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Characterization Of Tcl1-Tg:P53-/- Mice That Resemble Human Chronic Lymphocytic Leukemia With 17P-Deletion

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CHARACTERIZATION OF *TCL1-Tg:p53*$^{-/-}$ MICE THAT RESEMBLE HUMAN CHRONIC LYMPHOCYTIC LEUKEMIA WITH 17P-DELETION

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CHARACTERIZATION OF *TCL1-Tg:p53*$^{-/-}$ MICE THAT RESEMBLE HUMAN CHRONIC LYMPHOCYTIC LEUKEMIA WITH 17P-DELETION

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Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the United States and Europe. CLL patients with deletion of chromosome 17p, where the tumor suppressor p53 gene is located, often develop a more aggressive disease with poor clinical outcomes. However, the underlying mechanism remains unclear. In order to understand the underneath mechanism *in vivo*, I have recently generated mice with E_u-*TCL1*-Tg:*p53*$^{-/-}$ genotype and showed that these mice develop aggressive leukemia that resembles human CLL with 17p deletion. The E_u-*TCL1*-Tg:*p53*$^{-/-}$ mice developed CLL disease at 3-4 months, significantly earlier than the parental E_u-*TCL1*-Tg mice that developed CLL disease at 8-12 months. Flow cytometry analysis showed that the $CD5^+/IgM^+$ cell population appeared in the peritoneal cavity, bone marrow, and the spleens of E_u-*TCL1*-Tg:*p53*$^{-/-}$ mice significantly earlier than that of the parental E_u-*TCL1*-Tg mice. Massive infiltration and accumulation of leukemia cells were found in the spleen and peritoneal cavity of E_u-*TCL1*-Tg:*p53*$^{-/-}$ mice. *In vitro* study showed that the leukemia cells isolated from the E_u-*TCL1*-Tg:*p53*$^{-/-}$ mice were more resistant to fludarabine treatment than the leukemia cells isolated from spleens of E_u-*TCL1*-Tg mice. Interestingly, TUNEL assay revealed that there was higher apoptotic cell death found in the E_u-*TCL1*-Tg spleen tissue compared to the spleens of the E_u-*TCL1*-Tg:*p53*$^{-}$

$^{/-}$ mice, suggesting that the loss of p53 compromises the apoptotic process *in vivo*, and this might in part explain the drug resistant phenotype of CLL cells with 17p-deletion. In the present study, we further demonstrated that the p53 deficiency in the *TCL1* transgenic mice resulted in significant down-regulation of microRNAs miR-15a and miR16-1, associated with a substantial up-regulation of Mcl-1, suggesting that the p53-miR15a/16-Mcl-1 axis may play an important role in CLL pathogenesis. Interestingly, we also found that loss of p53 resulted in a significant decrease in expression of the miR-30 family especially miR-30d in leukemia lymphocytes from the E_u-*TCL1*-Tg:*p53*$^{-/-}$ mice. Such down-regulation of those microRNAs and up-regulation of Mcl-1 were also found in primary leukemia cells from CLL patients with 17p deletion. To further examine the biological significance of the decrease in the miR-30 family in CLL, we investigated the potential involvement of EZH2 (enhancer of zeste homolog 2), a component of the Polycomb repressive complex known to be a downstream target of miR-30d and plays a role in disease progression in several solid cancers. RT-PCR and western blot analyses showed that both EZH2 mRNA transcript and protein levels were significantly increased in the lymphocytes of E_u-*TCL1*-Tg:*p53*$^{-/-}$ mice relative to E_u-*TCL1*-Tg mice. Exposure of leukemia cells isolated from E_u-*TCL1*-Tg:*p53*$^{-/-}$ mice to the EZH2 inhibitor 3-deazaneplanocin (DZNep) led to induction of apoptosis, suggesting EZH2 may play a role in promoting CLL cell survival and this may contribute to the aggressive phenotype of CLL with loss of p53. Our study reveals that p53-miR15a/16-Mcl-1 axis & p53-miR30-EZH2 axis may contribute to the CLL pathogenesis, and EZH2 may be a potential target for CLL treatment.

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1. INTRODUCTION

1.1 Cellular origin(s) of chronic lymphocytic leukemia. Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world and accounts for about 30% of all adult leukemia cases. CLL is a disease of elderly people resulting from an accumulation of mature-looking neoplastic $CD5^+$ B lymphocytes in primary & secondary lymphoid tissues, and CLL cells express CD19, CD23 and CD20 as well (1-3). Despite the fact that over past years several cell types have been suggested as giving rise to CLL, the cellular origin of CLL is still unclear (4). The belief that the cellular origin of CLL is from follicular mantle B cells due to shared surface membrane expression of CD5 and CD23 was challenged by the DNA sequencing result showing that approximately 50% CLL cases have *IGHV* mutations, whereas follicular mantle B cells almost have unmutated *IGHV*s (5, 6). Another belief that CLL clones with either mutated or unmutated *IGHV*s derived from marginal zone (MZ) B cells defined as $IgM^{high}IgD^{low}$ cells as most CLL clones also faces difficulties because MZ B cells are $CD5^-CD23^-CD22^+$ differing from CLL cells (7, 8). A single-cell origin of CLL was challenged by the evidence for remarkably similar B-cell receptor (BCR) amino acid sequence and striking differences in poly-antigen and autoantigen-binding activity found in some CLL clones (9). The feature of CLL clones using either mutated or unmutated *IGHV* genes (M-CLL and U-CLL) gave rise to the hypothesis that 2 subgroups of CLL originated from distinct cell types considered as a 2-cell origin model (10, 11). However, the 2-cell origin model exhibited difficulties resulting from gene expression

profiling which revealed that only a relatively small number of genes have differences between U-CLL and M-CLL , whereas there are thousands of differences between normal B lymphocytes and either U-CLL or M-CLL (12). Therefore difference in cellular features between U-CLL and M-CLL could be explained by a sing-cell derivation with additional and nongenetic promoting factors. Regarding normal counterparts of CLL, leukemogenesis is at least a multistep process. In 2011, possibilities for single- and multiple-cell origin models offered by some researchers partially answered the question whether a single- or multiple normal counterparts of CLL were stimulated to evolve into CLL (9).

