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Review

Role of *miR-15/16* in CLL

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B-cell chronic lymphocytic leukemia (CLL) is the most common adult leukemia. The most common chromosomal abnormalities detectable by cytogenetics include deletion at 13q (55%), 11q (18%), trisomy 12 (12–16%) and 17p (8%). In 2002, we discovered that a microRNA cluster *miR-15a/miR-16-1* (*miR-15/16*) is the target of 13q deletions in CLL. MicroRNAs encoded by the *miR-15/16* locus (*miR-15* and *miR-16*) function as tumor suppressors. Expression of these miRNAs downregulated in CLL, melanoma, colorectal cancer, bladder cancer and other solid tumors. *miR-15/16* cluster targets multiple oncogenes, including *BCL2*, *Cyclin D1*, *MCL1* and others. The most important target of *miR-15/16* in CLL is arguably *BCL2*, as *BCL2* is overexpressed in almost all CLLs. In this review, we discuss the discovery, functions, clinical relevance and treatment opportunities related to *miR-15/16.*
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Facts

- *miR-15/16* is a first microRNA tumor suppressor located at 13q14 region deleted in CLL.
- *miR-15/16* exerts its tumor-suppressor function by targeting *BCL2*.
- *DLEU7* and *miR-15/16* are two cooperating tumor suppressors at 13q14.

Open Questions

- Is *BCL2* the only important target of *miR-15/16* in CLL?
- Can *miR-15/16* be used as a drug for CLL?
- Will Bcl2 inhibitors cure CLL?

MiR-15/16 at 13q14, Gene Discovery

Chronic lymphocytic leukemia (CLL) is the most common human leukemia. It accounts for ~12 000 newly cases diagnosed each year in the United States and represents one-third of all leukemia cases.[1] Most CLL patients can survive for several of years and show relatively mild symptoms.[1] Malignant CLL leukemic cells show morphologically mature appearance and typically do not proliferate *in vitro*.[1,2] CLL occurs in two forms, aggressive and indolent, and both forms show the clonal expansion of CD5-positive B cells.[1,2] In most cases, aggressive CLL is characterized by high zeta-chain-associated protein kinase 70 (ZAP-70) expression and unmutated IgH V_H (immunoglobulin heavy chain variable region genes), while indolent CLLs express low ZAP-70 levels and show mutated IgH V_H.[1,2]

Genomic aberrations are detected in >80% of CLL cases. The most common chromosomal abnormalities detectable by cytogenetics include deletion at 13q (55%), 11q (18%), trisomy 12 (12–16%), and 17p (8%).[3,4]

The most frequently deleted genomic region in CLL occurs at chromosome 13q14 and is associated with the indolent form of the disease.[4] The same region is frequently found as the sole cytogenetic abnormality in other types of cancers: 50% of mantle cell lymphomas,[5] ~30% of multiple myeloma,[6] and ~60% of prostate cancers,[7] indicating that 13q14 contains an important tumor suppressor involved in the pathogenesis of these malignancies. A number of years ago, several laboratories used positional cloning and sequencing of a region of >1 Mb at the deleted region to identify a tumor-suppressor gene at 13q14.[8,9] Detailed genetic analysis of protein-coding genes located in or close to the deleted region, including loss of heterozygosity (LOH) studies, mutation, and expression analysis, failed to show that any of these genes can function as tumor suppressors. And none of these known genes were found inactivated in CLL by mutations or deletions.[8–11] In 2001, we generated somatic cell hybrids between mouse and CLLs cells carrying 13q14 deletion and translocation. Analysis of these hybrids revealed that 13q14 tumor-suppressor gene lies within a 30-kb region between exons 2 and 5 of the *DLEU2* gene (Figure 1). However, *DLEU2* has been studied extensively and was excluded as a likely target of 13q14 deletions in CLL.[2,12,13] Interestingly, the 13q14 translocation breakpoint in one of the CLL cases was mapped in the same region.[12,13] Thus we continued to investigate this 30-kb genomic region and finally found that a cluster of two microRNA genes *miR-15a* and *miR-16-1* is located in the deleted region and very close to the translocation breakpoint.[12] We then investigated the expression levels of *miR-15/16* in CLL and found that *miR-15/16* cluster was deleted or its expression of both *miR-15* and *miR-16* was downregulated in two-thirds of CLL cases.[12]

