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Review

miR-15a and *miR-16-1* in cancer: discovery, function and future perspectives

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MicroRNAs (miRNAs) encoded by the *miR-15/16* cluster are known to act as tumor suppressors. Expression of these miRNAs inhibits cell proliferation, promotes apoptosis of cancer cells, and suppresses tumorigenicity both *in vitro* and *in vivo*. *miR-15a* and *miR-16-1* function by targeting multiple oncogenes, including *BCL2, MCL1, CCND1,* and *WNT3A*. Down-regulation of these miRNAs has been reported in chronic lymphocytic lymphoma (CLL), pituitary adenomas, and prostate carcinoma. This review summarizes the discovery, functions, and clinical relevance of these miRNAs in cancer, particularly CLL.
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MicroRNAs (miRNAs) are a relatively recently identified class of regulatory noncoding RNAs, typically 20–23 nt in length, which function primarily by targeting specific messenger RNAs (mRNAs) for degradation or inhibition of translation and hence decreasing the expression of the resulting protein.[1,2] Their regulatory functions involve a variety of biological processes, including development, differentiation, apoptosis, survival, senescence, and metabolism.[3,4] Several hundred miRNAs have been identified and studied so far, but it is predicted that there are many more miRNAs whose significance and role in homeostasis and pathology is yet to be identified. Intriguingly, a significant fraction of miRNA genes map to regions implicated in cancer susceptibility and at fragile sites, suggesting a role of miRNAs in carcinogenesis.[5–7] In 2002, we identified *miR-15* and *miR-16* as potential cancer genes in the pathogenesis of chronic lymphocytic leukemia (CLL).[8] Specifically, *miR-15a* and *miR-16-1*, located at 13q14.3, were frequently deleted and/or down-regulated in CLL.[8] This finding provided the first evidence that miRNA genes might be important for tumorigenesis.

To facilitate the efficient and comprehensive profiling of miRNA genes, a microarray chip defining the global levels for each known or predicted member of the miRNome was developed.[9] Other technologies, including macroarrays,[10] bead-based flow cytometric miRNA expression,[11] and quantitative reverse transcription PCR[12,13] also became available. Collectively, these approaches have shown the differential expression of miRNA in human tissues and identified significant miRNA genes that are of biological and clinical relevance in human diseases.[14] In particular, several research groups have sought to identify unique signatures or fingerprints of miRNAs in human malignancies in the hope that such knowledge would be helpful in exploring new venues and pathways that regulate the process of cancer evolution and would thus enable the design of novel diagnostic, prognostic, and therapeutic tools. Indeed, several miRNAs have been shown to have a promoting role in the development of primary tumors (reviewed in Ref.[15]); miRNAs that are amplified or overexpressed in cancer could act as oncogenes, whereas several miRNAs have been implicated as tumor suppressors based on their physical deletion or reduced expression in human cancer. Additionally, miRNAs have been implicated as modulators of tumor progression and metastasis (reviewed in Ref.[16]). As a result, we realize that deregulation of, at least some, miRNAs might also contribute to tumorigenesis.

In this review, we will discuss the classification of the *miR-15a* and *miR-16-1* cluster as cancer genes, the mechanisms by which *miR-15a* and *miR-16-1* genes exert their tumor suppressor function, and the clinical relevance of their expression in cancer.

Identification of *miR-15a* and *miR-16-1* as Potential Cancer Genes

Many miRNAs have been suggested as playing a key role in cancer owing to their location and expression profiles.[5–7] The first experimental evidence that miRNAs are involved in mammalian carcinogenesis was reported in CLL, the most common form of adult leukemia in the Western world.[8] Genetic alterations, including hemizygous and homozygous loss, at 13q14.3 occur in more than half of CLL cases and constitute the most frequent chromosomal abnormality in CLL.[17] Deletions at 13q14.3 have also been reported in other

