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MicroRNA dysregulation and multi-targeted therapy for cancer treatment

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Abstract

We established that loss of *miR-15a/16-1* genes on chromosome 13q14 is the most common alteration in Chronic Lymphocytic Leukemia (CLL) and that *miR-15/16* are crucial negative regulator of *BCL-2*, an antiapoptotic gene overexpressed in most CLLs and in many other malignancies. We have also shown that *miR-15/16* target ROR1, a cell surface receptor for Wnt5a which can enhance growth/survival of CLL cells. Interestingly, ROR1 is expressed by many cancers, but not by normal adult tissues. Moreover, Venetoclax, the anti-*BCL-2* drug, and Cirmtuzumab, the monoclonal antibody against ROR1, are synergistic in killing CLL cells.

Since an additional *miR-15/16* locus exists on chromosome 3q25 (*miR-15b/16-2*), we generated a knocked out mouse model to study its the role in cancer. We observed that the KO mice developed predominantly CLL. Thus, we generated a double knock out mouse model where both *miR-15/16* loci were deleted. Surprisingly we observed that 77% of double KO mice developed Acute Myeloid Leukemia (AML). Based on these evidences, we anticipate that also AMLs with low *miR-15/16* expression, overexpression of BCL2 and expression of ROR1, would show an excellent response to a combination therapy with venetoclax and monoclonal antibodies against ROR1, since both drugs target the same malignant cells that have lost *miR-15/16.*

Keywords

CLL; AML; *miR-15/16*; *BCL-2*; ROR1; Venetoclax; Cirmtuzumab

1. Introduction

In the last few years, two drugs have been developed to target specifically Chronic Lymphocytic Leukemia (CLL) cells: Venetoclax and Cirmtuzumab (Rassenti et al., 2017).

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Conflict of interest

There are no conflict of interest for any of the authors.

The design of Venetoclax, was based on the discovery of the key role of the antiapoptotic B-cell lymphoma 2 gene (*BCL-2*) and its dysregulation in follicular lymphomas and CLL (Calin et al., 2002; Croce and Reed, 2016; Tsujimoto et al., 1985; Tsujimoto and Croce, 1986; Tsujimoto et al., 1984). *BCL-2* is a master regulator of cell survival (Croce and Reed, 2016) and Venetoclax was developed to kill malignant cells driven by the overexpression this protein (Pekarsky et al., 2018; Pekarsky and Croce, 2019).

The development of Cirmtuzumab, was based on the discovery that the tyrosine kinase-like orphan receptor 1 (ROR1), a receptor of Wnt-5a, is frequently expressed on the surface of CLL cells but not in normal somatic tissues (Cui et al., 2016). Since Wnt5a-ROR1 binding on CLL cell surface initiates the non-canonical Wnt-5a pathway that promotes cell proliferation, Cirmtuzumab was developed as a humanized monoclonal antibody to compete with Wnt5a in the binding of ROR1, thus preventing the initiation of the proliferation signal (Pekarsky and Croce, 2019; Yu et al., 2017; Zhou et al., 2017).