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Abbreviations: TNF, tumor necrosis factor; KO, knockout; ZAP-70, zeta-chain-associated protein kinase 70; IgH V_H, immunoglobulin heavy chain variable region genes; 3′UTR, 3′ untranslated region
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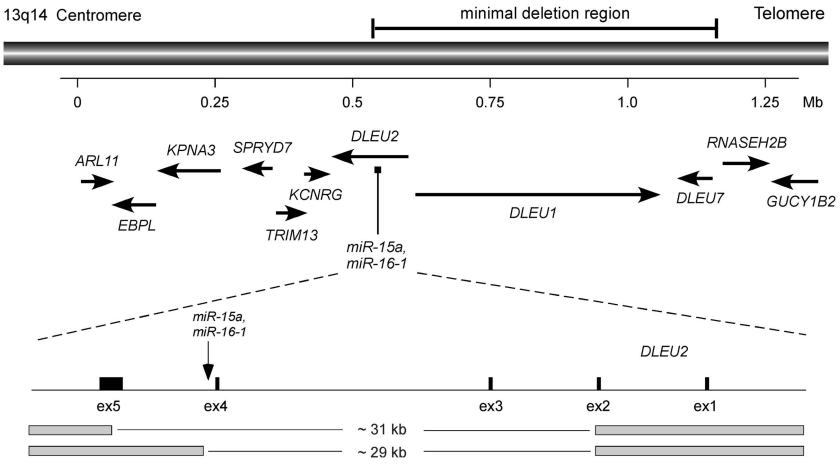

Figure 1 13q14 genomic region deleted in CLL

In contrast, expression of several protein-coding genes in the region in CLL was not affected by the 13q14 deletions.[8,9,13] This was the first demonstration of the involvement of non-coding RNA in human disease.[12]

The microRNAs are a large family of highly conserved non-coding genes thought to be involved in tissue-specific gene regulation.[14] microRNAs represent an evolving class of gene products that are usually excised from 70- to 80-nt stem-loop RNA precursor structures.[15] In mammals, single-stranded microRNA usually binds specific mRNAs (mainly in the 3′ untranslated (3′ UTR)) through sequences that are significantly, though not completely, complimentary to the target mRNA.[15] By a mechanism that is not fully characterized, this binding causes a block of translation and/or degradation of target mRNAs, resulting in decreased levels of the corresponding protein.[16] It is estimated that there could be up to 2000 microRNA genes in the human genome.[17]

Expression and Functions of *miR-15/16* in CLL

Since our initial observation that *miR-15/16* is deleted or downregulated in two-thirds of CLL samples, several other studies confirmed our results.[18,19] Tumor-suppressor genes in cancer are also frequent targets of mutations that can inactivate their function, and finding such mutations is a critical step in determining the contribution of microRNA or mRNA gene(s) in hematopoietic or solid malignancies. As *miR-15/16* is a target of 13q14 deletions in CLL, it is possible that mutations could have a role in inactivation of its expression. Our subsequent study analyzed 75 CLL cases and 160 normal controls for mutations in microRNA genes.[20] This study identified a germline mutation in the *pri-miR-15a/miR-16-1* sequence in 2 of the 75 CLL patients (a C/T substitution, only 7-bp 3′ to *miR-16-1* in the precursor), whereas no such mutation was found in 160 normal controls. Interestingly, this mutation significantly reduced the expression of both *miR-16* and *miR-15* in transfection experiments *in vivo*, indicating that this is the inactivating mutation.[20] As CLL often shows a familial association ($\sim$10% of patients have at least one first-degree relative with CLL), and CLL patients frequently have other cancers,[21] mutations found in CLL may predispose patients to other associated cancers. Interestingly, one of the two CLL patients harboring the C/T *miR-16-1* mutation was also diagnosed with breast cancer, her mother had CLL, and her sister had breast cancer.[20] Thus *miR-15/16* at 13q14 is deleted, mutated, and downregulated in a majority of CLL cases, indicating its contribution to the initiation and/or progression of CLL.

