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MicroRNAs in CLL: miRacle or miRage for prognosis and targeted therapies?

Katrien Van Roosbroeck[1] and **George A. Calin**[1,2,3]

[1]Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[2]Center for RNA Interference and Non-Coding RNAs, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[3]Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Abstract

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease and has a highly variable clinical course with survival ranging from a couple of months to several decades. MicroRNAs (miRNAs), small non-coding RNAs that regulate transcription and translation of genes, have been found to be involved in CLL initiation, progression and resistance to therapy. In addition, they can be used as prognostic biomarkers and as targets for novel therapies.

In this review, we describe the association between miRNAs and the cytogenetic aberrations commonly found in CLL, as well as with other prognostic factors. We describe the presence of miRNAs as extracellular entities in the plasma and serum of CLL patients and discuss their role in resistance to therapy. Finally, we will explore the potential of targeted miRNA therapy for the treatment of CLL, with a special emphasis on MRX34, the first miRNA mimic that is currently being evaluated for clinical use.

MicroRNAs (miRNAs) are small, non-coding RNAs of 19–25 base pairs long. Their main function is the regulation of gene expression by either mRNA degradation or inhibition of translation, but other functions, such as mRNA stabilization, translational activation and RNA decoy, have been described as well[1]. By now, it is well established that miRNAs are important in almost all cellular processes, including differentiation, proliferation, cell cycle regulation and apoptosis, processes that are deregulated in human cancers[2]. It was in chronic lymphocytic leukemia (CLL), the most common adult leukemia in the Western world, that the first miRNAs involved in human diseases were described. We showed that a cluster containing miR-15a and miR-16-1 was frequently deleted or downregulated in CLL, and that this downregulation correlated with allelic loss at 13q14, a region deleted in the majority of CLL cases[3]. After this initial report, many more studies correlated abnormal miRNA expression with cancer, resulting in more than 16,000 publications so far.

Correspondence: George A. Calin, M.D., Ph.D., Departments of Experimental Therapeutics and Leukemia, Unit 1950, The University of Texas MD Anderson Cancer Center, 1881 East Road, Houston, TX 77054; gcalin@mdanderson.org; Telephone: (713)-792-5461; Fax: (713)-745-4526.

In this review, we will describe the association between certain miRNAs and the cytogenetic aberrations commonly found in CLL, as well as with other prognostic factors. Furthermore, we will discuss their importance as circulating miRNAs in CLL and their role in resistance to chemotherapeutic agents that are used to treat CLL. Finally, we will describe the potential of targeted miRNA therapy for the treatment of CLL.

miRNAs associated with common cytogenetic aberrations in CLL

The prognosis and outcome of patients with CLL is highly variable and has been found to be largely dependent on cytogenetic abnormalities that occur in the tumors. The majority of patients with CLL (~80%) can be categorized in five distinct cytogenetic prognostic subgroups: deletion of the long arm of chromosome 13 (del13q), deletion of the long arm of chromosome 11 (del11q), deletion of the short arm of chromosome 17 (del17), trisomy of chromosome 12 (tri12) and normal cytogenetics and normal fluorescent *in situ* hybridization analyses (NL cyto/FISH). For some abnormalities, the target protein-coding or non-coding gene has been identified (Figure 1), while others are still under intensive investigation to unravel the biological significance of the aberrations.

Del13q is the most common cytogenetic abnormality (~55% of patients) and a good prognostic factor (median survival of 133 months)[4]. Detailed analyses of the deleted 13q14 region failed to demonstrate the presence of any protein-coding tumor suppressor gene in this region[5–8]. However, further investigation revealed the presence of a cluster containing two small non-coding miRNAs, miR-15a and miR-16-1[3]. Analysis of the expression of these miRNAs showed that both miR-15a and miR-16-1 were deleted or downregulated in the majority of CLL patients[3]. The miR-15a/16-1 cluster functions as a tumor suppressor by targeting various oncogenes, of which B-cell CLL/lymphoma 2 (*BCL2*) and myeloid cell leukemia 1 (*MCL1*) are the most important in CLL[9,10]. The role of miR-15a/16-1 in CLL has been reviewed elsewhere[11,12]. Recently, downregulation of miR-16 and miR-363 and upregulation of miR-146b-5p and miR-148a were found to be associated with del13q CLL[13].

