Chapter 5

Introduction to miRNAs in CLL
Introduction

Over the past few years it has become clear that microRNAs (miRNAs) play an important role in the pathogenesis of chronic lymphocytic leukaemia (CLL) and expression levels of some miRNA are correlated with previously published prognostic markers. However, there are some discordant results that may have been based on different study methods. Furthermore, although it has been elucidated that several miRNA play a crucial role in the disease, little is known about their molecular function.

In this chapter, we focus on miRNA detection methods and the contribution of deregulated miRNA expression in the pathogenesis of CLL.

miRNA biogenesis and function

MiRNAs are a form of small, single-stranded RNA molecules that are 18- to 25-nucleotides (nt) long. They are transcribed from genomic DNA, and instead of being translated into protein, they are processed to the small single-stranded RNA molecules that can regulate gene expression post-transcriptionally by affecting degradation or translation of target mRNAs.

Investigations on the biogenesis and function of miRNA still are in its infancy. In general, there are five steps involved in miRNA biogenesis of animal cells. First, miRNA is generated as a long primary (pri)-miRNA which is transcribed from the genome as a long RNA transcript of a few hundred to a few thousand base pairs. Second, the long pri-miRNA is excised by Drosha, which is a RNase III endonuclease, based on the presence of an imperfect stem-loop fragment to form a 60 to 70 nt precursor (pre)-miRNA.

Third, the pre-miRNA is exported out of the nucleus by Ran-GTP and Exportin-5. Fourth, in the cytoplasm, the pre-miRNA is cleaved to form the 18 to 25 nt long mature miRNA by Dicer. Last, the miRNA is incorporated into a Ribonucleoprotein (RNP) to form the RNA-induced silencing complex (RISC), which executes RNAi-related gene silencing. If there is a perfect complementarity between the miRNA and the targeted mRNA, mRNA degradation occurs similarly to that mediated by siRNA. If there is a non-perfect complementarity between the miRNA and the targeted mRNA, mRNA translation can be inhibited.

Perturbations in miRNA expression have been observed to affect the expression or function of targeted mRNA encoding proteins that have oncogenic or anti-oncogenic functions. In addition, animal studies have directly demonstrated a role of certain miRNA in oncogenesis. CLL is the first human tumor which was found to have a global miRNA profile with a potential clinical prognosis.

CLL biogenesis and immunoarchitecture
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CLL is characterized by the accumulation of mature CD5+CD19+CD23+ B lymphocytes in bone marrow, blood, lymph nodes and spleen. From a prognostic point of view CLL represents a heterogeneous disorder. Indolent versus aggressive forms of the disease can be discriminated by a magnitude of clinical, morphologic, kinetic, immunophenotypic, cytogenetic and molecular features. Recent reports showed that CLL with unmutated IgH genes and ZAP-70 positive CLL are associated with a more aggressive clinical behavior. Later on, two reports showed that CLL cases with unmutated IgH genes or ZAP-70 positive CLL cases have a distinct miRNA expression profile.

It is currently accepted that two interrelated neoplastic cell compartments exist: a proliferative compartment located in bone marrow, spleen and lymph node and an accumulative component represented by the cells that recirculate in the peripheral blood. In lymph nodes and bone marrow, so called proliferation centers (PCs) or pseudofollicles are the hallmarks of this lymphoproliferative disorder. These structures contain an increased number of T-cells and in a few cases also some dendritic cells. The neoplastic cells in these structures are more activated than the surrounding neoplastic cells and circulating cells, which is reflected by higher expression of a number of antigens including immunoglobulin, CD38, Ki67/MIB1, CD23 and MUM1/IRF4.

A recent study showed that the CD40L signaling pathway may play a key role in the proliferation of the tumor cells. Firstly, pseudofollicles contains not only proliferating tumor B lymphocytes but also a striking presence of CD4+ T cells closely in contact with Ki67+ CLL cells. Furthermore, CCL17, CCL22 and survivin are specifically expressed in the proliferating cells of the lymph node and bone marrow, but not in the accumulating cells of the peripheral blood. CD40 ligation of blood CLL cells induced mRNA expression of both CCL17 and CCL22. Furthermore, CD40 stimulation in vitro can induce the expression of survivin. Based on these facts, we can speculate that the accumulating malignant CLL cells can transform into proliferative cells by microenvironmental signals.

miRNA study methods in CLL

miRNA expression was initially determined by Northern blot hybridization. More recently, microarray technologies have been developed to analyze miRNA accumulation patterns. Both approaches examine expression at bulk tissue level and cannot be used to address where and in which cells miRNA are expressed. RNA-ISH can be used to detect miRNA expression patterns at a tissue level.