As we found that *miR-15/16* is a target of 13q14 deletions in CLL, it is very important to understand its functions relevant to CLL pathogenesis. Using bioinformatic tools, we searched for targets of these microRNAs focusing on oncogenes implicated in CLL. We found that *BCL2* oncogene was one of the top predicted targets of *miR-15/16*.[22] By analyzing homology between *miR-15/16* and the *BCL2* mRNA, we found that the first 9 nucleotides from the 5′-ends of both *miR-15* and *miR-16* are complementary to bases 3287 to 3279 in the 3′-end of the *BCL2* cDNA.[22] *BCL2* is a critical oncogene in a number of hematological malignancies as well as in solid tumors. It mostly functions by promoting survival and inhibiting cell death.[23,24] In B-cell malignancies, such as follicular lymphomas and diffuse B-cell lymphomas, *BCL2* is activated by the translocation t(14;18)(q32;q21), which places the *BCL2* oncogene in the proximity of immunoglobulin heavy-chain enhancers, resulting in overexpression of *BCL2*.[25,26] Interestingly, almost all CLLs overexpress Bcl-2,[27] but t(14;18)(q32;q21) translocations are very rare in CLL,[28] and no other mechanism for *BCL2* overexpression in CLL has been reported. We thus hypothesized that *miR-15/16* downregulation in CLL is responsible for the increased levels of *BCL2*. To demonstrate that overexpression regulation of *BCL2* is a direct effect of loss of *miR-15/16*, a 536-bp fragment of human *BCL2* interacting with the two microRNAs was fused to a luciferase reporter gene. Luciferase assay experiments showed that both *miR-15* and *miR-16* directly interact with 3′UTR of human *BCL2* but not with the mutated sequence lacking the region of homology.[22] This indicates that both microRNAs directly interact with and inhibit *BCL2* expression. In addition, we found that expression of both *miR-15* and *miR-16* is inversely correlated with Bcl2 expression in CLL samples and that Bcl2 repression by *miR-15/16* induced apoptosis in a leukemic cell line MEG-01 that carries alterations in both *miR-15/16* alleles and does not express any *miR-15/16*. In these experiments, we found MEG-01 cells

transfected with the wild-type *miR-15/16* rapidly undergo apoptosis evidenced by cleavage of pro-caspase 9 and of poly (ADP-ribose) polymerase, in a sharp contrast with MEG-01 cells transfected with the *miR-15/16* mutant found in CLL patients.[22] We concluded that *miR-15/16* targets *BCL2* expression in CLL and that loss of *miR-15/16* due to 13q14 deletions is the main reason of *BCL2* overexpression in CLL.

To further investigate the tumor-suppressor action of *miR-15/16* cluster, we tested its tumor-suppression function *in vivo*. MEG-01 cells, transfected *miR-15/16* cluster were inoculated into 'nude' mice. After 4 weeks days, tumor growth was completely suppressed in most of the mice inoculated with *miR-15/16*-transfected MEG-01, whereas we observed large tumors for the untreated and empty vector treated mice.[29] Results of these experiments showed the tumor-suppressor function of *miR-15/16* cluster in MEG-01 leukemia cells. Because most microRNAs inhibit translation of their target mRNAs, we also investigated the effects of *miR-15/16* on MEG-01 proteome. Among differentially expressed proteins, *BCL2* and *WT1* were validated *miR-15/16* targets. The targeted proteins have a variety of biological functions. Interestingly, 10 of the 27 proteins were involved in cell growth regulation or had anti-apoptotic function, and 8 of the 27 proteins were predicted targets of *miR-15/16*.[29]