Del11q occurs in ~18% of CLL patients and is associated with poor response to treatment and shorter progression free survival (median survival of 79 months)[4,14]. 11q is the genomic locus of ataxia-telangiectasia mutated (*ATM*), which is the major target of this deletion as mutations in *ATM* correlate with poor prognosis in CLL[15]. Often located in the 11q commonly deleted region is the miR-34b and miR-34c cluster. These miRNAs are targets of the tumor suppressor p53 (TP53)[16], which is often deregulated or mutated in CLL. miR-34b/c expression is significantly lower in CLL patients with del11q[17] and besides being deleted in CLL, miR-34b/c is also often epigenetically inactivated through hypermethylation[18,19]. Furthermore, an inverse correlation has been found between the presence of del11q and miR-34b/c hypermethylation, suggesting that there are different modes of silencing miR-34b/c that are independent of each other[18]. One of the main targets of the miR-34b/c cluster is 70 kD zeta-associated protein (*ZAP70*), a well-known prognostic factor in CLL[20]. Microarray miRNA profiling revealed a significant upregulation of two other miRNAs, miR-769-5p and miR-338-3p in CLL patients with del11q when compared to CLLs from other cytogenetic subgroups[13].

Del17p is the poorest prognostic factor, and is characterized by a median survival of 32 months and poor response to therapy[4]. This region harbors *TP53*, the most commonly deregulated (either by deletion or mutation) tumor suppressor in human cancers[21]. Mraz and colleagues compared the expression of 35 miRNAs in CLL samples with *TP53* abnormalities with wild type *TP53* CLL samples, and found that three miRNAs, miR-34a, miR-29c and miR-17-5p were significantly downregulated in CLL samples carrying *TP53* abnormalities[22]. It has been shown that the miR-34 family is regulated by TP53 through direct interaction[20,23]. Furthermore, TP53 is involved in a regulatory feedback loops with miR-15a/16-1[20]. Also, our group identified miR-15a, miR-21, miR-34a, miR-155 and miR-181b as being differentially expressed between CLL patients with del17p versus CLLs with normal 17p and normal karyotype[24]. When comparing CLL cases with del17p with CLL cases without this aberration, Negrini and colleagues found a significant downregulation of miR-34a and miR-150 and upregulation of miR-33b*, miR-96 and miR-21* in del17p cases[13]. Finally, miR-3676, located at 17p13 and co-deleted with *TP53* in CLL, has been found to directly target the T-cell leukemia/lymphoma 1 (*TCL1*) oncogene[25], which is involved in CLL cell survival, as well as the pathogenesis of leukemias[26]. miR-3676 is significantly downregulated in four cytogenetic subgroups (del11q, del13q, del17p and normal karyotype and normal FISH analysis (NL cyto/FISH)) compared with normal CD19+ B-cells, and is mutated in 1% of CLL patients[25].

Approximately 18% of patients do not have any apparent cytogenetic aberrations, as being evidenced by a NL cyto/FISH. In ~ 16% of CLL patients a trisomy of chromosome 12 (tri12) is found. Both are intermediate prognostic factors with median survival of 111 months and 114 months, respectively[4], but the genetic defects associated with both subgroups remain poorly understood. In the instance of tri12, there might be a gene dosage effect of a candidate oncogene, due to the presence of the extra chromosome 12. However, such a gene has not been identified so far[14]. A small gene expression study comparing four CLL samples with tri12 with 16 CLL controls identified four genes whose expression is significantly associated with tri12: Huntingtin interacting protein 1 related (*HIP1R*), myogenic factor 6 (*MYF6*), purinergic receptor P2Y, G-protein coupled, 14 (*P2RY14*) and cluster of differentiation 200 (*CD200*), but only *HIP1R* and *MYF6* are located on chromosome 12[27]. With regard to miRNA expression, CLL patients with tri12 showed significantly reduced expression of miR-155, miR-148a and miR-483-5p when compare to CLLs from other cytogenetic subgroups[13]. In CLL patients with NL cyto/FISH, miR-425*, miR-1237 and miR-484 were found to be downregulated[13].