1.2 Genetic abnormalities in CLL. Fluorescence in *situ* hybridization (FISH) analysis has revealed that up to 80% of CLL cases exhibited chromosomal abnormalities such as deletions of 13q14 in 55% CLL cases, 11q deletion in 12%, 17p deletion in 8% and trisomy of chromosome 12 in 15% of CLL cases (13, 14). Among the chromosomal abnormalities, deletions at 13q14 are the most frequent chromosomal abnormalities and present in more than half of CLL cases. Some of the cytogenetic changes are associated with poor prognosis and aggressive disease progression. Chromosome 17p deletion (17p-) is the most recognized cytogenic alteration in CLL associated with aggressive disease progression, resistance to chemotherapy, and poor clinical outcome (15). Since the tumor suppressor p53 gene is located in human chromosome 17p (16), it is suspected that the loss of p53 function in CLL cells with 17p- may be responsible for the poor prognosis of this subgroup of CLL patients (17, 18).

Initially, TP53 was considered as an oncogene. However, since 1989 it has been found that p53 functions as a tumor suppressor and is frequently mutated in human cancers (19-21). More than half of human tumors have a p53 deletion or mutation (22). What is the p53 status in CLL? Mutations of TP53 are found in 4% to 37% of patients with CLL (23). Patients with fludarabine-refractory CLL have the highest incidence of TP53 mutation (24). Interestingly, recent study suggested that a very high concordance (over 70%) in 17p-deletion and mutations in the remaining p53 allele (25). Furthermore, p53 dysfunction may also arise via alternative mechanisms such as functional inactivation, which may explain certain CLL with poor prognosis but without apparent structural changes in p53 gene such as 17p-deletion or mutations (26). Thus, it is clear that the loss of p53 function has profound effect on the CLL disease progress and treatment outcomes. However, the underlying mechanisms remain to be elucidated.

1.3 CLL microenvironment. The microenvironment, a complication of accessory cells that within individual organs, provides growth factors, nutrients and immune tolerance for the survival and propagation of malignant cells in solid tumors through cell-cell contact and active molecular crosstalk (27). In CLL, the interactions between the malignant cells and the microenvironment *in vivo* provide the proliferative drive for the malignant cells through the external signals from the microenvironment such as antigens, cytokines and cell-cell contact. *In vitro*, CLL cells undergo spontaneous apoptosis, suggesting that CLL cell survival depends on microenvironment signals (28, 29). There is

growing evidence suggesting that CLL cells are protected from spontaneous apoptosis and conventional drug *in vitro* by various stromal cells including mesenchymal marrow stromal cells (MSCs) (30, 31), monocyte-derived nurselike cells (NLCs) (30), and follicular dendritic cells (32) through co-culture that partially mimics the CLL microenvironment. Interactions between CLL cells and MSCs provide CLL survival and drug resistance by promoting CLL cell migration which depends on CXCR4 and VLA-4 expression by CLL cells (33). There are several common stromal cell lines used for protecting CLL cells through co-culture, including human stromal cell lines HS5 & NK Tert and mouse stromal cell line KUSA-H1. NLCs protect CLL cells from spontaneous apoptosis and conventional drugs through secreting CXCL12 (30), CXCL13 (34), B cell-activating factor of the tumor necrosis factor (BAFF), CD31 and plexin-B (35). T cells play several roles in CLL. In one hand, some subsets of T cells may overcome the antitumor effect derived from other T-cell subsets and favor disease progression. In the other hand, the significant immune deficiency, which is typical in CLL, resulted from T-cell abnormalities (36, 37). The overall number of circulating T cells stimulate CLL cell growth and survival by secreting interleukin-4 (IL-4) and tumor necrosis factor-α (TNF- α) (27).

1.4 CLL treatment. CLL treatment includes chemotherapy with agents such as fludarabine, chlorambucil, and bendamustine (38). Fludarabine, a purine analog, generated a significant improvement in overall responses compared with chlorambucil, an alkylating agent, in the CLL treatment (39). Up to 37% of untreated patients with CLL do not respond to fludarabine treatment and up to

76% of patients with CLL become refractory to the fludarabine treatment (40). Fludarabine and chadribine in combination with cyclophosphamide are equally effective for progressive CLL. However, both combinations are not effective in patients with 17p13 deletion (41). Bendamustine agent, a cytotoxic hybrid of an alkylating agent and a purine analog, has improved overall response rates (42). The major issue for the purine analog therapy is lack of response in patients with 17p- CLL. Histone deacetylase inhibitors (HDACIs) can induce acetylation of histone and nonhistone proteins (43). The HDACI, valproate (VPA), synergizes with purine analogues to induce apoptosis in CLL cells (44). β-Phenylethyl isothiocyanate (PEITC), which is a natural compound found in cruciferous vegetables, effectively eliminates fludarabine-resistant CLL cells through ROS accumulation and glutathione depletion (45). It could be possible that PEITC only or PEITC combined with other chemotherapy agents will take a big step in the CLL treatment. Recent studies have shown that the compound PEITC overcomes resistance to HDACI in human leukemia through redox modulation (46). Despite the improvement of chemotherapy in CLL, relapse is frequent. Rituximab (anti-CD20), the first approved therapeutic antibody in cancer treatment, has been used in refractory CLL (47). Chemoimmunotherapy improves overall therapy in patients with CLL and alemtuzumab (anti-CD52) based chemoimmnotheray has improved responses in relapsed/refractory disease (38, 48). Recently protein kinases have been considered as therapeutic targets in CLL due to the fact that an imbalanced functional response of the B-cell receptor (BCR) signaling axis results in the deregulation of gene expression

in CLL. Spleen tyrosine kinase (SYK) is critical for B-cell development and is essential for the survival and maintenance of malignant B cells (49, 50). SYK inhibition in human CLL cells led to the downregulation of Erk, Akt, and Mcl-1, demonstrating that SYK regulates CLL survival (51-53). Bruton tyrosine kinase (BTK) plays an essential role in B-cell signaling and development. BTK are overexpressed in CLL lymphocytes as compared with normal B lymphocytes at both protein and mRNA levels (54). Ibrutinib, a specific inhibitor of BTK, can disrupt tumor microenvironment interactions, inhibit cellular migration and adhesion and induce apoptosis in malignant B-cells (55). Despite those significant improvements and potential therapeutic strategies in clinic, CLL still remains an incurable disease. Novel therapeutic agents targeting different signaling pathways will bring a new way for the future treatment of CLL and fludarabine-resistant/refractory CLL.