malignancies, such as multiple myeloma, mantle cell lymphoma, and prostate carcinoma.[18] Together, these observations and others suggested that the 13q14.3 genomic region harbors one or more tumor suppressor genes that are involved in the pathogenesis of some malignancies. Nonetheless, the identification of cancer genes involved in the 13q14.3 deletions in CLL failed, despite the extensive effort of several research groups. A region of more than 1.0 Mb has been fully sequenced and characterized in detail and identified several genes (Figure 1), but none of them were found to be involved in the initiation or progression of CLL or other human tumors.[19] These findings led to the hypothesis that 13q14.3 is dispensable for CLL development and that deletions at this region do not confer any selective advantage to the malignant cells. Another possibility would be that haploinsufficiency itself predisposes to CLL.[20] Alternatively, unknown genes, such as those spanning large genomic regions with huge introns or, possibly, extremely small genes perhaps not detected by classic cloning methods, might reside in this region and be the real target of genomic alteration.

To pursue this latter hypothesis and to identify putative tumor suppressor genes at 13q14.3, we completely sequenced and characterized a segment of 790 kb spanning the minimal region of loss,[21] and we also generated somatic cell hybrids between mouse LM-TK⁻ and CLL cells carrying

13q14.3 translocations and/or deletions.[8] Subsequent analysis of an ~30-kb deletion in one case of CLL associated with retinoblastoma (*RB*) and the chromosomal breakpoint translocation t(2:13)(q32;q14) in a different patient led to the identification of the critical region that contained two tightly linked miRNAs, *miR-15a* and *miR-16-1* (Figure 1), involved in tumor suppression in CLL. Northern blotting and microarray analyses have shown that the *miR-15a* and *miR-16-1* genes are ubiquitously and highly expressed in normal CD5⁺ lymphocytes,[8] the cells from which CLL originates. These data suggested that these genes might play an important role in normal CD5⁺ B-cell homeostasis and that they could be the genomic targets of 13q14.3 in CLL (see below). It is worth noting that a second cluster including *miR-15b/miR-16-2* is located on chromosome 3, though these genes are expressed at a much lower level in B cells and in CD5/CD19 positive cells as the chromosome 3q26.1 cluster.[8] Further refined quantitative RT-PCR studies should investigate the specific expression of these two similar clusters in various human normal and pathologic states.

The identification of *miR-15a* and *miR-16-1* as candidate cancer genes led to the question of how general the involvement of miRNAs is in human cancer. Numerous studies have used high-throughput microarrays to identify cancer-specific miRNA fingerprints in every type of cancer analyzed, including breast,[22] hepatocellular,[23,24] lung,[25–27]
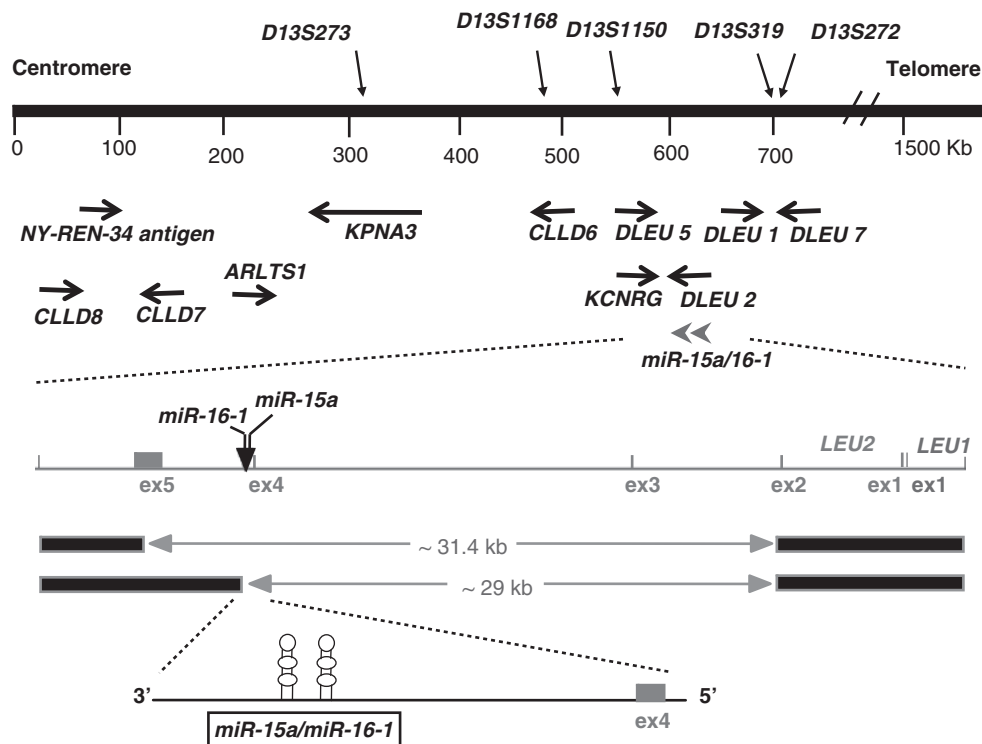