2. *BCL-2* and Venetoclax

In 1984 we investigated the t(14;18) chromosomal translocations, the hallmark of follicular lymphoma. These studies led to the discovery and characterization of the B-cell lymphoma 2 gene (*BCL-2*). In follicular lymphoma, the t(14;18) translocation places the *BCL-2* gene from chromosome 18 next to the immunoglobulin heavy chain locus on chromosome 14, prompting to an excessive transcription and expression of *BCL-2* (Tsujimoto et al., 1985; Tsujimoto et al., 1984). The mechanism of action of this oncogene, however, remained unknown until 1988 when a functional study revealed that, in IL-3-dependent haematopoietic progenitor, exogenous expression of Bcl-2 increased cell survival when the essential grow factor was removed. In this experiment, both *BCL-2* overexpressing cells and control cells went into cycle arrest when IL-3 was removed. However, while control cells died within 72 hours, cells overexpressing *BCL-2* did not (Vaux et al., 1988). These evidences indicated that *BCL-2* overexpression supports cancer cell survival by preventing cell death rather than enhancing proliferation. This effect was noted in human cancers as well. For instance, in small low grade breast cancer with positive estrogen receptor/progesterone receptor (ER/PR) status and negative human epidermal growth factor receptor 2 (HER2) status, high expression of *BCL-2* correlates with favorable prognostics (Dawson et al., 2010). However, high expression of *BCL-2* is an independent unfavorable prognostic factor in patients with ER-negative/PR-negative or triple-negative breast cancer (Abdel-Fatah et al., 2013; Honma et al., 2015). Likewise, in prostate cancer, the expression level of Bcl-2 is increased during progression from androgen-dependent to androgen-independent growth stage (Lin et al., 2007; McDonnell et al., 1992). In this context, the survival advantage provided by *BCL-2* overexpression, facilitate the accumulation of additional lesions that may lead to full malignant transformation. *BCL-2* transgenic mice, show accumulation of B lymphoid cells, but the incidence of lymphoma is low and delayed (McDonnell et al., 1989) while Myc transgenic mice develop lymphomas, but only after the 6^{th} week of age and following a period of benign lymphocytosis (Harris et al., 1988; Langdon et al., 1986), double transgenics for Bcl-2 and c-Myc, show a very rapid lymphoma development, caused by the combination of Myc-driven enhanced proliferation, with the Bcl-2-driven apoptosis evasion (Strasser et al., 1990).

To generate an effective anti-Bcl-2 compound is essential to understand its mechanism of action. High expression of *BCL-2* protect cells from the effects of cellular stresses that would otherwise induce apoptosis (Fulda, 2010; Fulda et al., 2010). The mechanism can be summarized as Bcl-2 binding and inactivating Bcl-2-related proteins which would trigger apoptosis by damaging the mitochondrial outer membrane (Kroemer et al., 2007). Bcl-2 related proteins contain highly conserved *BCL-2* homology (BH) domains and are divided in 3 subgroups (Gillies and Kuwana, 2014): (1) Apoptosis initiators, or BH3 proteins, such as Bim, Bid, Puma and Bad, which detect cellular stress and activate apoptosis; (2) Apoptosis effectors, or multi-BH proteins, such as Bax or Bak, which permeabilize the mitochondrial outer membrane; and (3) Antiapoptotic members, such as Bcl-2 Mcl-1, Bcl-xl, Bfl-1/a1, Bcl-w, Bcl-b, which bind and inactivate BH3 and multi-BH proteins to halt apoptosis. This system is designed to finely regulate apoptosis initiation by inducing or inhibiting Bak and Bax in response to cell conditions. Thus, maintaining the correct balance in the expression of all these proteins is essential to ensure apoptosis activation in response to cell damage. The observation that *BCL-2* overexpression prevents apoptosis by binding to BH3-protein, led to the generation of BH3-mimetic compounds that would hinder Bcl-2 to allow apoptosis initiation (Hata et al., 2015). It took over 20 years from our discoveries on *BCL-2*, to the generation of the first anti-Bcl-2 drugs, ABT-737 and ABT-263, developed by the pharmaceutical company Abbott the in the early 2000. Unfortunately, these compounds, would not only inhibit Bcl-2 but also Bcl-xL, which is essential for the survival of platelets, causing serious thrombocytopenia (Croce and Reed, 2016). Thus, a specific Bcl-2-inhibitor, ABT-199 (Venetoclax), was generated in 2013, that showed striking success in causing CLL cell death (Croce and Reed, 2016; Souers et al., 2013). In 2016, 32 years after the discovery of *BCL-2* and 11 years after the elucidation of its microRNA-driven dysregulation mechanism in CLL, FDA approved the use of Venetoclax as a single treatment agent for relapsed/refractory CLL (Croce and Reed, 2016; Stilgenbauer et al., 2018) and in May 2019, Venetoclax was approved as a Chemotherapy-Free combination regimen for previously untreated CLL patients (Fischer et al., 2019). Remarkably, in 2018, the FDA also granted accelerated approval to use Venetoclax in combination with Azacitidine, Decitabine or Cytarabine to treat newly-diagnosed AML patients who are ineligible for chemotherapy (DiNardo et al., 2019).