We recently investigated transcriptional regulation of *miR-15/16* in CLL. Several TP53-binding sites were found upstream of the *miR-15/miR-16* chromosome 13q14 and on a homologous *miR-15/16* cluster chromosome 3.[30] Chromatin immunoprecipitation analysis revealed that TP53 directly binds to its predicted binding sites on both chromosome 13q14 and chromosome 3, both in cell lines and in primary CLLs with normal cytogenetic profiles. A luciferase reporter assay showed that TP53 significantly increased the luciferase reporter activity of all the binding site containing vectors.[30] In MEG-01 cells, TP53 transactivation of the *miR-15/16* cluster was also confirmed by real-time reverse transcription-PCR. Similarly, doxorubicin-mediated TP53 activation in MEG-01 cells increased the expression of *miR-16*, an effect that was abolished when TP53 was silenced by an anti-TP53 oligonucleotide.[30] We also identified a binding site for both *miR-15* and *miR-16* inside the 3′UTR of *TP53*. A luciferase reporter assay showed that both *miR-15* and *miR-16* directly target the identified *TP53*-binding site and significantly reduced the luciferase reporter activity compared with a scrambled oligonucleotide-negative control. This effect was completely abolished when the binding site was either deleted or mutated. We concluded that *miR-15/16* and *TP53* are engaged in feedback circuitry loop in CLL: p53 transactivates *miR-15/16*, while increased levels of *miR-15/16* target *TP53* expression (Figure 2).[30]

Although *BCL2* is arguably the most important target of *miR-15/16*, several other oncogenes targeted by *miR-15/16* were recently identified. For example, it has been shown that *miR-15/16* inhibits *MCL1* oncogene in CLL *BMI1* oncogene in mantle call lymphoma.[29,31] Two other recent reports demonstrated that *miR-15/16* directly target critical cell cycle regulator *Cyclin D1* in bladder cancer and osteosarcoma.[32,33] Another study showed that *miR-15/16* expression is down-regulated in malignant pleural mesothelioma (MPM).[34] Administration of synthetic mimics to restore *miR-15/16* expression led to growth inhibition in MPM cell lines and in xenograft-bearing nude mice; administration of miR-15/16 mimics led to consistent inhibition of MPM tumor growth.[34]

miR-15/16 Cooperates with *DLEU7* in CLL Pathogenesis

A recent study reported a high-resolution map of 13q14 deletions using 171 CLL samples.[35] Interestingly, in addition to *miR-15/16*, this region also contained *DLEU7* gene (that was previously identified as a candidate tumor-suppressor gene at 13q14) located telomeric to *miR-15/16*.[35,36] We investigated whether *DLEU7* can also function as a tumor suppressor and cooperate with *miR-15/16*,[37] as *DLEU7* is the only protein-coding gene located within reported deleted region. Among the 25 CLL samples examined for *DLEU7* expression, 24 showed lower expression compared with normal $CD19^+$ B cells, while 13 of the 25 samples showed decreases of $\geq$10-fold. Expression of *miR-15/16* was also decreased in almost all CLL examined.[37] Recent studies of CLL mouse demonstrated the importance of the NF-kB pathway. (reviewed in Pekarsky *et al.*[38]). For instance, transgenic mice expressing APRIL (a proliferation-inducing tumor necrosis factor (TNF) ligand), a member of the TNF superfamily involved in NF-kB activation showed significant expansions of $CD5^+$ B cells. We thus investigated whether Dleu7 functions as an inhibitor of NF-kB, in particular if its function is related to APRIL. APRIL and BAFF are two members of the TNF superfamily and show abnormally high expression levels in a number of B-cell malignancies, including mantle cell lymphoma, diffuse large cell lymphoma, and CLL.[39] APRIL binds to two of its receptors, TACI and BCMA (B-cell maturation antigen).[39] TACI and BCMA interact with various TRAF family members and activate the NF-kB pathway.[39] Thus, NF-kB activation through TACI and BCMA may be an important pathway and is important in CLL pathogenesis.[37] We investigated whether *DLEU7* expression has an effect on NF-kB activation by TACI and BCMA. Our results showed that indeed Dleu7 expression inhibited NF-kB activation by BCMA over fivefold and by TACI was inhibited over fourfold.[37] We concluded that Dleu7 functions as an