Figure 1 Schematic diagram showing the 13q14.3 deletions in CLL cells targeting the *miR-15a/miR-16-1* cluster. Eleven genes were cloned in the 13q14.3 region (upper panel), inside or very close to the deleted region. The lower panel shows the minimal region of deletion from two CLL patients,[8] identified by cell hybrid experiments and containing the *miR-15a/miR-16-1* cluster. The genomic markers and gene orientation are indicated. The gene symbols are as follows: *DLEU7* (deleted in lymphocytic leukemia 7), *DLEU1* (deleted in lymphocytic leukemia 1), *DLEU2* (deleted in lymphocytic leukemia 2), *miR-15a* and *miR-16-1* (microRNA genes 15a and 16-1), *KCNRG* (potassium channel regulator), *DLEU5* (*RFP2*, ret finger protein 2), *CLLD6* (*C13orf1*, chromosome 13 open reading frame 1), *KPNA3* (karyopherin alpha 3, importin alpha 4), *ARLTS1* (ARL11, ADP-ribosylation factor–like 11), *CLLD7* (*RCBTB1*, regulator of chromosome condensation [RCC1] and BTB [POZ] domain containing protein 1), *NY-REN-34 antigen* (*PHF11*, PHD finger protein 11), and *CLLD8* (*SETDB2*, SET domain, bifurcated 2)

colon,[28,29] and gastric[30] carcinomas. These studies clearly showed that the spectrum of miRNAs expressed in malignant cells is significantly different from that in healthy counterpart cells, suggesting that alterations in miRNA genes play a critical role in the pathophysiology of many, and perhaps all, human cancers.

Deregulation of *miR-15a* and *miR-16-1* in Cancer

The importance of miRNAs in cancer, in general, is highlighted by the observation that half of all miRNA genes are located in cancer-associated genomic regions or fragile sites,[6,7] which are frequently altered in cancer. Global repression of miRNA processing machinery, including the enzymes Drosha, Pasha, and Dicer, promotes cellular transformation in mouse models, implicating the role of mature miRNA genes in cancer-related processes.[31] Furthermore, large-scale profiling of miRNAs has revealed that miRNA deregulation is a common event in cancer (reviewed in Ref. [32]). Although much is known about the aberrant expression of miRNAs in human cancer, much less is known about the functional relevance of such aberrations.

The mapping of *miR-15a* and *miR-16-1* in a region that is frequently altered in cancer suggested that these two miRNAs could be the target genes of 13q14.3. Several studies of CLL (reviewed in Refs. [33,34]) and solid tumors[35,36] have shown that *miR-15a* and *miR-16-1* are deleted or down-regulated in tumor cells, suggesting that these two miRNA genes could be 'hot spots' in cancer transformation.[33] A recent analysis of *miR-15a* and *miR-16-1* expression in prostate cancer revealed these genes' consistent down-regulation in ∼80% of tumor samples compared with that in healthy tissues.[35] No correlation between loss of the *RB* gene, which is located in the vicinity, and deregulation of *miR-15a* and *miR-16-1* has been reported, indicating that the loss of *miR-15a* and *miR-16-1* expression is often independent of the absence of the gene encoding RB. *miR-15a* and *miR-16-1* have been also shown to express at lower levels in pituitary adenomas than in normal pituitary tissue.[36] These data led to the hypothesis that *miR-15a* and *miR-16-1* function as tumor suppressors and that their inactivation by allelic loss may contribute to tumorigenesis.