3. ROR1 and Cirmtuzumab

ROR1 is transmembrane protein within the receptor tyrosine kinase (RTK) family. ROR1 was initially discovered in early 1990 in a neuroblastoma cell line as a cell surface receptor with a key role in the neurite growth in the central nervous system (Reddy et al., 1997). ROR1 gene is highly expressed during early embryonic development, playing an important role in regulating embryonic muscle and skeletal development (Borcherding et al., 2014). During fetal development though, the expression of ROR1 is turned off, thus normal adult cells and tissues do not express this protein. In sharp contrast with the negligible expression of ROR1 in normal adult tissues, significant expression of ROR1 has been detected in several cancers, suggesting that this transmembrane protein could be considered as a tumor-specific cells surface antigen (Zhang et al., 2012). Expression of ROR1 was undoubtedly associated with ovarian cancer stem cells (Zhang et al., 2014) and CLL (Cui et al., 2016) and

it seems to play a functional role in promoting migration/invasion by activating the non-canonical Wnt5a pathway (Hasan et al., 2019). Studies on ROR1 expression in CLL patients showed that only ~10% of CLL patients have leukemic cells (CD19/CD5 double positive) expressing negligible level of ROR1 like normal CD19+ B cells (Rassenti et al., 2017). In ~90% of patients (Rassenti et al., 2017), ROR1 is highly and consistently expressed in malignant cells (~ 97% of CD19/CD5 double positive B-CLL cells show high expression of ROR1 as surface antigen) (Uhrmacher et al., 2011). No significant difference in ROR1 expression on CD19/CD5 double positive cell surface was found when comparing ROR1+ indolent and ROR1+ aggressive patients and its expression on CLL cell surface is not influenced by treatment. Indeed even after 6 cycles of therapy, the residual CD19/CD5 double positive leukemic cells are still ROR1 positive (Uhrmacher et al., 2011). This indicates that ROR1 can be a diagnostic marker not only for initial diagnosis but also as treatment-independent indicator of the clinical stage and minimal residual disease. Thus, in 2016, a humanized mAb specific for ROR1 (UC-961 or Cirmtuzumab) was developed and preclinical studies showed that cirmtuzumab is specific and safe (Choi et al., 2015). Cirmtuzumab is currently being evaluated in clinical trials for therapy in CLL and since ROR1 is broadly expressed in many types of cancer, but not their normal tissue counterparts, breast cancer clinical trials have started to test effectiveness of Cirmtuzumab in combination with chemotherapy agents (Zhang et al., 2019).

4. *miR-15/16* expression in leukemia

In 2000, we investigated the deletion affecting chromosome 13q often observed in patients with chronic lymphocytic leukemia. This genomic region was also found deleted or mutated in other malignancies (Chen et al., 2001; Dong et al., 2001) and thus we believed that it was hosting an important tumor suppressor gene. Surprisingly, in 2002, we discovered that, instead, a cluster of two microRNA genes, *miR-15a* and *miR-16–1*, was the target of 13q deletions in CLL (Calin et al., 2002). Remarkably, functional analysis showed that microRNA are non-coding RNAs capable to bind the 3’ UTRs of mRNAs in a sequence specific fashion (Ambros, 2004) or guide Poly(A)-specific Ribonuclease (PARN) to a target specific mRNA (Gomez-Cambronero et al., 2018), inducing mRNA decay and/or inhibition of translation. Thus, microRNAs were already thought to be involved in cellular processes as negative regulators of gene expression. However, we were the first to demonstrate that an alteration in the non-coding genome was effectively involved in cancer pathogenesis (Calin et al., 2002). Indeed, we proved that the deletion of a microRNA can lead to the aberrant overexpression of important genes by showing, in 2005, that *miR-15/16* function as tumor suppressors in CLL by directly targeting *BCL-2*: the loss of these negative regulators of *BCL-2* expression in 13q- CLL results in inhibition of apoptosis and CLL onset (Cimmino et al., 2005). More recently we discovered that ROR1 is also a target of *miR-15/16* (Rassenti et al., 2017) and we showed that, in patients where ROR1 expression is lower, *miR-15/16* expression is higher. Furthermore, we confirmed that CLL cells expressing high levels of ROR1 also expressed high levels of *BCL-2* (Rassenti et al., 2017). Several reports revealed that loss of *miR-15a/16–1* expression is a hallmark of almost all CLL cases: about 55% of CLL cases lose *miR-15a/16–1* expression as a consequence of the chromosomal deletion at 13q14 detectable by FISH analysis (Döhner et al., 2000); about 15% of CLL cases that do