1.5 Treatment resistance in CLL-the role of the p53 pathway. Despite the improvements of chemotherapy strategies by novel therapeutic agents or their combinations in the CLL treatment, relapse is frequent. The appearance of effective first-line chemoimmunotherapy such as retuximab in CLL improved the response rates for treatment-refractory patients. Genetic abnormalities such as 13q14 deletion, un-mutated *IGHV* gene status, 11q deletion, particularly 17p deletion seem to be more important in determining the treatment outcome than initial treatment choice (56-58). CLL patients with 17p deletion have been shown to poorly respond to conventional chemotherapies such as fludarabine (57), probably because that the remaining allele contains a TP53 mutation or

deletion in the most cases. Are CLL patients with TP53 mutation in the absence of 17p deletion resistant to chemotherapy? The answer is positive. TP53 mutation without 17p deletion corresponds to the development of chemotherapy-resistant disease and is associated with poor survival (59). More than half of human tumors have a p53 deletion or mutation (22). Mutations of TP53 are found in 4% to 37% of patients with CLL (23). Patients with fludarabine-refractory CLL have the highest incidence of TP53 mutation (24). Taking together, p53 seems to play a central role in the drug resistance of CLL.

1.6 Animal models that resemble human CLL and underlying mechanisms. Animal model tools are important to investigate disease processes and associated pathological mechanisms *in vivo*. Currently there are several CLL mouse models, which include the E_{μ}-*TCL1* transgenic (*TCL1*-Tg) mice (60), the April transgenic mice (61), the TRAF2DN/bcl2 transgenic mice (62), the miR-155 mouse model (63), the NZB mouse model with miR-16 alteration (64), and the miR-29 transgenic mice (65), and so on. The *TCL1*-Tg mouse model, which was created by the insertion of the human *TCL1* gene under the control of the immunoglobulin heavy chain variable region promoter and immunoglobulin heavy chain enhancer, represents a well characterized mouse model that develops leukemia resembling human CLL (60). Why TCL1 causes leukemia? TCL1 physically interacts with Akt through PH domain of Akt. This interaction enhances Akt kinase activity and promotes Akt nuclear translocation (66). It has been reported that transgenic mice expressing constitutively activated Akt in T cells develop T cell leukemia (67), whereas transgenic mice

expressed constitutively activated Akt in B cells do not develop B cell leukemia (68). Those reports suggest that Akt activation causes leukemia in T cells initiated by TCL1. However TCL1 deregulation in B cells causes CLL not through Akt activation. Overexpression of TCL1 in B cells causes CLL by enhancing NF-κB activity and inhibiting AP-1 (69). The *TCL1*-Tg mice develop human CLL-like disease at 8 to 12 months of age and exhibit features of human CLL with expanded $IgM^{+}CD5^{+}$ CLL population in peritoneal cavity (PC), spleen and bone marrow (60). The CLL cells isolated from *TCL1*-Tg mice undergo rapid cell turnover with high levels of proliferation and apoptosis (70). *TCL1*X*BAFF*-Tg mice generated by crossing *TCL1*-Tg with *BAFF*-Tg mice, which express high levels of CD257, develop CLL-like disease around 4.5 months of age with more rapid disease progression and shorter survival that *TCL1*-Tg mice (70). BAFF and APRIL are recent members of TNF superfamily and show increased expression levels in various B cell malignancies. BAFF and APRIL bind to two receptors BCMA & TACI and stimulate NFκB pathway through interactions with TRAFs, contributing to CLL pathogenesis in *TCL1*X*BAFF*-Tg mice and April transgenic mice (71). APRIL transgenic mice had increased white blood cell count and did not develop any hematopoietic malignancy probably due to lack of second hit such as TCL1 overexpression (61). It has been reported that miR-29 is upregulated in indolent human CLL with low ZAP-70 expression and mutated *IGHV* (72). MicroRNAs are regulatory non-coding RNAs with 20 to 25 nucleotides in length. The primary function of microRNAs is to target specific messenger RNA for degradation or

inhibition of translation, leading to downregulation of the target proteins (73, 74). Recent studies have shown that the regulatory functions of microRNAs are involved in various cellular processes including development, differentiation, apoptosis, survival and metabolism (75, 76). It has been reported that microRNA signatures are associated with CLL progression and prognosis (77, 78). Desregulation of some microRNAs have been viewed as a contributing factor for CLL apoptotic defect. For example, upregulated Bcl-2 resulted from downregulation of miR15a/16-1 (79). In 2006, Dr. Croce's group reported that E_{μ}-miR155 transgenic mice develop a B cell malignancy with a preleukemic pre-B cell proliferation (63). In 2010, Dr. Croce's group also reported that E_{μ}-miR-29 transgenic mice develop a disease that resembles human indolent CLL phenotype probably through targeting TCL1 and Mcl-1 by miR-29 (80, 81). Previous studies have shown that over-expression of Bcl-2 family members occur in many cases of CLL and this is correlated with resistance to therapy and a poor prognosis (82). A constitutive increase of Bcl-2 through a deletion of miR-15a/16-1 cluster contributes to CLL pathogenesis (62). Bcl-2 transgenic mice showed polyclonal expansion of B cells and increased cell survival without developing any tumor (83). In contrast, most of TRAF2DN/Bcl2 double transgenic mice develop B cell leukemia resembling human CLL with severe splenomegaly and significant high number of white blood cells (62). As mentioned before, TRAF2, a TNF receptor associated factor 2, interacts with TNF receptor family members such as BCMA and activates NF-κB and JNK signaling pathways (84). Although Bcl-2 or TRAF2 single transgenic mice

failed to develop any hematological malignancy, the TRAF2DN/Bcl2 double transgenic mice had rapid disease progression and developed human CLL-like disease over time and died at 6 to 14 months of age. NF-κB activation and overexpression of anti-apoptotic Bcl-2 could contribute to the disease progression in the TRAF2DN/Bcl2 double transgenic mice. Among the anti-apoptotic members of Bcl-2 family, the myeloid cell leukemia-1 (Mcl-1) has been demonstrated as an important anti-apoptotic protein in CLL both *in vitro* and *in vivo* (85). It has been shown that Mcl-1 promotes CLL cell survival by inhibiting the intrinsic Bak/Bax-mediated apoptotic pathway (86). Loss of p53 function in cancer cells has also been associated with decrease in apoptotic response and drug resistance (59), and mice with p*53*$^{-/-}$ genotype are highly susceptible to the development of a variety of tumors (87). However, currently it is unclear if there is a link between the loss of p53 and over-expression of Mcl-1 in CLL cells. As mentioned before, there is no doubt that the loss of p53 function has profound effect on the CLL disease progress and treatment outcomes. However, there is no any animal model available for studying the effect of p53 deletion in CLL biology and pathogenesis. *TCL1*-Tg mouse model closely recapitulates human CLL disease. This mouse model can not be used to study CLL with p53 gene deletion. To generate an animal model that closely resembles human CLL patients with p53 deletion, we generated a mouse colony with *TCL1* transgenic and p53-deletion (*TCL1*-Tg:*p53*$^{-/-}$) genotype by crossing the *TCL1*-Tg mice with *p53*$^{-/-}$ mice. We hypothesized that loss of p53 causes rapid disease progression, treatment resistance and shorter survival time in