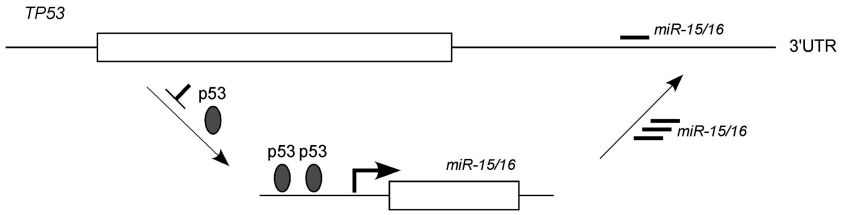

Figure 2 *TP53–miR-15/16* circuitry in CLL

inhibitor of the NF-kB pathway, a critical pathway in CLL development (Figure 3). Nuclear factor of activated T-cells (NFAT) is a hallmark of unstimulated CLL cells and can also be activated by TACI and BCMA.[40–42] NFAT is also involved in transactivation of CD5 promoter in B cells.[41,42] Since Dleu7 inhibits function of TACI and BCMA in NF-kB activation, we investigated whether Dleu7 can also inhibit NFAT activation by TACI and BCMA. Our results showed that Dleu7 expression can inhibit NFAT activation by TACI and BCMA approximately eightfold. To demonstrate tumor suppressor function of Dleu7 we used adenovirus expressing Dleu7 or empty adenovirus as a control. In these assays, Dleu7 expression resulted in over twofold decrease of cells in the S phase and in over threefold induction in apoptosis.[37] Our current view is that 13q14 region deleted in CLL contains two tumor suppressors, *miR-15/16* and *DLEU7*. *DLEU7* inactivation results in NF-kB and NFAT activation, while *miR-15/16* inactivation causes constitutive activation of Bcl2 and Mcl1.[29,37] Interestingly, transgenic mouse models confirmed that 13q14 region contains two tumor suppressors cooperating tumor suppressors. Transgenic mice expressing *BCL2* or *TRAF2DN* (a molecule downstream of TACI and BCMA) do not develop CLL.[43,44] After the cross, *TRAF2DN/BCL2* double transgenic mice developed CLL-like CD5-positive CLL phenotype.[45] Thus, *miR-15/16* and *DLEU7* are inactivated by the 13q14 deletions. *DLEU7* deletions cause the activtion of TNF signaling through TRAFs, while *miR-15/16* deletions cause a constitutive increase of Bcl2 expression. These two events are very similar to the oncogenic mechanism causing CLL development in *TRAF2DN/BCL2* transgenics (Figure 4).[37]