not show such chromosomal abnormality, carry smaller deletions (not FISH detectable) or mutations in the *miR-15a/16–1* genomic region (Calin et al., 2005); lastly we proved that p53 is a positive activator of *miR-15/16*, and since p53 is deleted/mutated in 7–10% of CLL samples, loss of p53 may lead to down-regulation of *miR-15/16* (Fabbri et al., 2011). Thus, up to 80–90% of CLL cases show a low expression of *miR-15/16* (Rassenti et al., 2017). This evidences are consistent with the observation that ~90% of CLL patients bear leukemic cells showing a high expression of ROR1. All of these striking results indicate that, in CLL, lack of *miR-15/16* cells causes a combination of Bcl-2-driven apoptosis evasion and ROR1-driven enhanced proliferation that lead to cancer development and progression. Since, in this scenario, the selection of a *BCL-2* or a ROR1 mutant clone resistant to monotherapy is a possible event, we believe that CLL patients should be treated with a combination therapy of Venetoclax and Cirmtuzumab, to elicit enhanced anticancer effect and protect from selection of resistant subclones. Indeed this formulation would target two cancer drivers upregulated as a consequence of the same alteration: *miR-15/16* loss. In support of this idea, we proved that Cirmtuzumab could enhance the in vitro cytotoxicity of Venetoclax for CLL cells with high-level ROR1 (Rassenti et al., 2017). Since *BCL2* and ROR1 dysregulation has been observed also in other malignancies such as breast, prostate and ovarian cancer (Abdel-Fatah et al., 2013; Bonci et al., 2008; Lagadinou et al., 2013; Lin et al., 2007; Zhang et al., 2014), we believe that this formulation would be effective for treatment of several other types of cancers in reducing the possibility of resistance/relapse to virtually zero.

Interestingly, an additional locus of *miR-15/16* exists on chromosome 3 (3q25), encoding for *miR-15b/16–2*. Thus, in 2015 we generated a knocked out mouse model to study its role in lymphomagenesis. These mice developed mainly CLL and few diffuse large B cell lymphoma at higher penetrance and earlier age than the *miR-15a/16–1* KO mice described by Klein et al (Klein et al., 2010; Lovat et al., 2015). In humans, *mir-16–1* and *miR16–2* mature sequences are exactly the same while *miR15a* and *miR15b* have slightly different sequences but maintain the same “seed region” responsible for mRNA targeting, thus we were able to investigate the expression of *miR-15/16* locus on 3q25 in CLL, by evaluating the expression of *miR-15b* in B-CLL cells from 13q- and 11q-deleted patients (Lovat et al., 2015). We compared these results with *miR-15b* expression in normal CD19+ B cells: *mir-15b* is downregulated 1.6 folds in 13q-CLL vs normal CD19+ B cells and 3.4-folds in 11q-CLL vs normal CD19+ B cells. Additional analysis revealed that in 13q− CLLs patients, the lack of *miR-15a/16–1* cluster seem to be compensated by modulating the expression of *pri-miR-15b* and *miR-15b*, suggesting a possible level of miRNA “regulation” that warrant further study. Following these experiments, we generated a double knocked out mouse where both *miR-15/16* loci were deleted (Lovat et al., 2018). Surprisingly, these mice developed an aggressive AML (77%) and, more infrequently, B cell lymphomas (23%). This was an unexpected exciting result, indicating that the combined loss of *miR-15a*, *miR-15b*, and *miR-16* and overexpression of their targets starting from animal development lead to acute myeloid leukemia. Remarkably, previous studies suggested that, in AML patients, *BCL-2* is overexpressed in Leukemia Stem Cells (LSCs) (Lagadinou et al., 2013), and showed that overexpression of Bcl-2 or Mcl-1 is associated to chemoresistance, failure to achieve complete remission and relapse (Ricciardi et al., 2017). Our *miR-15/16* double KO AML mouse model suggests that *BCL-2* overexpression in LSCs is very likely induced by