TCL1-Tg mice by modulating CLL cell proliferation and apoptosis. Our preliminary data showed that the *TCL1*-Tg:$p53^{-/-}$ mice develop leukemia that resembles human aggressive CLL disease around 3-4 months. The leukemia cells from *TCL1*-Tg:$p53^{-/-}$ mice exhibited higher proliferation, higher survival capacity, and more resistant to drug treatment with fludarabine than the leukemia cells from the *TCL1* transgenic mice with wild-type p53. We further demonstrated that the loss of p53 led to a significant increase of Mcl-1 expression, likely through the expression of miR-15a and miR-16-1 expression. Our microRNA array data showed significant downregulation of miR15a/16-1, miR-30a, miR-30d and miR-30e, especially miR-30d, in both human and mouse CLL cells with p53 deficiency, and such downregulation of those microRNAs were confirmed by RT-PCR. As mentioned before, mir-15a and miR-16-1, located at 13q14, were deleted or downregulated in approximately 66% CLL cases (88). Recent studies have shown that the miR15a/16-1 cluster function as tumor suppressor by targeting Bcl-2, Mcl-1, CCND1 and WNT3A (89). The association between the loss of p53, the decrease in miR15a/16-1 and the increase in Mcl-1 was further confirmed in primary leukemia cells from CLL patients with chromosome 17p deletion. The role of miR30 family in CLL is still unknown. It has been reported that miR-30d targets the polycomb protein enhancer of zeste 2 (EZH2) which is involved in repressing gene expression through methylation of histone H3 on lysine 27 and upregulated in anaplastic thyroid carcinomas (90). Over years, many studies have established that EZH2 is overexpressed in various cancers including some hematologic malignancies,

and such overexpression is associated with aggressiveness and progression (91, 92). DZNep, an S-adenosylhomocysteine hydrolase inhibitor, induce apoptosis in cancer cells such as AML through inhibiting S-adenosyl-L-methionine-dependent methyltransferases such as EZH2 (93). A recent report showed a therapeutic strategy for lymphoma with EZH2-activating mutations through EZH2 inhibition (94). The role of EZH2 in CLL has yet to be examined *in vitro* and *in vivo*. Another target of miR-30 is B-Myb, which expression can be regulated by miR-30 and miR-29 during cellular senescence (95). B-Myb, a transcription factor, is involved in cell proliferation and transcription and carcinogenesis. B-Myb overexpression presents in various cancers and is associated with aggressive tumor growth and poor outcomes (96-98). However, the role of B-Myb in CLL is still unknown. This study provides *in vivo* evidence to support that p53→miR15a/16→Mcl-1 & p53→miR30d→ EZH2 & B-Myb axis may contribute to the pathogenesis of aggressive CLL.

2. SPECIFIC AIMS

Chronic Lymphocytic Leukemia (CLL) is the most common leukemia in Western countries, and the disease is very heterogeneous in disease progression and response to drug treatment. Genetic aberrations such as chromosome deletion or gene mutation are frequently observed in CLL (99). A subtype of CLL with chromosome 17p deletion is associated with accelerated disease progression, drug resistance, and poor prognosis due to loss of p53 gene (100).

The subtype of CLL patients with 17p deletion or with TP53 mutations have much shorter survival time than other CLL patients without 17p deletion and TP53 mutations (101) . To develop efficient therapeutics for the CLL subtype with 17p deletion or TP53 mutations, it is critical to understand how exactly loss of p53 contributes to CLL disease progression and drug resistance. MicroRNAs (miRNAs), a group of short non-coding RNA molecules, regulate target gene expression via translational repression or transcriptional degradation (74). Several miRNAs such as miR-15a, miR-16 and miR-34a have been implicated in CLL pathogenesis and prognosis (102, 103). Interestingly, p53 can upregulate miR-34 gene and miR-34 overexpression in turn induces senescence, apoptosis or cell cycle arrest by regulating proteins such as Bcl-2, Cyclin D1, CDK4 and c-MYC etc (104). Moreover, deregulated miRNA expression also mediates drug resistance in CLL patients with 17p deletion (105-109). Overall, p53 and miRNAs have been closely linked in CLL pathogenesis and prognosis. However, it remains unclear how loss of p53 affects miRNAs and alters the regulatory function of miRNAs in CLL. As mentioned before, the *TCL1*-Tg mice develop human CLL-like disease at 8 to 12 months of age with expansion of $IgM^+/CD5^+$ CLL population in PC, spleen and bone marrow (60). However, this *TCL1*-Tg mouse model can not be used to study the effect of p53 deficiency in CLL pathogenesis and microRNA deregulation. Therefore, we hypothesized that loss of p53 causes rapid disease progression and shorter survival time in *TCL1*-Tg mice by modulating CLL cell proliferation and apoptosis. To address this hypothesis, the *TCL1*-Tg:$p53^{-/-}$

mouse model is generated by our group. This model is expected to be a good research model for providing potential therapeutic strategies and novel chemotherapies in the treatment of CLL subtype with 17p deletion, and for studying CLL biology and pathogenesis. It is also anticipated that the proposed studies will bring new insights into the relationship between loss of p53 and microRNA deregulation in CLL. In the proposed studies, 3 specific aims will be accomplished to address the hypothesis.

Specific Aim 1: Generation and characterization of *TCL1*-Tg:*p53*$^{-/-}$ mice.