Abnormally expressed cancer genes are frequently targets for mutations that can activate or inactivate their function.[37] Therefore, screening of mutations in miRNAs has been a critical step in determining the contribution of this new class of genes to cancer development and in identifying the genetic mechanisms responsible for their deregulation. Given that *miR-15a* and *miR-16-1* have been suggested to act as tumor suppressors, it is possible that genetic mutations could inactivate their expression. Indeed, as we reported in one study, analysis of 75 CLL cases identified germline and somatic mutations in 5 of 42 sequenced miRNAs in 11 of 75 CLL patients, but no such mutations were found in 160 subjects without cancer ($P < 0.0001$).[38] Of note, a germline mutation in the *pri– miR-16-1* sequence of two CLL patients— a C→T homozygous substitution, 7 bp in the 3′ direction after the precursor—have been found.[38] This mutation has been found to reduce the expression of *miR-16-1 in vivo*, as CLL cells expressing these mutants display reduced expression of

miR-16-1 compared with expression by normal CD5^{+} cells. Furthermore, ectopic expression of mutated *miR-16-1 in vitro* in HEK293 cells revealed reduced expression compared with that of a wild-type construct.[38] Intriguingly, in the New Zealand Black mouse, which is characterized by spontaneously occurring late-onset CLL, Raveche *et al.*[39] described a point mutation in 3′ DNA adjacent to the *miR-16-1* region. This particular mutation resulted in the down-regulation of *miR-16-1* expression in this CLL-prone mouse model, further indicating the tumor suppressor function of *miR-16-1* in CLL. These observations indicate that mutations in the *miR-16-1* gene could be responsible, at least in part, for its altered expression. Other mechanisms, including loss of heterozygosity, epigenetic regulation, and defects in the miRNA biogenesis machinery, could also contribute to the deregulation in miRNA expression.[40]

Given that CLL is a disease with a frequent familial association (10–20% of patients who have at least one first-degree relative with CLL) and that other cancers also occur frequently in persons with CLL,[41] miRNA mutations may be a predisposing factor for cancers associated with CLL. In fact, one of the CLL patients harboring the germline C→T *miR-16-1* germline mutation not only had been diagnosed earlier with breast cancer but also her mother died of CLL and her sister of breast cancer.[38] Altogether, these findings may suggest that the *miR-15a* and *miR-16-1* sequence could be a target of inactivation by point mutations that contribute to the initiation and/or progression of cancer.

miR-15a and *miR-16-1* Function in Cancer Cells

miRNA profiling was instrumental in identifying the differential expression of these genes both in homeostasis and carcinogenesis.[32] These profiling efforts led to the identification of miRNA oncogenes (oncomirs), referring to elevated levels of miRNAs in human tumor cells compared with normal cells. Oncomirs target the 3′ untranslated region (UTR), the 5′ UTR, or the coding sequence of mRNA of tumor suppressor genes and thus contribute to their down-regulation. On the other hand, up-regulation of oncogenes could be due to loss or reduced expression of miRNAs that function as tumor suppressors.[42] After we found that *miR-15a* and *miR-16-1* expression is lost or down-regulated in CLL cells, we hypothesized that these two genes may target the expression of a known oncogene or survival factor that is essential for CLL development.

Using bioinformatics tools, we found that the *miR-15a* and *miR-16-1* sequences and the *BCL2* mRNA sequence share a complementary homology, which suggests that the Bcl2 oncoprotein may be a target of posttranscriptional repression by *miR-15a* and *miR-16-1* tumor suppressors.[43] Bcl2 is a central player in the genetic program of eukaryotic cells, favoring survival by inhibiting cell death.[44] Overexpression of the Bcl2 protein has been reported in many types of human cancers, including leukemias, lymphomas, and carcinomas.[45] In follicular lymphomas and in a fraction of diffuse B-cell lymphomas, the mechanism of *BCL2* activation has been found to be the translocation t(14;18)(q32;q21), which places the *BCL2* gene under the control of immunoglobulin heavy-chain enhancers, resulting in deregulated expression of the

gene.[46,47] B cell-CLL overexpress Bcl-2[48]; however, with the exception of the fewer than 5% of cases in which *BCL2* is juxtaposed to immunoglobulin loci,[49] no mechanism has been discovered to explain *BCL2* deregulation in CLL.