The miRNA microarray technology makes use of a microarray that contains several hundred different oligos corresponding to the antisense human pre- or mature miRNA sequences. Two miRNA profiling studies on CLL have been published by the same
group. In the first study\textsuperscript{10} miR-15a, miR-21 and miR-92 were among the miRNA that could discriminate between ZAP-70 positive and ZAP-70 negative cases. In the second study\textsuperscript{25}, miR-15a and miR-16 were also differentially expressed between the two main CLL clusters, but miR-21 and miR-92 were not. Part of these discrepancies might be related to the poorer quality of the first generation of miRNA microarrays that also bind some precursor miRNA. This reflects the general difficulty in reliable assessing mature miRNA levels on a microarray. Although microarrays could improve the throughput of miRNA profiling, the method is limited in terms of sensitivity and specificity. Low sensitivity becomes a problem for miRNA quantification because it is difficult to amplify these short RNA molecules. Furthermore, low specificity may lead to false positive signals from closely related miRNAs, precursors and genomic sequences. Recently, a new platform of microarray which can discriminate single-base difference miRNA expressions by using microarray Probe Design Guru was set up\textsuperscript{28}. This suggests that miRNA arrays pose new promises and challenges.

The cloning of small RNA is achieved by ligating adaptors to both ends of the RNA molecules by using T4 RNA ligase, which is followed by reverse transcription and derivation of a double-stranded complementary DNA (cDNA) library. Recently, two miRNA expression profiling studies by cloning have been published for CLL\textsuperscript{29,30}. They reported similar miRNA expression profiles in CLL, such as high levels of miR-142-3p, miR-142-5p, miR-150 and miR-155. In the second report, Marton et al\textsuperscript{30} also cloned five novel miRNA candidates from CLL samples (i.e. miR-1200, let-7i, miR-1201, miR-1202, miR-1203). The main advantage of the cloning method is the fact that knowledge of miRNA sequences is not a requirement. This approach also can be seriously considered when the organism was never studied before and no genomic or miRNA information exists. The major disadvantages of the method are its low speed and low sensitivity. The construction of the libraries is a lengthy procedure and many thousands of sequencing reactions must be performed on each library to obtain enough data. Although with current technology, sequencing does not seem to be a limited step anymore. The low sensitivity stems from the fact that most sequences are derived from the very few most abundantly expressed miRNA, which account for a large majority of the sequences that will be obtained using this cloning strategy.

In 2005, Chen\textsuperscript{31} et al reported a quantification method of microRNA by stem–loop RT–PCR. This method includes two steps: First, a stem–loop RT primer is hybridized to a miRNA molecule and used as a starting point for cDNA syntheses using reverse transcriptase. Next, the RT products are quantified using conventional quantitative PCR. This method has been widely used in CLL studies to detect the mature miRNA expression level\textsuperscript{29,30}. Quantitative PCR has become the gold standard of nucleic acid
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quantification due to the specificity and sensitivity of the technique. TaqMan miRNA assays are specific for mature miRNA and discriminate among related miRNA that differ by as little as one nucleotide. Furthermore, they are not affected by genomic DNA contamination. Thus, this method enables fast, sensitive and specific miRNA expression profiling and is suitable for facilitation of high-throughput detection and quantification of miRNA expression. Furthermore, the concept of stem-loop RT primer design can be applied in small RNA cloning and multiplex assays for better specificity and efficiency.

MiRNA staining has been performed by in situ hybridization of a LNA probe antisense to the miRNA on fixed cells or tissue sections. By using RNA-ISH, van den Berg et al. showed that expression of BIC is specific for Reed-Sternberg cells of HL and is expressed weakly in a minority of germinal center B cells of normal tissue. In situ hybridization is an important tool for analyzing gene expression patterns and developing hypotheses about gene functions.

miRNAs in CLL

The first study demonstrating involvement of miRNAs in the pathology of CLL is on miR-15a and miR-16-1, a miRNA cluster located at chromosomal region 13q14. This region is deleted in more than half of the CLL cases. Later on, by using miRNA microarray, Calin et al. demonstrated that there was a unique miRNA expression profile which can distinguish the poor prognostic group (high ZAP-70 expression and unmutated IgV_H) from the good prognostic group (low ZAP-70 expression and mutated IgV_H). Recently, more and more other miRNA associated with prognostic markers or showing deregulated expression were identified in CLL using different methods (Table 1).

Table 1: Overview of miRNA expression and correlation with prognosis in CLL

<table>
<thead>
<tr>
<th>MiRNA</th>
<th>expression in CLL with poor prognosis</th>
<th>chromosome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-15a</td>
<td>High</td>
<td>13q14.3</td>
<td>25</td>
</tr>
<tr>
<td>miR-16-1/2</td>
<td>High</td>
<td>13q14.3, 3q26</td>
<td>25</td>
</tr>
<tr>
<td>miR-155</td>
<td>High</td>
<td>21q21</td>
<td>25,30</td>
</tr>
<tr>
<td>miR-92</td>
<td>High</td>
<td>13q31</td>
<td>10</td>
</tr>
<tr>
<td>miR-150</td>
<td>Low</td>
<td>19q13.3</td>
<td>29</td>
</tr>
<tr>
<td>miR-223</td>
<td>Low</td>
<td>Xq12-13.3</td>
<td>25,29</td>
</tr>
<tr>
<td>miR-29bc</td>
<td>Low</td>
<td>1q32.2-32.3</td>
<td>25,29</td>
</tr>
</tbody>
</table>

Bad prognosis: VH unmutated and / or ZAP-70+
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MiR-15-16 cluster