General strategy. *TCL1*-Tg homozygous mice (B6C3 mice) were provided by Dr. Carlo M. Croce. They are crossed with *p53*$^{-/-}$ mice (B6C3) which were from Jennifer Alana (a microinjection specialist in MDACC). Then the second generation of *TCL1*-Tg:*p53*$^{+/-}$ mice were intercrossed to get *TCL1*-Tg:*p53*$^{-/-}$ CLL mouse model. Afterwards, *TCL1*-Tg:*p53*$^{-/-}$ mice were intercrossed with each other to obtain pure *TCL1*-Tg:*p53*$^{-/-}$ mice for research studies (Figure 1). All mice were housed under conventional barrier protection in accordance with University of Texas MD Anderson Cancer Center guidelines, and mouse protocols were approved by University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Validation of p53 deletion and TCL1 overexpression in *TCL1*-Tg:*p53*$^{-/-}$ mice. Prior to any phenotypic analysis, the mice are genotyped for *TCL1* transgene and p53 mutant alleles. Furthermore, to confirm the inactivation of p53 protein expression, Western blot analysis of p53 will be performed using

splenocytes and peritoneal cavity cells from mice with respective genotypes. The *TCL1*-Tg mice don't develop CLL disease until 13 months old, and $p53^{-/-}$ mice may develop tumors at 6 months old. Most of *TCL1*-Tg:$p53^{-/-}$ are fertile and we don't expect any embryonic lethality or pup survival problem of these mice. Indeed, we have obtained the *TCL1*-Tg:$p53^{-/-}$ mice in the laboratory.

Characterization of CLL phenotype of *TCL1*-Tg:$p53^{-/-}$ mice. The *TCL1*-Tg mice develop CLL disease featured by $CD5^+$ B cell accumulation in bone marrow, spleen, and PC. To determine the percentage of $CD5^+$ B cells at different ages, I will isolate cells from bone marrow, spleen, and peritoneal cavity of mice with difference genotypes at 2 month, 4months, 5 months, 7 months of age, and perform FACS analysis using anti-CD5 and anti-IgM antibodies. Meanwhile, blood smear will be performed to monitor white blood cell expansion in these mice at different ages. To further characterize the phenotype of the *TCL1*-Tg:$p53^{-/-}$ mice, Hematoxylin and eosin (H&E) staining of mouse tissues such as spleen and liver will be performed. Mouse survival will be monitored for three groups of mice for up to 2 years and each group contains more than 20 mice.

Specific Aim 2: Determine the effect of p53 deficiency on proliferation and apoptosis of splenocytes and investigate underlying mechanisms.

Determine p53 deficiency on proliferation and apoptosis of splenocytes. For years, CLL has been viewed as a tumor with low level of cell turn over. Consistently anti-apoptotic Bcl-2 family members are overexpressed in most CLL cases (82). Recently it was found that CLL cells from *TCL1*-Tg mice

undergo rapid cell turnover that can be offset by extrinsic CD257 to favor disease progression (70). We expect that p53 deficiency will accelerate CLL disease progression in *TCL1*-Tg mice by modulating cell proliferation and apoptosis. One of the complications of CLL is an enlarged spleen called splenomegaly. To determine the effect of p53 deficiency on cell proliferation, Bromodeoxyuridine (*BrdU*), a synthetic thymidine analog, incorporation assay will be performed for splenocytes isolated from *p53*$^{-/-}$ mice, *TCL1*-Tg mice, *TCL1*-Tg:*p53*$^{+/-}$ and *TCL1*-Tg:*p53*$^{-/-}$ mice. To further confirm the proliferation result, Ki67 (a proliferation marker) staining of spleen isolated from different types of mice will be performed. To determine the effect of p53 deficiency on apoptosis of splenocytes, Annexin V/Propidium Iodide (PI) staining will be performed for splenocytes isolated from p*53*$^{-/-}$ mice, *TCL1*-Tg mice, *TCL1*-Tg:*p53*$^{+/-}$ and *TCL1*-Tg:*p53*$^{-/-}$ mice at 0, 24h and 72h after isolation. Terminal deoxynucleotidyl transferase dUTP nick end labeling (*TUNEL*) assay will be performed on spleen tissues isolated from different types of mice to confirm the apoptosis results obtained from the Annexin-V/PI analysis.

Investigate the mechanistic link between p53 deficiency and altered proliferation & apoptosis rates of splenocytes. P53 functions as a transcription factor and regulates the expression of a number of genes involved in cell-cycle arrest, apoptosis and senescence (21, 110). P53 is either mutated or deleted in more than 50% of human cancers and plays an tumor suppressor role in cancer (22). However, it is unknown how loss of p53 affects altered proliferation & apoptosis and pathogenesis in CLL. It has been reported that

anti-apoptotic Bcl-2 family members are overexpressed in most CLL cases, which is associated with chemotherapy resistance and poor prognosis (82). Particularly Mcl-1 plays an important role for CLL survival in both *in vitro* and *in vivo* (85). We first determine the expression of anti-apoptotic Bcl-2 family members in mouse splenocytes and PC cells by Western blot analysis at protein levels. PCR experiments will be also performed to determine the mRNA levels of anti-apoptotic Bcl-2 family members. Both protein and mRNA levels of anti-apoptotic Bcl-2 family members will also be determined in human CLL patient samples with 17p deletion or without 17p deletion. We expect elevated expression levels of anti-apoptotic Bcl-2 family members in CLL cells with p53 deficiency compared to CLL cells with p53 wt. How does p53 deficiency cause alteration of anti-apoptotic Bcl-2 family members? It could be through microRNA regulation. Several miRNAs such as miR-15a, miR-16 and miR-34a have been implicated in CLL pathogenesis and prognosis (102, 103). However, it remains unclear how loss of p53 affects miRNAs and alters the regulatory function of miRNAs in CLL. To address that question, miRNA array will be performed for both mouse and human RNA samples with p53wt or p53 deficiency. Regard the array data, RT-PCR experiments need to be performed to confirm those miRNA alterations. Those miRNA targets observed may explain the alteration of anti-apoptotic Bcl-2 family members in mouse and human CLL cells with p53 deficiency. The mRNA and protein levels of other downstream molecules of those miRNAs will be determined by Western blot analysis respectively. To further confirm that p53 regulates those miRNAs, p53 will be

knocked down in some B cell lines such as EBVB. Then the expression of those miRNAs, Bcl-2 family and the downstream molecules of miRNAs will be determined in the B cell lines with p53 knock-down. In summary, we expect that p53 deficiency regulates some specific miRNAs which resulted in upregulation of anti-apoptotic Bcl-2 family members, leading to hyperperliferation, reduced apoptosis and rapid disease progression in *TCL1*-Tg mice.

