenotype in these mice was more pronounced. Therefore additional tumor suppressive functions for residual parts of the MDR on 13q14, including *DLEU2* and potentially parts of *DLEU5* in 13q14 deletions exceeding the MDR, can be assumed (Klein et al. 2010).

In the work of Ulf Klein and colleagues, the *miR15a/16-1* cluster has been shown to influence growth, cell-cycle control and/or apoptosis, although the exact regulatory function by which *miR15a/16-1* exert its pathogenic effects in CLL. However the group found limited evidence of the *miR-15a/miR-16-1* to regulate BCL-2 (Cimmino et al. 2005; Fulci et al. 2007; Linsley et al. 2007; Klein et al. 2010; Calin et al. 2008).

Patients with a monoallelic 13q14 deletion seem to express higher *miR-15a/16-1* levels than patients with a biallelic 13q14 deletion (Fulci et al. 2007) and patients with a monoallelic del13q14 show slower lymphocyte growth kinetics than patients with biallelic deletions (Pfeifer et al. 2007), implicating mechanism which regulate the gene-dosage of these miRs. Nonetheless, questions remain as for example the question if and how *miR-15a/16-1* levels could be deregulated in cases without 13q deletion.

There appears to be some functional redundancy among members of the *miR-16* family (Linsley et al. 2007) and there are hints for further, potentially overlapping regulatory mechanisms modulating the expression levels (Zhao et al. 2009) and primary effect and of the *miR-15a/16-1* cluster.

12.4.2 MiRNA Microarray Screens Identifying Signatures and Subgroups for Prognostication

Although distinctive markers have been successfully identified to subgroup different CLL entities, further insights are needed to develop robust prognostic and predictive models. Estimations for the clinical course are currently based on the Binet classification and Rai staging system, age, beta2-microglobulin/thymidine kinase levels and genetic features (Zenz et al. 2010).

An adverse clinical course in CLL-patients has been proven for genetic factors such as unmutated *IGHV* genes associated with high ZAP-70 expression, *V3-21*-gene usage and particularly for genomic aberrations as 17p- and 11q- deletions or *TP53* mutation, which frequently lead to a treatment-refractory course (Zenz et al. 2010).

Microarray technology and global gene expression profiling (GEP) has been a successfully applied method to identify gene-signatures associated with specific biological processes, clinical stages and outcome in different cancer types (Quackenbush 2006). However, GEP performed on CLL samples mostly failed to identify specific expression-patterns (if unsupervised analysis was performed) that could differentiate CLL-subtypes. It was only after supervised analysis based on *IGHV*-mutation status that differentially expressed mRNAs were identified (Rosenwald et al. 2001; Klein et al. 2001; Jelinek et al. 2003; Durig et al. 2003; Stratowa et al. 2001).

MiRNAs profiling studies were quickly initiated using either microarrays (Calin et al. 2005; 2004) or PCR based approaches (Fulci et al. 2007).

Besides revealing distinct signatures that differ between CLL tumor-samples and normal $CD5^+$ B cells, characteristic signatures with respect to *IGHV* mutation status and/or ZAP-70 expression were observed in these early studies again based on supervised analysis (Calin et al. 2004; 2005).

Although the initially reported gene-signatures could not be completely reproduced by subsequent studies (Fulci et al. 2007; Marton et al. 2008), down-regulation of *miR-223* and members of the *miR-29* family were independently reported to be associated with unmutated *IGHV* genes and disease progression (Fulci et al. 2007;

Table 12.2 Selected microRNAs with characteristic signatures and potential prognostic relevance in chronic lymphocytic leukemia

MicroRNA signatures	Reference prognostic markers	Relevance	References
miR-15a, *miR-195*, *miR-221*, *miR-23b*, *miR-155*, *miR-24-1*, *miR-146*, *miR-16-1*, *miR-16-2* (all up) *miR-223*, *miR-29a-2*, *miR-29b-2*, *miR-29c* (all down)	Unmutated *IGHV*, ZAP-70	Association with adverse markers	Calin et al. (2005)
miR-29c, *miR-223*, *miR-150*, *miR-29b* (all down)	Unmutated *IGHV*	Disease progression	Fulci et al. (2007)
miR-181a, *let-7a*, *miR-30d* (all down) *miR-155* (up), *miR-29* (down)	Unmutated *IGHV*	Disease progression	Marton et al. (2008)
miR-29c, *miR-223*	Significantly associated with Binet stage, sCD23, beta2-M; ZAP-70, LPL *IGHV* mutational status, CD38 expression, and cytogenetics	Disease progression score combining *miR-29c*, *miR-223*, ZAP70, and LPL stratifies survival	Stamatopoulos et al. (2009)
miR-223, *miR-29b*, *miR-29c*, *miR-181* family (all down)	17p deletion	Potential subdivision of CLL with 17p deletion	Visone et al. (2009)
miR151-3p, *miR-29c*, *miR-34a* (all down)	17p deletion		
miR-29b (down), *miR-155* (up)	11q deletion	"Karyotype specific" microRNA	Visone et al. (2009)
miR-640/miR-148a (down), *miR-146b-5p/miR-146a* (up)	Trisomy 12		
miR-148a (up)	Normal karyotype		
miR-155 (down), *miR-640* (up)	13q deletion		

Marton et al. 2008) (Table 12.2). Further evaluation of these miRNAs in a retrospective study showed that low *miR-223* and *miR-29c* levels were associated with poor prognosis by showing shorter treatment free survival and reduced overall survival. A qPCR-score developed in this study integrated ZAP-70, LPL, *miR-223* and *miR-29c* and was able to discriminate prognostic subgroups of CLL patients (Stamatopoulos et al. 2009).