the absence of *miR-15/16* which could also induce ROR1 expression as well. Thus, since relapse in AML is typically caused by the development of treatment-resistant clones derived from LSCs, a Venetoclax-Cirmtuzumab combination treatment could be successful in targeting LSCs, and prevent the occurrence of drug resistance development and relapse.

5. Future perspectives and concluding remarks: identification of novel mechanism of gene expression dysregulation in cancer, efficient stratification of patient and multi-target combination therapy

In this review, we discussed the role of microRNA dysregulation in cancer development, and its involvement in the identification of genes that can be targets for novel drugs. It is important to highlight that the dysregulation of other small non-coding RNAs may affect gene expression regulation in cancer as well (Pekarsky and Croce, 2019). For instance, our most recent studies showed that a novel class of small non-coding RNAs generated through the processing steps of tRNA molecules and named tsRNAs, can inhibit key genes in the development of CLL and possibly other cancers (Balatti et al., 2017a; Balatti et al., 2017b; Balatti et al., 2015; Pekarsky et al., 2016). The mechanism of action of tsRNAs is still under investigation but they seem to be able to affect gene expression both pre and post transcriptionally (Pekarsky et al., 2016). These molecules could represent additional markers for diagnosis and their study could lead to the identification to novel targets for therapy, as previously done for microRNAs.

MiR-15a/16–1 deletion on chromosome 13q14 is a driver event for the development of CLL since it leads to the overexpression of *BCL-2* and of ROR1, an anti-apoptotic gene and an initiator of proliferation signals, respectively (Cimmino et al., 2005; Rassenti et al., 2017). Thus, the discovery of *miR-15/16* dysregulation in CLL provided the basis for development of Venetoclax and Cirmtuzumab. Remarkably, in the last decade, many other types of cancers were shown to be ROR1 positive (Zhang et al., 2012) and to overexpress *BCL-2 (Abdel-Fatah et al., 2013; Lin et al., 2007)*, while the expression of *miR-15/16* is still under investigation. Furthermore, the mouse model where the additional locus for *miR-15/16* on chromosome 3q25 (*miR-15b/16–2*) is knocked out along with the *MiR-15a/16–1* locus, develops for the most part Acute Myeloid Leukemia (Lovat et al., 2018). Based on these observation, the study of genetic alterations involving *miR-15/16* in CLL-B cells from CLL patients and LSC from AML patients may represent a valuable tool to stratify patients for more effective therapy. *MiR-15/16* main target genes, *BCL-2* and ROR1 should be therapeutically pursued (Pekarsky et al., 2018) in patients where *miR-15/16* are downregulated. Indeed, a combination therapy of Venetoclax and Cirmtuzumab would be quite efficient because it would target both drivers upregulated as a consequence of the same alteration: *miR-15/16* loss. Additionally, both drugs show very modest side effects. Lastly, it is important to notice that the identification of *miR-15/16* as the cause of the dysregulation of these two druggable targets, also offers the opportunity to identify additional targets for the development of new compounds.