Specific Aim 3: **Determine the effect of p53 deficiency on CLL treatment response on cellular level and animal level.** As mentioned in the introduction part, CLL patients with 17 deletion or TP53 mutation are resistant to conventional chemotherapies in the CLL treatment. To confirm that, CLL patient samples with or without 17p deletion will be appropriately treated with conventional chemotherapies such as fludarabine, chlorambucil and so on. 48h later, Annexin-V/PI analysis will be performed for those treated cells to detect apoptosis. Annexin-V/PI analysis will be also performed for similarly treated mouse splenocytes isolated from *p53*$^{-/-}$ mice, *TCL1*-Tg mice, *TCL1*-Tg:*p53*$^{+/-}$ and *TCL1*-Tg:*p53*$^{-/-}$ mice. We expect that both human CLL cells with 17p deletion and mouse CLL cells with p53 deficiency are resistant to those conventional chemotherapies. To overcome the treatment resistance of CLL cells with p53 deficiency, a novel chemotherapy agent-NL-101 is studied in the CLL treatment. NL-101 contains suberoylanilide hydroxamic acid (SAHA) and Bendamustine moieties in 1:1 ration. We hypothesized that HDACI such as SAHA can overcome the resistance of alkylating agents such as bendamustine

by reducing CLL migration to the microenvironment and induce more DNA damage than alkylating agents on CLL cells. Recent studies have shown that SAHA induces apoptosis in CLL by downregulating the CXCR4 chemokine receptor, leading to impaired migration of CLL cells (111). Human CLL cells with 17p deletion or without 17p deletion will be treated with single agents such as NL-101, bendamustine, chlorambucil, SAHA or their combinations for 48h, and followed by Annexin-V/PI analysis. To investigate underlying mechanisms of the novel agent NL-101 on CLL treatment, the expression levels of cytokines in stromal cell lines treated with HDACI such as SAHA will be determined by RT-PCR, and followed by Enzyme-linked immunosorbent assay (ELISA) assay. Comet assay will be performed to determine DNA damage rate in CLL cells treated with SAHA, bendamustine, SAHA+Bendamustine and NL-101. We expect that HDACIs will reduce some cytokine levels such as CXCL12 secreted in stromal cell lines. We also expect more DNA damage caused by NL-101 or SAHA+bendamustine than single bendamustine treatment on CLL cells. PEITC can effectively eliminate fludarabine-resistant CLL cells through ROS accumulation and glutathione depletion (45). Therefore, we will try PEITC treatment or combination with other chemotherapies in both human and mouse CLL cells with or without p53 deficiency. To determine the effect of p53 deficiency on CLL treatment response on animal level, 20 *TCL1*-Tg:$p53^{-/-}$ mice will be treated with nano-PEITC weekly through iv injection. Another 20 *TCL1*-Tg:$p53^{-/-}$ mice will be treated with NL-101 2 times/month through iv injection.

We expect that both PEITC and NL101 will prolong the survival time of *TCL1*-Tg:*p53*$^{-/-}$ mice.

In summary, the major objective of the proposed researches is to establish a CLL mouse model with p53 deficiency. This *TCL1*-Tg:*p53*$^{-/-}$ mouse model will be utilized for testing novel therapeutic strategies and studying the effect of p53 deficiency on CLL biology and pathogenesis. These proposed studies will provide new insights into how p53 deficiency affects expression of some specific miRNAs and their regulatory functions.

Figure 1

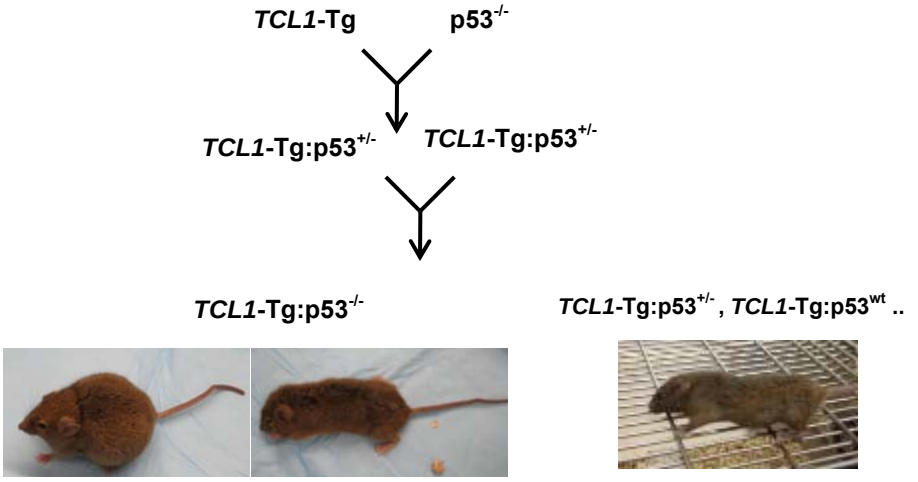

TCL1-Tg
p53$^{-/-}$
TCL1-Tg:p53$^{+/-}$
TCL1-Tg:p53$^{+/-}$
TCL1-Tg:p53$^{-/-}$
TCL1-Tg:p53$^{+/-}$, *TCL1*-Tg:p53^{wt} ..

Figure 1. Generation of *TCL1*-Tg:*p53*$^{-/-}$ mice. *TCL1*-Tg homozygous mice are crossed with p53$^{-/-}$ mice. Then the second generation of *TCL1*-Tg:*p53*$^{+/-}$ mice are intercrossed to get *TCL1*-Tg:*p53*$^{-/-}$ mice, *TCL1*-Tg:*p53*$^{+/-}$ mice, *TCL1*-Tg:*p53*wt mice and non-*TCL1*-Tg mice.