That the *BCL2* gene is overexpressed in 65–70% of B cell-CLLs, and that deletions or down-regulation of the *miR-15a* and *miR-16-1* cluster have been reported in the same proportion of CLL samples led us to hypothesize that *miR-15a* and *miR-16-1* could be responsible for the increased levels of Bcl2 in CLL.[43] Indeed, our data showed that *miR-15a* and *miR-16-1* expression are inversely correlated with Bcl2 expression in CLL samples and that both miRNAs negatively regulate Bcl2 at a posttranscriptional level. Bcl2 repression by these miRNAs induced apoptosis in a leukemic cell line model. In MEG-01 cells transfected with the wild-type miR-15/16, we observed an increased rate of apoptosis mediated by cleavage of pro-caspase 9 and of poly (ADP-ribose) polymerase, indicating that the reduction in Bcl2 protein levels caused by miRNAs is sufficient to initiate the apoptotic process.[43] Given that Bcl2 is overexpressed in the majority of CLL malignant lymphocytes and no genetic alteration can explain this aberrancy, control of Bcl2 expression by the *miR-15/16* cluster can be considered as one of the main molecular mechanisms of this aberration.

To further shed light on the tumor suppressor action of *miR-15a* and *miR-16-1* cluster, we analyzed the effects of both miRNAs on transcriptome and proteome in CLL cells.[50] Ectopic expression of *miR-15a* and *miR-16-1* led to the up-regulation of 265 genes and down-regulation of 3307 genes, among which the *MCL1* anti-apoptotic gene and others are predicted targets of *miR-15* and *miR-16* (Table 1). Of note, ~20% of the down-regulated genes had AU-rich elements (AREs), which could further support the finding that ARE-mediated instability is implicated in the regulation of gene expression by *miR-16-1*.[51] Analysis of the proteomic changes

after overexpression of *miR-15a* and *miR-16-1* cluster revealed 27 different proteins that exhibited at least four-fold change with respect to the control group. Intriguingly, the proteins Bcl2 and Wilms tumor 1, a confirmed and predicted target, respectively, were among the proteins with prominent changes. Other proteins found are involved in cell growth, the cell-cycle, oncogenesis, tumor suppression, and anti-apoptosis.[51] These observations clearly support the fact that a single miRNA, for example, *miR-15a* or *miR-16-1*, can have far-reaching effects on the overall function of the cell.

The tumor suppressor function of *miR-15a/16-1* has also been addressed *in vivo*. In immunocompromised nude mice, ectopic expression of *miR-15a/16-1* was found to cause dramatic suppression of tumorigenicity of MEG-01 leukemic cells exhibiting a loss of endogenous expression of *miR-15a/16-1*.[50] This observation confirms that *miR-15a* and *miR-16-1* genes are tumor suppressors and are highly effective in preventing the growth of leukemic xenografts.

The *miR-15a/16-1* cluster targeting of *BCL2* at the posttranscriptional level implies that miRNA genes play an important role in regulating apoptosis. Our data show that in cancer cells lacking these miRNAs, restoration of *miR-15a* and *miR-16-1* triggers apoptosis and suppresses tumorigenicity.[43] On the other hand, oncomirs such as *miR21* and the *miR17-92* cluster inhibit the expression of tumor suppressor genes such as *PDCD4*[52] and *BIM*,[53] reducing apoptosis and enhancing proliferation (reviewed in Ref.[54]) and thus contributing to tumorigenesis. Deregulation of apoptosis is an important step in cancer, as it allows genetically unstable cells to survive and accumulate further mutations that eventually lead to tumorigenesis. Therefore, it is possible that perturbation of the expression of oncosuppressor miRNA genes, such as *miR-15a* and *miR-16-1*, may lead to tumorigenesis.