Involvement of miR-15a and miR-16-1 was first published by Calin et al. The authors showed frequent deletions and downregulation of these two miRNA in CLL\textsuperscript{22}. Deletion of chromosome band 13q14 is the most frequent aberration found in CLL and was shown to be associated with reduced levels of miR-15a and miR-16 by Northern blot\textsuperscript{22}. Besides this cluster on chromosome 14, there is also a second homologous cluster on chromosome 3. MiR-16-1 and miR-16-2 are identical and cannot be detected separately, whereas miR-15a and miR-15b differ in 4 nucleotides and can be detected separately. Later on, Ouillette et al\textsuperscript{39} reported that marked reductions in miR15a/miR16 expression were only found in 15\% of CLL cases and were not significantly associated with bi-allelic 13q14 loss by qRT-PCR. This discrepancy may be accounted for by the different methods to quantify mature miRNA expression levels or by differences in expression levels of the chromosome 3 cluster.

In 2005, BCL-2 was identified as a miR-15a and miR-16 target in CLL\textsuperscript{40}, but later on, Ouillette\textsuperscript{39} et al did not find any correlation of miR-15a and miR-16 expression with BCL-2 expression in CLL samples. By using a mouse model, two groups\textsuperscript{41,42} showed that exogenous miR-16 delivered to an NZB malignant B-1 cell line resulted in cell-cycle alterations and increased apoptosis, suggesting that reduced expression of miR-16 might be an important molecular lesion in CLL.

MiR-17-92 cluster

The miR-17-92 cluster is located in an intron of the C13ORF25 gene, which is located at 13q31.3, a locus involved in many aggressive B-cell lymphomas\textsuperscript{43,44}. The C13ORF25 region contains the polycistronic miRNA cluster, miR-17/miR-18a/miR-19a/miR-20a/miR-19b-1/miR-92-1. Based on reports that the miR-17-92 cluster acts as an oncogene\textsuperscript{9}, one could expect its upregulation in CLL compared to healthy controls. However, Fulci et al\textsuperscript{29} found that miR-92 was 2-fold lower in CLL cells compared to the normal CD19\+ B cells. Fulci et al did not study the expression level of other miRNA of the miR-17-92 cluster. The function of this miRNA cluster in CLL should be further studied.

BIC/miR-155

BIC was originally identified several years ago as a noncoding RNA from a common retroviral integration site in avian leukosis virus-induced lymphomas and was the first miRNA-encoding RNA shown to participate in oncogenesis\textsuperscript{45-47}. Lagos-Quintana et al\textsuperscript{48} identified miR-155, which originates from the phylogenetically conserved region of BIC. Later on, several studies\textsuperscript{26,49} showed that BIC and miR-155 are overexpressed in human lymphomas including Hodgkin lymphoma, primary mediastinal and some
samples of diffuse large B cell lymphoma.

To investigate miR-155 in B-cell malignancies, transgenic mice were generated that overexpressed this miRNA under the control of a \( V_\text{H} \) promoter-Ig heavy chain \( E_\mu \) enhancer\(^8\). Transgenic mice with miR-155 expression targeted to B cells initially exhibited preleukemic pre-B-cell proliferation, which was evident in spleen and bone marrow, and later developed B-cell malignancies. On the other hand, by generating miR-155/\( BIC \)-/- mice, two groups\(^{50,51}\) reported that \( BIC \)/miR-155 also has an important role in normal immunological functions, especially by regulating the germinal center response. B and T lymphocytes from \( BIC \)-deficient mice had diminished responses to T cell-dependent antigens. Microarray analysis of \( BIC \)-deficient Th1 and Th2 cells revealed a wide variety of candidate miR-155 targets, including c-Maf, a potent transactivator of the IL4 promoter and thus Th2 responses\(^50\). Flowcytometric analysis of miR-155 -/- mice and mice with miR-155 -/- B cells, as well as RT-PCR and Northern blot analysis of wildtype mice, demonstrated that activated germinal center (GC) B cells expressed miR-155 and that GC formation required miR-155\(^{51}\).

**MiR-150**

miR-150 was originally cloned from mouse\(^{48}\). Later on, by using miRNA arrays and quantitative RT-PCR analysis, Zhou et al\(^{52}\) detected abundant expression of miR-150 in lymph nodes and spleen, with lower levels in thymus, heart, and brain. Fulci et al reported that miR-150 was also 2-fold overexpressed in CLL, which is the first report linking this miRNA directly to cancer. Recent reports in HeLa cells have demonstrated that inhibition of miR-150 expression causes a repression of cell growth, which suggested that miR-150 may act as an oncogene\(^{53}\).

By using a mouse model, Zhou et al\(^{52}\) found that expression of mouse miR-150 increased during B- and T-cell development. Overexpression of miR-150 in hematopoietic stem cells led to impaired formation of mature B cells, but not other lymphoid and myeloid cell populations. Premature expression of miR-150 blocked transition from pro-B to pre-B lymphocytes. In a transgenic miR-150 mouse model, miR-150 controlled B-cell differentiation by targeting the c-Myb transcription factor\(^{54}\).
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