3. MATERIAL AND METHODS

3.1 Generation of *TCL1*-Tg:*p53*$^{-/-}$ mice. *TCL1*-Tg mice (B6C3) were kindly provided by Dr. Carlo M. Croce. They were crossed with *p53*$^{-/-}$ mice (B6C3) which were from Jennifer Alana (a microinjection specialist in MD Anderson Cancer Center). Then the second generation of *TCL1*-Tg:*p53*$^{+/-}$ mice were intercrossed to get *TCL1*-Tg:*p53*$^{-/-}$ CLL mouse model. For research studies, pure *TCL1*-Tg:*p53*$^{-/-}$ mice were obtained by intercrossing *TCL1*-Tg:*p53*$^{-/-}$ mice (Figure 1). All mice were housed under conventional barrier protection in accordance with University of Texas MD Anderson Cancer Center guidelines, and mouse protocols were approved by the University of Texas MD Anderson Cancer Center Institutional Animal Care Committee. Survival data were obtained by observing mice up to 2 years (n=33 for *TCL1*-Tg:*p53*$^{-/-}$ mice and n=20 for *TCL1*-Tg mice). For mouse genotyping, small segments of mouse tail tips were collected from littermates at the age of 3-4 weeks, and were digested in 200 μL direct PCR lysis reagent with 5 μL proteinase K at 56ºC in a water bath for overnight, followed by a 5-minute incubation at 95ºC and then cooled on ice. After removal of tissue debris by centrifugation, 2 μL supernatant was used in a PCR reaction for genotyping. All primers were purchased from Sigma.

3.2 Blood smear. Around 250 μL blood/mouse was collected from mouse tail of 5 mice for each genotype and put in an EDTA-coated tube. All blood samples were mixed well upon collection. Complete Blood Count (CBC) service and WBC differential count service were provided by Department of

Veterinary Medicine & Surgery Section of Veterinary Laboratory Medicine in MD Anderson Cancer Center.

3.3 Histopathology. Mouse spleen, liver and lymph node tissues from 6 mice of each type were fixed in neutral buffered 10% formalin solution. After 24h, the tissues were transferred to 70% alcohol. Within 3 months after fixation in 70% alcohol, the fixed tissues were sent to the histology core laboratory in MD Anderson Cancer Center for preparing histology slides. Extra sections of the tissues were embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) or Ki67 by the histology core laboratory in MD Anderson Cancer Center. H&E stained tissue slides were analyzed by Dr. Hesham M. Amin from the department of hematopathology in MD Anderson Cancer Center.

3.4 Polymerase Chain Reaction (PCR). For mouse genotyping, TCL1 and p53 DNA contents were amplified using their specific primers. The TCL1 DNA amplifying protocol was provided by Dr. Croce's group. Primers for TCL1 are: sense, 5'GCCGAGTGCCCGACACTC3' and antisense, 5'CATCTGGCAGCAGCTCGA3' The PCR using TCL1 primers was conducted at 94°C for 5 min for an initial denaturation step followed by 30 cycles for 30 sec of denaturation at 94°C, 30 sec of annealing at 65°C, and 30 sec of extension at 72°C, and a final extension step of 7 min at 72°C. The p53 DNA amplifying protocol was provided by Chad Smith (Transgenic Core Facility of MD Anderson Cancer Center). Primers for p53 are: p53-X7, 5'GGATGGTGGTATACTCAGAGCC3', p53-X6, 5'AGCGTGGTGGTACCTTATGAGC3' and neo19,

5’GCTATCAGGACATAGCGTTGGC3’. The PCR for amplifying p53 was performed at 95°C for 5 min for an initial denaturation process followed by 35 cycles for 1 min of denaturation at 95°C, 1min of annealing at 60-62°C, and 3 min of extension at 72°C, and a final extension step of 7 min at 72°C. The PCR results are ready for DNA gel electrophoresis or saved at -20°C. 1.8% agarose gel was prepared for electrophoresis. After DNA gel electrophoresis, the PCR results were separated on agarose gel and visualized on alpha-imager.

3.5 Isolation of CLL cells and cytotoxicity assays. Primary leukemia cells were isolated from the peripheral blood samples of CLL patients diagnosed according to the NCI criteria (112). Proper informed consents under a research protocol approved by the Institutional Review Board (IRB) of MD Anderson Cancer Center were obtained from all patients before the collection of blood samples. Specimens from CLL patients with or without 17p deletion were all used for comparison. CLL cells were isolated from blood samples by density gradient centrifugation as described previously (45), and incubated in RPMI 1640 medium supplemented with 10% FBS and Penicillin (100 U/ml) + Streptomycin (100 ug/ml) overnight before testing drug sensitivity by incubation with CLL chemotherapies such as fludarabine. PC cells and splenocytes were isolated and treated with ACK cell lysis buffer for 2 minutes on ice to remove red blood cells. After lysis, RPMI medium with 10% FBS was added to the cells to stop the lysis. Afterwards, the cells were washed once by PBS and filtered through cell strainer with 40 μM nylon mesh (Fisher Scientific, Pittsburgh, PA) for single cell preparation and cultured in the same medium as

human CLL cells. B cells were purified from CLL cells by using CD19 microbeads, and incubated in RPMI 1640 medium supplemented with 10% FBS and Penicillin (100 U/ml) + Streptomycin (100 ug/ml). At the same day, those B cells were treated with fludarabine or oxaliplatin for 48h. Cell viability and cellular sensitivity to drug treatment *in vitro* were determined by flow cytometry after double staining of $1x10^6$ cells with annexinV-fluorescein isothiocyanate (FITC) and PI analysis.

3.6 Reagents. 9-β-*D*-arabinofuranosyl-2-fluoro-adenine (F-ara-A, the nucleoside form of fludarabine), oxaliplatin, SAHA, PEITC, chlorambucil, 3-DZNep, PI and PCR primers were purchased from Sigma-Aldrich (St. Louis, MO). Direct PCR lysis reagent was purchased from Viagen Biotech Inc. (Los Angeles, CA). ACK lysis buffer was from Lonza Houston, Inc. (Houston, TX). Ficoll-lite Lympho H (Fico) was from Atlanta Biological (Lawrenceville, GA). CD19 microbeads were purchased from MACS Miltenyi Biotech Inc. (Auburn, CA). Annexin V-FITC was from BD Biosciences (San Jose, CA). NL-101 compound was from Northlake Biosciences LLC (Lexington, MA). TUNEL staining kit was obtained from Roche Applied Science (Indianapolis, IN). Antibodies against Bcl-XL, Bcl-2, EZH2 and β-actin were purchased from Cell Signaling Technology Inc. (Danvers, MA). Anti-b-Myb antibody was from EMD Millipore (Billerica, MA). Anti-Mcl1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