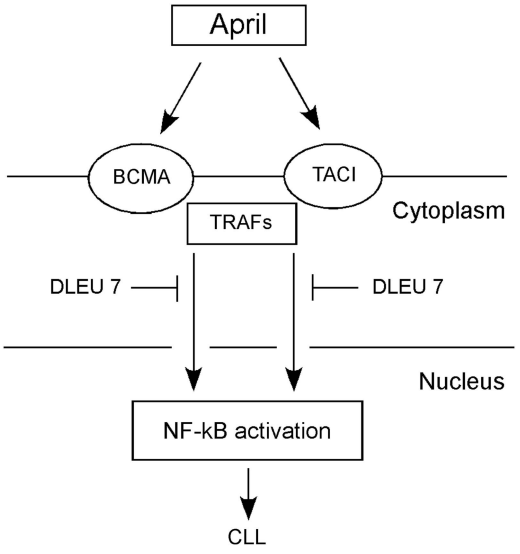

Figure 3 *DLEU7* inhibits NF-kB activation in CLL

miR-15/16 in CLL Mouse Models

There is only one natural mouse strain, as opposed to models of CLL that are genetically engineered, used as a model of disease presenting with a late-onset CLL due to slow progression of the leukemia induced by B-cell hyperproliferation: the New Zealand Black (NZB) strain.[46] Aging NZB mice are characterized by a sub-population of B-1 B cells that expands clonally and resembles that found in the human counterpart.[46] An experiment of genome-wide linkage scan conducted in the laboratory of Dr. Raveche identified the loci associated with CLL development in this strain.[47] Thirty-five percent of the animals generated from the backcross between NZB and the control strain DBA/2 presented with lymphoproliferative disease (LPD) of B cells. Three loci located on chromosomes 14, 18, and 19 were found to be associated with this disease. Half of all cases of human CLL show a deletion on chromosome 13q14.3 whose region is syntenic with the region on NZB chromosome 14 where the locus associated with LPD was found.[47] The *miR-15/16* cluster, contained in an intron of the gene *DLEU2*, is present in both human and mouse regions of synteny. Interestingly, by DNA sequencing of multiple NZB tissues, a point mutation in the 3′-flanking segment of the precursor miRNA, *miR-16-1*, was found to resemble the C→T point mutation previously identified in the *miR-16-1* 3′-flanking region of human patients.[20,47] No other strain of mice, including the NZW strain, the closest relative of NZB, was found to carry the same mutation. As expected, analysis of the levels of mature miRNA

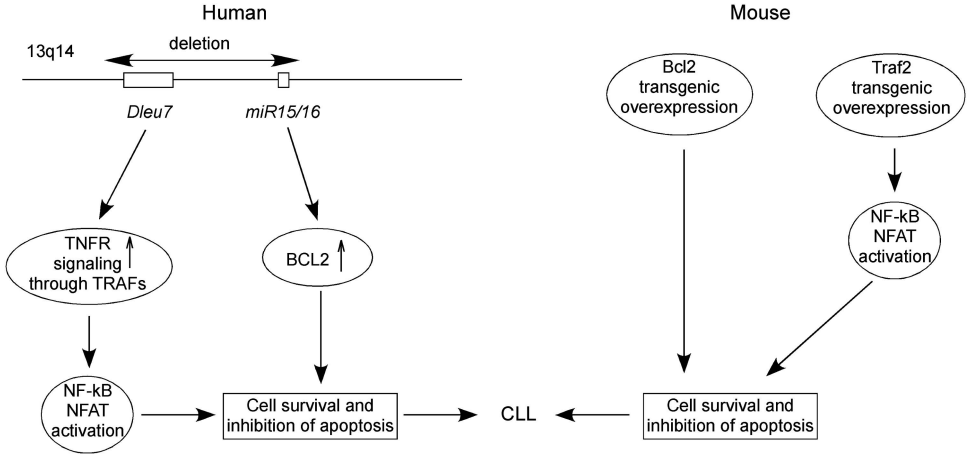

Figure 4 *DLEU7* and *miR-15/16* are two cooperating tumor suppressors at 13q14

revealed a reduced expression of *miR-16* in lymphoid tissues of NZB mice. In addition, cell cycle alterations such as G1 arrest and decrease in S phase cells were induced by exogenous *miR-16* delivery into a NZB malignant cell line.[47]