Genome-wide miRNA expression analysis of these five main cytogenetic subgroups identified 32 miRNAs that were able to discriminate del13q, NL cyto/FISH, tri12, del11q and del17p subtypes of CLL[28]. In addition, it was shown that disease progression in del17p cases was strongly associated with low expression of miR-223, miR-29b/c and miR-181 family, unmutated immunoglobulin heavy chain variable region (IGHV) and low expression of ZAP70, two prognostic factors in CLL[28] (see next section).

miRNAs associated with other prognostic factors in CLL

Besides the presence of cytogenetic aberrations, there are several other markers that can be used to predict the prognosis of CLL patients, including the mutational status of IGHV, expression levels of ZAP70, CD38 expression levels and expression of beta-2-microglobulin (B2M). Typically, the presence of unmutated IGHV genes, high expression of ZAP70 (>20%), high levels of CD38 (≥30%) and elevated B2M expression levels (>2x upper limit of normal, ULN) are indicators for poor prognosis[29]. Several groups have established miRNAs and miRNA signatures associated with these prognostic factors in CLL.

In 2005, our group performed genome-wide miRNA expression analysis and identified a 13 miRNA signature consisting of miR-15a, miR-195, miR-221, miR-23b, miR-155, miR-223, miR-29a-2, miR-24-1, miR-29b-2, miR-146, miR-16-1, miR-16-2 and miR-29c that was able to differentiate CLL cases with low ZAP70 expression from those with high ZAP70 expression, and CLL cases with unmutated IGHV from those with mutated IGHV[30]. Several of the miRNAs from this original signature could be confirmed, including increased expression of miR-15a[31,32] and miR-16[31], and decreased expression of miR-29a[31] and miR-29b[33,34] as being associated with unmutated IGHV, and decreased levels of miR-29c[32,33,35,36] and miR-223[32,33,35–37] as being associated with unmutated IGHV and with increased expression of ZAP70, CD38 and B2M. One miRNA that was not included in the original signature, but that was repeatedly reported to correlate with poor prognostic markers (unmutated IGHV and increased expression of ZAP70, CD38 and B2M) is miR-150. MiR-150 is generally found to be downregulated in poor prognosis patients[32,33,38], but Li and colleagues found that increased expression of miR-150 was associated with high expression of ZAP70[35]. In addition, a recent study found opposite prognostic significance of cellular and serum circulating miR-150 in CLL, where decreased levels of miR-150 in CLL B-cells, but increased levels of miR-150 plasma levels were associated with tumor burden, disease aggressiveness and poor prognostic factors, such as unmutated IGHV and increased expression of ZAP70, CD38 and B2M[39].

A few miRNA-based scoring systems have been proposed to aid prognosis and stratification of CLL survival. Stamatopoulos and colleagues[40] developed a quantitative PCR (qPCR) score based on the individual prognostic markers ZAP70, lipoprotein lipase (LPL) and miR-29c to predict overall survival. The score ranges from 0 (most favorable prognosis) to 3 (most unfavorable prognosis) and the presence of a poor prognostic factor (high expression of ZAP70 or LPL, and low miR-29c expression) increases the score with 1 point. This qPCR score was able to significantly predict treatment-free survival and overall survival by dividing patients into 3 groups (score 0/3, 1–2/3 and 3/3). Rossi and colleagues[24] developed a 21FK score based on expression of miR-21, FISH and karyotype that stratifies patients according to overall survival. The score ranges from 0 (low risk) to 2 (high risk) and also here, a poor prognostic factor (high miR-21, del17p on FISH or karyotype) increased the score with 1 point. When comparing the power of 21FK score with this of the classic prognostic factors, such as B2M, ZAP70, IGHV, and CD38, the score was found to be the best performer.