Table 1 Examples of identified targets of the *miR-15a/16-1* cluster as identified by proteomics and transcriptome analyses in MEG-01 leukemia cells and CLL cells

Gene symbol	Full name	Identification	Gene summary
BCL2	B cell CLL/lymphoma 2	Proteomics	An integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells such as lymphocytes
CDC2	Cell division cycle 2, G1 to S and G2 to M	Proteomics	A member of the Ser/Thr protein kinase family this protein is a catalytic subunit of the highly conserved M-phase promoting factor (MPF), which is essential for G1/S and G2/M phase transitions of eukaryotic cell cycle
ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	Transcriptoma	A transcriptions factor that regulates numerous genes and is involved in stem cell development, cell senescence and death, and tumorigenesis
JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	Transcriptoma	Encodes a protein that interacts directly with specific target DNA sequences to regulate gene expression
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	Transcriptoma	A member of the Bcl-2 family that enhances cell survival by inhibiting apoptosis
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (*E. coli*)	Transcriptoma	A human homolog of the *E. coli* mismatch repair gene *mutS*, identified as a locus frequently mutated in hereditary nonpolyposis colon cancer consistent with the characteristic alterations in microsatellite sequences (RER+ phenotype)
PDCD6IP	Programmed cell death 6 interacting protein	Transcriptoma	Encodes a protein thought to participate in programmed cell death and binding to the product of the *PDCD6* gene, a protein required for apoptosis
RAB9B	RAB9B member RAS oncogene family	Proteomics	A member of the RAS oncogene family
WT1	Wilms tumor 1	Proteomics	A transcription factor that contains four zinc-finger motifs at the C terminus and a proline/glutamine-rich DNA-binding domain at the N terminus. It has an essential role in the normal development of the urogenital system, and it is mutated in a small subset of patients with Wilm's tumors

An miRNA gene could have several targets; therefore, it is likely that *miR-15a* and *miR-16-1* may target other oncogenes that are relevant to cancer. Recently, Bonci *et al.*[35] reported that the *miR-15a* and *miR-16-1* cluster targets not only *BCL2* but also *CCND1* (encoding cyclin D1) and *WNT3A* mRNA, which promote several prostate tumorigenic features, including survival, proliferation, and invasion. *In vivo* knockdown of *miR-15a* and *miR-16-1* has been shown to result in prostate hypoplasia associated with Ccnd1 and Wnt3a upregulation, indicating that the loss of these miRNAs could be a relevant pathogenic event in prostate carcinogenesis.[35] Although *BCL2*, *CCND1*, and *WNT3A* may play a key role in various types of human cancer driven by loss of *miR-15a* and *miR-16-1* expression, there could be hundreds of predicted targets for these miRNAs that might affect the process of carcinogenesis. Together, these data suggest that *miR-15a/16-1* genes are natural anti-sense interactors with *BCL2* and probably other oncogenes and that they can be used to suppress tumor growth (Figure 2).
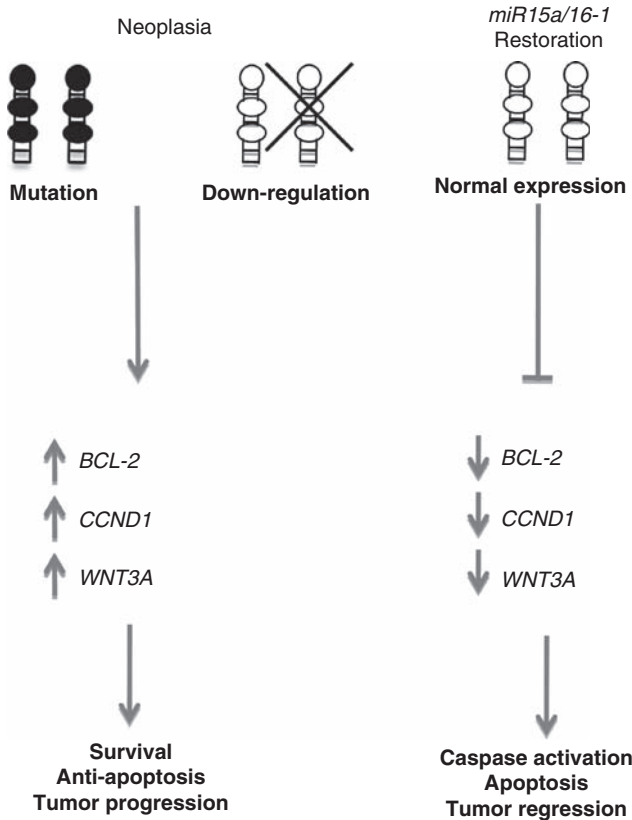