To confirm the importance of *miR-15/16* deletion in the pathogenesis of CLL, Dr. Dalla-Favera's laboratory generated the first genetically manipulated mice.[48] This model was based on conditional alleles mimicking the loss of either the minimal deleted region (*MDR*) that spans the entire gene *Dleu2*, as was characterized in human CLL, or only the *miR-15/16* cluster with no altered expression of *Dleu2*. At 1 year of age, while control animals displayed 15% of $CD5^+$ $B220^+$ B cells among mononuclear cells in the peritoneum, both *Mdr* and *miR-15/16* knockout (KO) strains showed 50% of the same cell population.[48] Overall, between 15 and 18 months, CLL affected 21% of *miR-15a/16-1* KO and 27% of *Mdr* KO mice, but some type of clonal B-cell proliferation involved 26 and 42% of *miR-15/16* KO and *Mdr* KO animals, respectively.[48] To confirm that the *Mdr* KO phenotype was more severe than the *miR-15/16* KO, the survival of the latter was not statistically different from that of wild-type siblings, while the former lived less than WT littermates, at the end succumbing to their leukemias.[48] These findings likely point to other genetic elements with tumor-suppression function, including the gene *DLEU2* itself, that are part of the *MDR* locus.[48]

Different approaches were also used to study B-cell proliferation mechanisms. For example, experiments of BrdU incorporation into DNA synthesis revealed that WT B cells started DNA synthesis later than *miR-15/16* KO B cells.[48] Expression levels of the phosphorylated retiniblastoma (p-Rb) protein indicate the entry into the cell cycle. In WT mitogen-stimulated B cells, p-Rb was produced at later time points than in B cells from *miR-15/16* KO or *Mdr* KO animals. Investigators were also able to dissect the single contributions of *miR-15/16* cluster and *DLEU2* gene to the lymphoproliferation. In a human cell line derived from a 13q14 KO CLL, an inducible system was generated where the two genetic elements underwent separate *in vitro* re-expression. Results showed that, with higher fraction of cells in G0/G1 phase, in *miR-15/16*-expressing cells impaired proliferation occurred, whereas it did not in those expressing *DLEU2*.[48] Thus a possible control of G0/G1 phase transition by *miR-15/16* has been suggested.[48]

Treatment Implications

Due to high costs and difficulties in intracellular delivery, *miR-15/16* itself cannot be easily used as a drug for CLL, at least currently. However, Bcl2, its arguably most important target in CLL, represents a very attractive molecular target. ABT-199 is a first-in-class orally bioavailable *BCL2* selective inhibitor that was recently developed, and very recently, Souers *et al.*[49] evaluated the ability of ABT-199 to suppress tumor growth *in vivo* in a broad spectrum of human hematological tumor xenografts established in immunocompromised mice. After a single oral dose of 12.5 mg/kg body weight in xenografts derived from RS(4;11) leukemia cells, ABT-199 caused a maximal tumor growth inhibition of 47% and tumor growth delay of 26%. The magnitude and durability of the response increased in a dose-dependent manner, with the highest dose of 100 mg/kg body weight tumor growth inhibition increased to 95% and a tumor growth delay to 152%.[49] In addition to showing preclinical efficacy in *BCL2*-dependent cell lines and tumor xenograft models, ABT-199 demonstrated immediate anti-leukemic activity after a single dose in three patients with refractory CLL while causing only minor changes in platelet counts.[49] Thus potent and selective *BCL2* inhibitors such as ABT-199 could be efficiently used as CLL drugs.

Conclusions

Despite numerous studies of clinical features and genomic rearrangements in CLL, molecular mechanisms resulting in disease development are still not clearly understood. Our discovery that *miR-15/16* is a main target of 13q14 deletions in CLL was the initial step in uncovering of one critical mechanism in CLL development as well as a first example of a major involvement of microRNAs in cancer pathogenesis. The fact that *miR-15/16* targets *BCL2* expression was a first link between major tumor-suppressor microRNA and major oncogene in CLL, and these findings increased interest in finding drugs inhibiting *BCL2*. These efforts recently resulted in the development of ABT-199, a very potent oral drug showing great promise in CLL treatment.

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