Circulating miRNAs in CLL

Most studies focused on miRNA expression analysis in CLL B-cells, but circulating miRNAs can also be detected in plasma and serum of CLL samples. In fact, the number of circulating miRNAs found in CLL plasma samples was almost one third higher compared with normal, control plasma[41]. A series of 14 plasma miRNAs (of which miR-150, miR-150*, miR-29a, miR-135a* and miR-195 were the most differentially expressed) were able to discriminate CLL samples from normal plasma, multiple myeloma and hairy cell leukemia[41]. When the expression of these differentially expressed miRNAs was analyzed in the corresponding CLL B-cells, the obtained profiles were distinct and clearly different, suggesting that circulating miRNAs may be released by other cell types besides CLL B-cells. In addition, a higher number of miRNAs could be detected in plasma of ZAP70+ CLL samples than in ZAP70− CLL samples, and different miRNAs were expressed. For example, miR-19b and miR-144* levels were higher in ZAP70+ samples, while expression of miR-205, miR-29a and miR-652 was higher in ZAP70− CLL plasma samples[41].

miRNA plasma levels can also predict response to CLL therapy as evidenced by miR-155 expression levels in CLL samples collected before treatment was initiated. In these samples, miR-155 was found to be significantly higher expressed in patients who fail to achieve a complete remission, when compared to those experiencing a complete response[42].

miRNAs and CLL therapy resistance

Although CLL is generally an indolent disease, a significant number of patients show an aggressive clinical course with resistance to therapy or relapse after initial treatment. That resistance to therapy is a significant medical issue in CLL is underlined by the fact that the 5-year progression-free survival of patients receiving the standard of care chemotherapy-based fludarabine, cyclophosphamide and rituximab (FCR) treatment is less than 50%[43]. When looking at chemotherapy-refractory CLL cases, 30–40% seem to be caused by deletions and/or mutations of the tumor suppressor *TP53*[44], while approximately one-third have a del11q/*ATM*. In the remaining cases, the cause for therapy refraction still remains unclear. As miRNAs, including miR-34, miR-155 and miR-181, have been demonstrated to be involved in chemoresistance and therapy refraction in many types of cancer, including CLL, resistance in these cases might be explained by abnormal expression of miRNAs.

MiR-34a is a tumor suppressor miRNA, which is downregulated in CLL cases with del17p and/or mutated TP53[22,45], the poorest prognosis subgroup characterized by poor response to therapy. In addition, it is expressed at significantly lower levels in fludarabine-refractory CLL than in CLL cases without refractory disease and this was irrespective of the 17p/TP53 status[44]. In TP53 wild-type patients, miR34a is expressed at variable levels, which prompted Asslaber and colleagues to study the correlation of a single nucleotide polymorphism (SNP309) in the intronic promoter of *MDM2*, a gene upstream of *TP53*[45]. They found that patients with the GG genotype showed significantly lower expression of miR-34a when compared to patients with the TT genotype, and that these low levels of miR-34a were associated with shorter time to treatment. Upregulation or reintroduction of miR-34a induces the pro-apoptotic Bax and cell cycle regulator p21[44], as well as apoptosis[45]. Therefore,

miR-34a is a promising candidate for targeted anti-cancer therapy, which will be discussed in more detail in the next section.

The miR-181 family, and more precisely miR-181a and miR-181b, have been found to be involved in therapy resistance in CLL as well. We showed that miR-181a is downregulated in therapy-refractory CLL and that low expression of miR-181b in therapy-refractory cases predicts treatment-free survival[24]. Moreover, CLL patients with progressive disease show decreasing levels of miR-181b expression, whereas in patients with stable disease miR-181b levels remained constant[28,46]. In contrast, miR-181a and miR-221 were found to be upregulated and miR-29a was found to be downregulated in pretreatment samples of fludarabine-resistant CLL patients as compared to sensitive patients[47]. Finally, reintroduction of miR-181a and miR-181b enhances drug sensitivity in primary CLL cell cells through direct targeting of the anti-apoptotic genes *BCL2*, *MCL1* and X-linked inhibitor of apoptosis, E3 ubiquitin protein ligase (*XIAP*)[48].

miR-155 is a well-known oncogenic miRNA that is overexpressed and associated with poor prognosis in many types of cancer[49]. In addition, miR-155 has been demonstrated to be involved in chemoresistance[50,51]. Recently, it was suggested that Toll-like receptor 9 (TLR9) stimulation leads to protection of CLL cells from fludarabine-induced apoptosis in patients bearing adverse prognostic factors, and that this is marked by upregulation of miR-155-3p, the strand that is generally degraded[52].