Figure 2 Genetic alteration in *miR-15a/16-1* cluster triggers apoptosis, proliferation, and tumor progression. The main mechanisms of *miR-15/16* alteration are deletion, down-regulation, and mutation. Experimentally proven targets of *miR-15a* and *miR-16-1* are shown: the *BCL2* antiapoptotic gene, cyclin D1 (*CCND1*), and the WNT gene *WNT3A*. In tumor cells, *miR-15/16* genes are altered, which affects the expression of their targets and leads to enhanced survival, increased proliferation, and tumor progression. Restoration of *miR-15/16* genes in tumor cells should inhibit expression of these oncogenes, resulting in caspase activation, apoptosis, and tumor regression

Deregulation of *miR-15a* and *miR-16-1* in Cancer is Associated with Clinical Parameters

In addition to their role in promoting the development of primary tumors, miRNAs might also guide the choice of the best treatment strategy by providing prognostic information. CLL is characterized by the progressive accumulation of mostly nondividing CD5$^+$ B lymphocytes.[17,41] Several clinical parameters are used to determine prognosis and the therapeutic course in CLL. For example, few or no mutations in the immunoglobulin heavy-chain variable-region gene (IgV_H) or high expression of the 70-kD zeta-associated protein (ZAP-70 +) predict an aggressive course, whereas mutated IgV_H CLL clones or low ZAP-70 + expression predicts an indolent course.[17] Analysis of miRNA expression profiles of 94 samples of CLL cells defined a unique miRNA expression signature composed of 13 genes (of 190 analyzed) that can differentiate between low and high ZAP-70 expression and between unmutated and mutated IgV_H.[38] The corresponding miRNA signature was associated with the presence or absence of disease progression. Given that allelic loss of 13q14.3 has been shown to be associated with a favorable outcome in B-cell–associated CLL, we speculated that deletion or down-regulation of *miR-15a* and *miR-16-1* could also be associated with improved prognosis. In fact, deregulation of *miR-15a* and *miR-16-1* in CLL has been correlated with a better prognosis.[38]

Lower levels of *miR-15a* and *miR-16-1* have also been reported in pituitary adenomas, and their expression was inversely correlated with tumor diameter and tumor growth.[36] Bonci *et al.*[35] also noted that deletion of *miR-15a* and *miR-16-1* genes was associated with the presence of more-advanced prostate tumors. Therefore, accumulating evidence suggests that the miRNA expression profile not only is different between malignant cells and their normal counterparts but also is associated with prognostic factors in human cancer.

Future Perspectives

We envision the possible future use of miRNAs or compounds interacting with miRNAs as new therapeutic agents in cancer. Compared with other RNA inhibition techniques (such as anti-sense oligonucleotides (ASOs), ribozymes, and siRNAs), the major advantage of using miRNAs to treat cancer patients is that a specific miRNA targets several genes involved in the same pathway. For example, among the components of the *miR-15a/16-1* signature in CLL, a disease in which the main altered cell program is apoptosis, we observed significant enrichment in cancer genes (such as *MCL1*, *BCL2*, *ETS1*, and *PDCD6IP*) (Table 1) that directly or indirectly affect apoptosis and the cell cycle. Therefore, by targeting miRNAs, a more effective functional restoration of the abnormal pathway can be achieved than can be achieved with the usual one-agent, one-target effect characteristic of all other RNA inhibition agents. Given recently published results showing the efficacy of oblimersen sodium in patients with advanced CLL that is refractory to fludarabine,[55] it would be feasible to design regimens using a cocktail of anti-*BCL2* ASOs and miRNAs targeting *BCL2*, such as *miR-15* and *miR-16*, for indolent CLL or of anti-*MCL1* miRNAs, such as *miR-29* and *miR181*, for

aggressive forms of CLL. In this way, multiple drugs targeting the anti-apoptotic overexpressed regulators Bcl2 and Mcl1 could have a better chance of consistently and robustly reducing these proteins' expression levels. Given that many more miRNAs have been predicted *in silico* and will probably be proven experimentally to interact with *BCL2*, the possible combinations of RNA inhibitory drugs will increase in the near future.

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