In a study from Pallasch and colleagues, 50 treatment-naïve CLL patients and peripheral B cells of 14 healthy donors were assessed (Pallasch et al. 2009). In CLL

cells, a set of 7 up- and 19 down-regulated miRNAs was identified including the up-regulation of *miR-155*. Among the miRNAs down-regulated in CLL cells, 6 of 10 miRNA promoters examined showed gain of methylation compared with normal B cell controls suggesting that methylation plays an important role in controlling miR expression. Subsequent target prediction of deregulated miRNAs suggested significant binding prediction at the 3′ untranslated region of the pleomorphic adenoma gene 1 (*PLAG1*) oncogene. *PLAG1* was shown to be regulated by *miR-181a*, *miR-181b*, *miR-107*, and *miR-424*.

Another profiling-study identified a karyotype-specific signature consisting of 32 miRNAs of which 9 miRs validated by qRT-PCR were associated with common cytogenetic features (Visone et al. 2009). This study confirmed previous reports of low expression levels for *miR-29b* and *miR-29c* in CLL with 11q deletion (Pekarsky et al. 2006) and the significance of *miR-29c* expression for poor prognostic CLL subgroups (Stamatopoulos et al. 2009). Moreover, it was possible to further subdivide the group of patients with 17p- based on the down-regulation of *miR-223*, *miR-29b*, *miR-29c*, and the *miR-181* family (Visone et al. 2009) (Table 12.2). In a similar study by Rossi et al. the authors were able to show that miRs correlated with 17p status and specifically were down- (*miR-34a*, *miR-181b*, *miR-497*) or up-regulated (*miR-15a*, *miR-21*, *miR-155*) in CLL with 17p deletion. In addition, *miR-21* and *miR-181b* expression were shown to be associated with overall and progression free survival respectively. These data added important information to other recently published studies showing that *miR-34a*, *miR-29c*, *miR-17-5p*, and *miR 151-3p* are differentially expressed based on 17p status (Mraz et al. 2009; Visone et al. 2009; Zenz et al. 2009. To address the mechanisms underlying the deregulation of the miRNAs, the authors have taken advantage of a cell line model where *p53* was silenced to assess if the miRNAs are direct *p53* targets. In accordance with prior studies the authors show evidence that both *miR-34a* and *miR-155* are dependent on *p53* as these were up- (*miR-34a*) and down-regulated (*miR-155*) after silencing of *p53*.

MiR-29c and *miR-181* have been shown to regulate the *TCL1*-oncogene, which is highly expressed in CLL samples with 11q deletions (Pekarsky et al. 2006) or CLL with high ZAP-70 levels and unmutated *IGHV*-genes (Herling et al. 2006). Other studies report regulatory functions of the *miR-29* family by suppression of MCL1, a member of the bcl-2 family with anti-apoptotic function, which is correlated with classic adverse prognostic factors and unfavourable disease course (Pepper et al. 2008). Enforced expression of *miR-29* family members in lung cancer cell lines and AML restores normal patterns of DNA methylation by targeting DNA methyltransferases (DNMTs) and leads to normal DNA methylation patterns with consequent re-expression of silenced tumor suppressor genes (Fabbri et al. 2007; Garzon et al. 2009b).

12.5 Summary

The growing understanding of miRNA deregulation has greatly advanced our understanding of leukemia. While a great deal of mechanistic insight into the precise

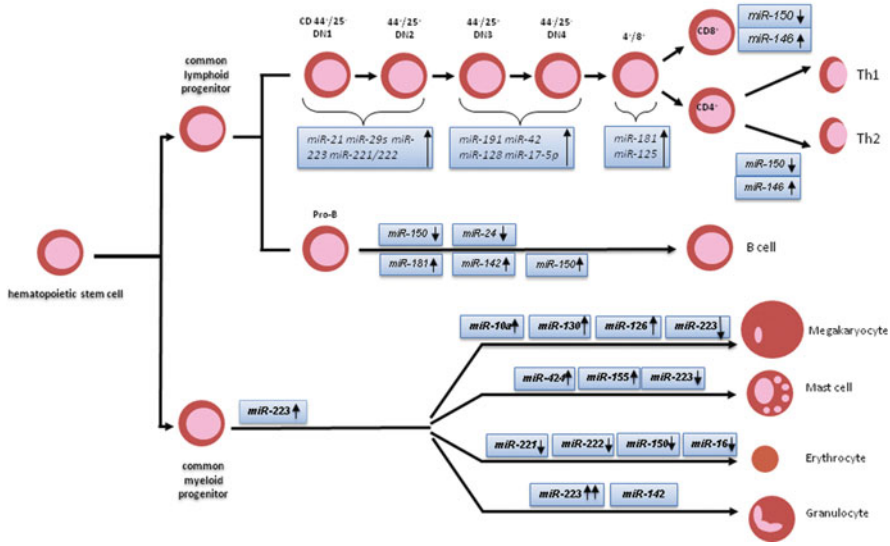

Fig. 12.1 MicroRNAs are involved in the differentiation of hematopoietic progenitors. Multiple miRNAs of this process have also been shown to be deregulated in leukemia (e.g. *miR-155*, *miR29s*, and *miR-223*).

function of deregulated miRNA remains to be discovered, there are a number of miRNAs (e.g. *miR-223*, *miR-34a*, *miR-15a/miR16-1*) with a clearer pathogenetic role in leukemogenesis. The near future is thus likely to yield a translation of these discoveries into targeted treatment approaches in leukemia and other cancers. This translation could relate to the use of the miRNA expression and a prognostic or predictive marker (e.g. *miR-34a* in *TP53* mutant cases) or *miR-15a/miR-16-1* as a therapeutic target in CLL.

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