To establish a miRNA signature associated with fludarabine resistance, Ferracin et al compared the expression profiles of 723 human miRNAs before and 5 days after fludarabine treatment in a set of patients, which either responded or were refractory to fludarabine treatment[53]. They found that high expression of miR-148a, miR-221 and miR-21 was able to differentiate refractory from sensitive CLL samples. Knock-down of miR-221 and miR-21 *in vitro* increased caspase activity, suggesting that those miRNAs may be involved in the development of fludarabine resistance.

miRNA-based targeted therapy for the treatment of CLL

miRNAs are very attractive targets for novel therapeutics due to their prevalence in physiological processes and widespread involvement in human diseases, including cancer. Given that miRNAs can either function as a tumor suppressor gene or an oncogene[54], targeted strategies are based on either re-expression of downregulated tumor suppressor miRNAs or silencing of oncogenic miRNAs (Figure 2; reviewed by[1,2,55]). There are several advantages associated with the use of miRNAs as therapeutic tools: they exist as short sequences, can be easily chemically modified and are able to target multiple genes that are involved in different signal transduction pathways[56]. The latter can also be a challenge, as modulating aberrantly expressed miRNAs may result in the deregulation of pathways that were not affected in the first place, and this can be accompanied by off-target effects. This is especially true for miRNA replacement therapy, as reintroduction of a miRNA may lead to supra-physiological conditions in which the normal targets are saturated or overloaded, redirecting the excess of miRNAs to secondary targets[57]. The main major challenge, however, remains successful *in vivo* delivery. The ideal delivery system should be efficient,

stable, safe and tumor-specific. Introduction of naked RNA molecules in the human body leads to rapid degradation, which makes encapsulation in some kind of carrier necessary. In this regard, nanocarriers seem to be promising delivery vehicles for oligonucleotide-based therapeutics (reviewed by[58]).

One very promising miRNA to be used as a therapeutic target in CLL is miR-34a. It is a key tumor suppressor downregulated in a plethora of human cancers, including neuroblastoma, glioblastoma and cancers of the ovary, colon, liver, lung, breast, prostate, pancreas, kidney, bladder, skin, esophagus, cervix and urothelium (reviewed by[59,60]). As already mentioned in the previous section, in CLL, miR-34a is downregulated in cases with del17p and/or mutated TP53[22,45], which makes sense since miR-34a is a major downstream target of TP53[23]. In addition, low expression of miR-34a is associated with worse prognosis and fludarabine-refractory disease[44]. Re-expression of miR-34a in primary CLL patient cells resulted in a significant increase in apoptosis when wild-type TP53 was expressed, but not when TP53 was attenuated[61]. The widespread involvement of miR-34a in human cancer makes it an ideal candidate for miRNA replacement therapy. In fact, MRX34, containing a miR-34a mimic, is the first miRNA mimic to enter clinical trials and is currently being evaluated in a multicenter Phase I study in patients with liver cancer or those with liver metastases from other cancers, as well as in patients with hematological malignancies, including CLL (ClinicalTrials.gov identifier NCT01829971).