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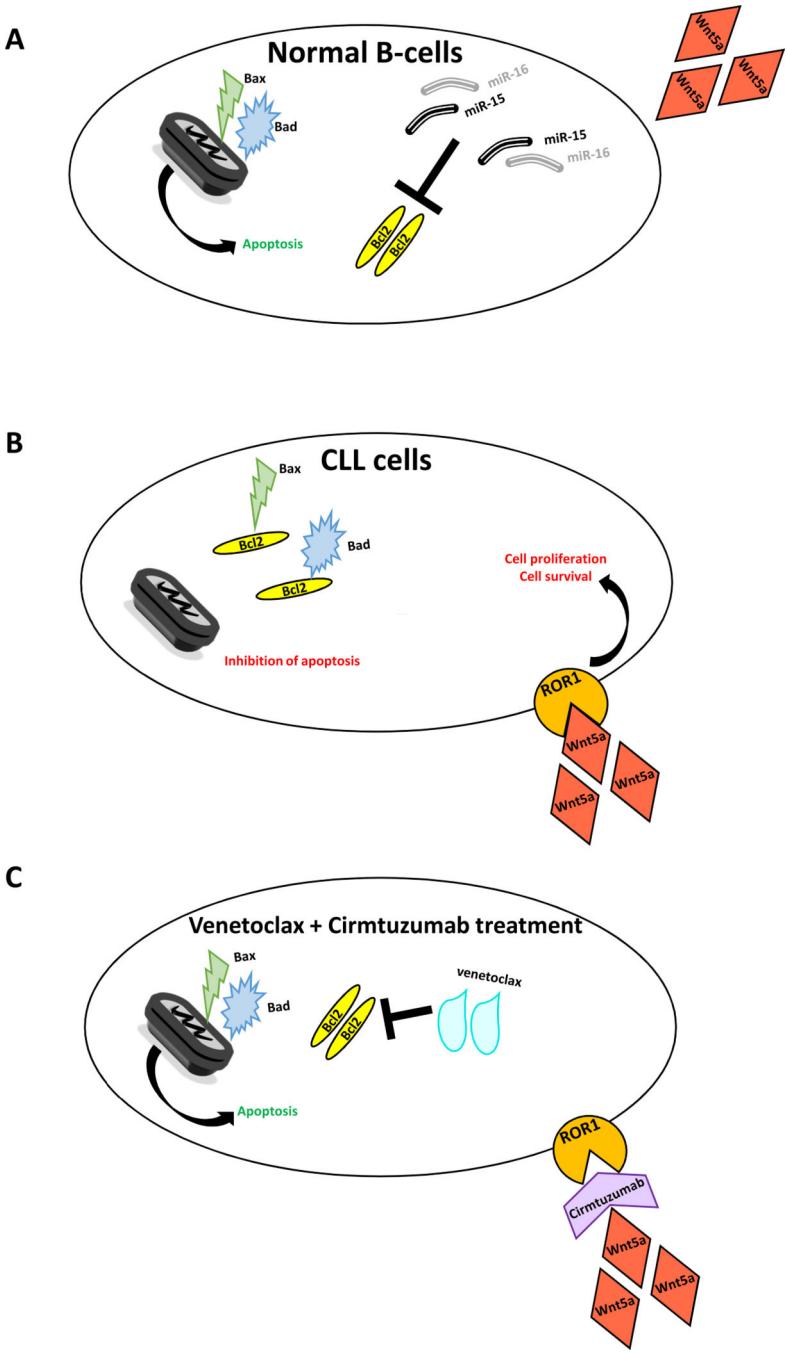

Figure 1.

In normal B cells, *Mir-15/16* target Bcl2. BAC and BAD can activate apoptosis. ROR1 is not expressed thus Wnt5a cannot activate the proliferation pathway (A). In leukemic cells, lack of *miR-15/16* is the cause of Bcl2 overexpression that leads to apoptosis inhibition. ROR1 is expressed in the surface of leukemic cells thus wnt5a activates the proliferation pathway (B). Therapeutic implication of combined treatment with Venetoclax, that targets Bcl2 and

Cirmtuzumab that binds to ROR1: the antiapoptotic pathway is initiated and the cell proliferation pathway is inhibited (C).