MRX34 (Mirna Therapeutics) is a double-stranded miR-34 mimic, which is encapsulated in SMARTICLES (ionizable liposomes that form particles of ~120 nm in diameter; Marina Biotech, Bothell, WA) for safe and efficient delivery[60]. Preclinical *in vivo* experiments in an orthotopic mouse model of hepatocellular carcinoma showed significant tumor regression and prolonged survival without notable drug-related side effects upon treatment with MRX34[62,63]. A more than 100-fold increase in miR-34 expression was detected in liver tumor cells and resulted in reduced expression of miR-34 oncogenic targets[64]. Currently, MRX34 is being tested in a Phase I clinical trial with the primary objectives to establish the maximum tolerated dose and the recommended Phase II dose in a variety of cancers. For CLL patients, MRX34 is administered intravenously and a treatment schedule of five days in a row with two weeks off in 21-day cycles is being evaluated (ClinicalTrials.gov Identifier NCT01829971). The secondary objectives are to investigate safety, tolerability and pharmacokinetic profile, as well as to assess any biological activity and clinical outcomes. Interim data on safety and preliminary efficacy for 52 patients were recently released and showed that MRX34 has a manageable safety profile. Main treatment emergent adverse events consisted of infusion reactions (such as fever, chills, nausea, vomiting, back and flank pain) and fatigue, diarrhea, headache, dehydration, elevation of liver enzymes, decreased albumin, hyponatremia, lymphopenia, thrombocytopenia, and neutropenia[64]. The Phase I study is expected to be completed by the end of 2015.

Discussion and future directions

CLL is a heterogeneous disease and has a highly variable clinical course with survival ranging from a couple of months to several decades. A number of biomarkers with prognostic value have been identified, such as cytogenetic abnormalities (del13q, tri12,

del11q, NL cyto/FISH and del17p), IGHV mutation status, ZAP70 expression, B2M expression and CD38 expression, but the underlying molecular mechanisms are not always that well-defined. Over recent years, miRNAs have been identified that can contribute to CLL initiation, progression and resistance to therapy, or can be used as biomarkers. High-throughput techniques, such as microarrays and RNA-sequencing, make it possible to generate a massive amount of data and identify a large number of differentially expressed miRNAs. Based on the platforms and techniques being used, the outcome can greatly vary, making it hard to reproduce the results. However, several miRNAs, such as miR-34a, miR-155, miR-29c and miR-223 keep popping up, regardless of the applied techniques and platforms. These may be further evaluated as "true" biomarkers or disease contributors, and led to the first clinical trials using a miRNA mimic for miR-34a in CLL. So far, MRX34 is the only miRNA-based therapeutics ready for clinical evaluation in cancer; however, a miR-122 inhibitor used to treat patients with chronic hepatitis C virus (HCV) infection has been found to be safe and efficient in a Phase 2a clinical trial[65]. Although the preliminary results of MRX34 are promising and the non-coding RNA research community is getting excited about the use of a first miRNA mimic in the clinic, there is still a long way to go before this new drug can be successfully used for the treatment of patients with cancer.

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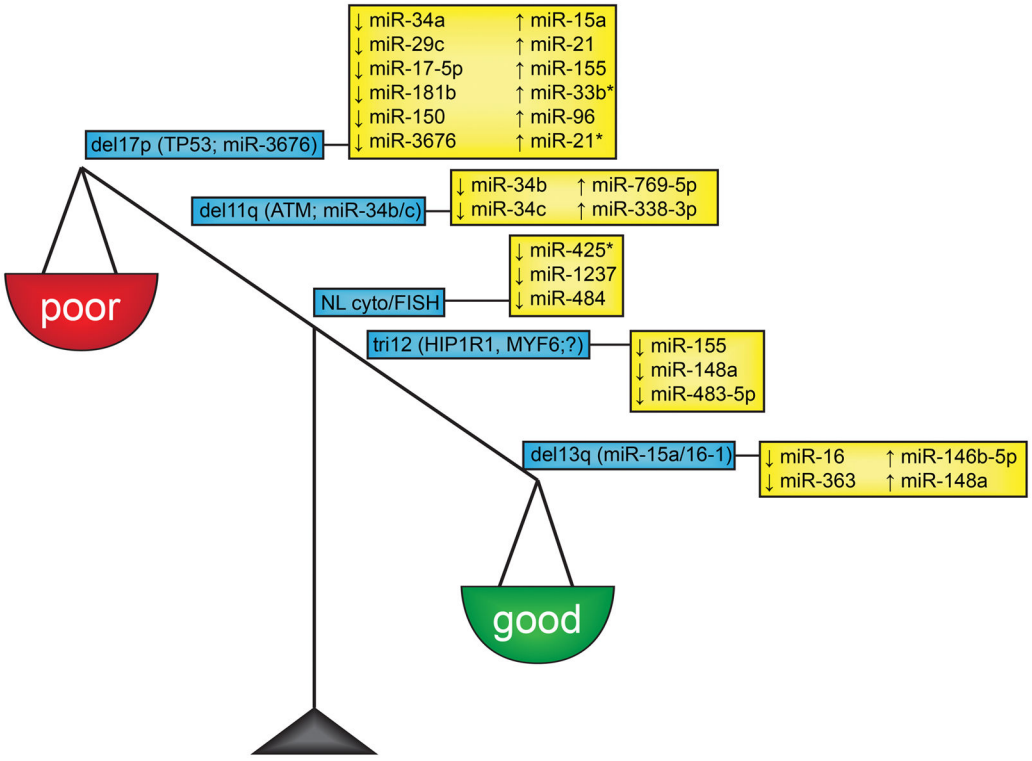

Figure 1. MicroRNAs associated with common cytogenetic aberrations in CLL

CLL patients an be categorized in different cytogenetic subgroups based on prognosis: del13 is a good prognostic factor, tri12 and NL cyto/FISH are intermediate prognostic factors and del11q and del17p are associated with poor prognosis. The protein-coding and non-coding genes between brackets in the blue boxes represent targets of the cytogenetic aberration; the miRNAs highlighted in the yellow boxes represent miRNAs that have been associated with the specific cytogenetic aberration.

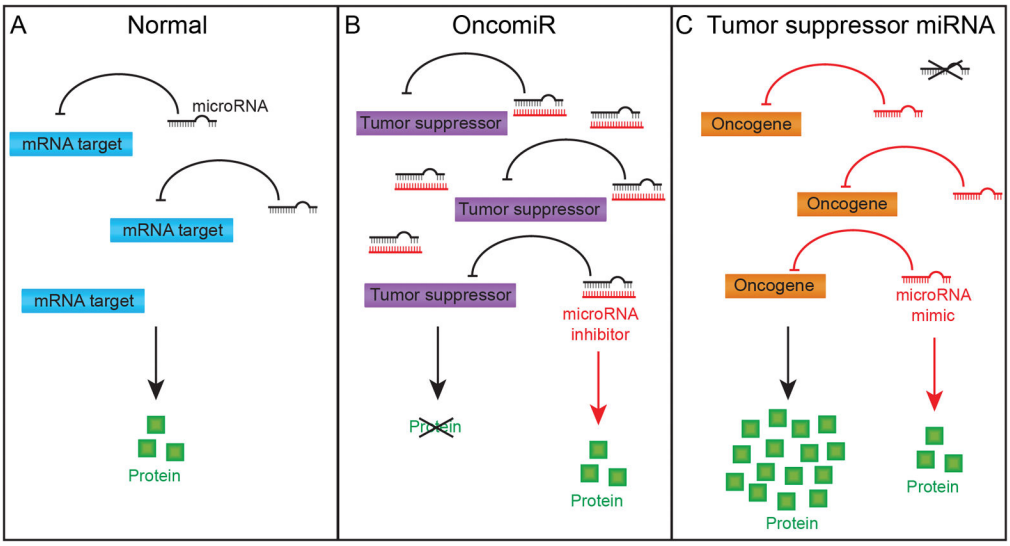

Figure 2. Therapeutic strategies to target oncogenic microRNAs (or oncomiRs) and tumor suppressor microRNAs

(A) Under normal conditions, miRNAs repress their mRNA targets resulting in reduced expression of proteins. (B) When an oncomiR is overexpressed, its tumor suppressor target is blocked, resulting in inhibition of protein expression. An oncomiR can be targeted by the introduction of miRNA inhibitors, which prohibit miRNA-based targeting and results in re-expression of the tumor suppressor target (marked in red). (C) When a tumor suppressor miRNA is downregulated, its oncogenic target loses its repression, resulting in overexpression of the oncoprotein. Re-expression of a miRNA mimic will block the translation of the oncogene, resulting in normal levels of protein expression (marked in red). Verliezen