3.7 Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA from $1X10^7$ splenocytes isolated from *TCL1*-Tg, *TCL1*-Tg:*p53*$^{+/-}$

and *TCL1*-Tg:*p53*$^{-/-}$ mice (4 for each genotype), were extracted, purified (RNeasy Mini kit, Qiagen) and quatified by Ultrospec 3300 pro UV/visible spectrophotometer. First-strand cDNA was synthesized from 0.5 μg total RNA by using a commercial kit (RevertAid First Strand cDNA Synthesis Kit-Fermentas) according to the manufacturer' instructions. Real-time PCR was performed with 7900 GT Sequence Detection System (ABI PRISM). All human and mouse primers for Bcl-XL, Mcl-1, Bcl-2, EZH2, b-Myb and CXCL12 were purchased from Sigma. Each PCR was performed in a 25-μL volume on a 96-well optical plate for 2 minutes at 50ºC, followed by 10 minutes at 95ºC, then followed by 40 cycles of 95ºC for 30 seconds, 60ºC for 30 seconds and 72ºC for 1minute and followed by 10 minutes at 72ºC. To independently validate the individual miRNA expression pattern from the miRNA array result, total RNA was isolated from patient white blood cells, and spleen cells from *TCL1*-Tg, *TCL1*-Tg:*p53*$^{+/-}$ and *TCL1*-Tg:*p53*$^{-/-}$ mice by using miRNA isolation kit (Ambion). Then cDNA synthesis was carried out using Taqman MiRNA Reverse Transcription kit and specific Taqman RT primers (Applied Biosystems). MiRNA-specific real-time PCRs were performed using Taqman Universal PCR Master Mix and Taqman small assays according to the manufacturer's protocol. The relative expression of specific miRNAs was calculated by the delta (deltaCt) method.

3.8 Immunoblotting. Primary CLL cells were isolated from patient blood using Fico as described above. Mouse splenocytes and PC cells were purified by ACK lysis buffer. B cells were purified by CD19 microbeads. Cell number

was determined by a Coulter Z2 particle count and size analyzer (Beckman Coulter, Inc., Fullerton, CA). The mouse splenocytes/PC cells or human CLL cells /B cells with same amount were then lysed in protein lysis buffer containing a cocktail of protease inhibitors. The nuclei and cell debris were removed by centrifugation at 4°C (13,000 rpm for 5 min), and the supernatants were collected as protein lysates. The protein lysates were then heated at 95°C for 5-15 min and separated by SDS-PAGE followed by Western blot analyses with antibodies specific for Bcl-XL, Mcl-1, Bcl-2, EZH2 and B-Myb.

3.9 Flow cytometry. Single-cell suspensions were made from mouse spleen, bone marrow and PC as described above, and stained for surface expression with FITC-labeled anti-IgM, allophycocyanin (APC)-labeled anti-CD5 antibodies (Ebioscience, San Diego, CA). Annexin V and propidium iodide (PI) were used to monitor cell death.

3.10 Cell lines and cell culture. Stromal cell lines including human stromal cell lines NKTert & HS5 and mouse stromal cell line Kusa.H1, EBVB and Raji were cultured in RPMI 1640 medium supplemented with 10% FBS. Human white blood cells were isolated from CLL patients' blood using Fico and cultured in RPMI 1640 medium supplemented with 10% FBS and Penicillin (100 U/ml) + Streptomycin (100 ug/ml) (PS). Mouse splenocytes were cultured in the same medium as used for human CLL cells. If co-culture applied, human CLL cells and mouse splenocytes were co-cultured with appropriate amount of stromal cells in 24-well plates one day before treatment with chemotherapies.

3.11 Analysis of cell proliferation and apoptosis. Mouse spleen sections were fixed in neutral buffered 10% formalin solution for 24h and then incubated in 70% alcohol. The fixed tissues were sent to the histology core laboratory in MD Anderson Cancer Center for preparing histology slides. Cell proliferation was estimated by Ki67 immunostaining using Ki67 specific antibody and a horseradish peroxidast (HRP)-conjugated secondary antibody to reveal the diaminobenzidine (DAB) staining (Ki67 staining service ordered from the histology lab in MD Anderson Cancer Center). Terminal deoxynucleotidyl transferase deoxyuridine-triphosphatase nick-end labeling (TUNEL) assays were performed with an In Situ Cell Death Detection kit (Roche Applied Science, Indianapolis, IN) according to manufacturer's instruction and visualized under fluorescent microscopy. Annexin-V/PI double-staining and flow cytometry analysis were used to monitor cell death.

3.12 Enzyme-linked immunosorbent assay (ELISA). SDF-1α (CXCL12) ELISA kit was purchased from Fisher Scientific (Pittsburgh, PA). CXCL12 levels in drug treated NKTert, Kusa.H1 and HS5 stromal cells were determined by ELISA assay according to manufacturer's instruction.

3.13 Comet assay. $5X10^6$ human CLL cells were treated with 3μM SAHA, 3μM NL-101, 10μM bendamustine and 3μM SAHA+10μM bendamustine for 24h. The cells were collected and mixed with 37°C Low Melting Poit (LMP) agarose in dark. Then the cells were placed onto slides with coverslip for 5 min. The slides were put into cold, freshly made lysing solution at 4°C for at least 1h. The slides were gently removed from the lysing solution and briefly rinsed with

neutralization buffer, and put in alkaline butter for 15 min in dark to allow for unwinding of the DNA and expression of alkali-labile damage. Electrophoresis was performed for the slides at 25v/300mA for 20 min. After electrophoresis, the slides were dropwisely added neutralization buffer for 5 min and drained, and coated with cold 100% ethanol and allowed drying. The dry slides were stained with SYBR green and visualized under the fluorescent microscope. Those slides were rinsed in 100% ethanol and stored after drying.

3.14 Statistical analysis. Student *t* tests were used for testing the statistical difference between two groups of samples. Mouse survival curves by Kaplan-Meier plots were generated by Graphpad Prism software (GraphPad, San Diego, CA), and the statistical significance was analyzed by the log-rank (Mantel-Cox) test. A *p* value of less than 0.05 was considered statistically significant.

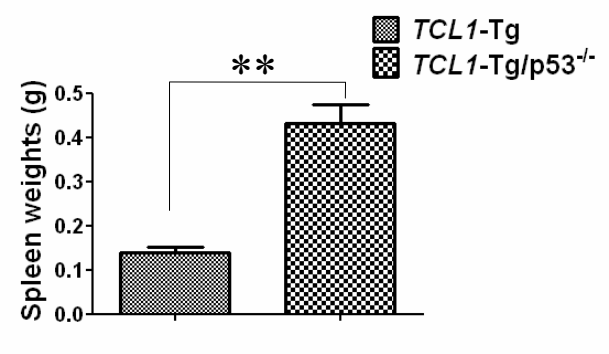
4. RESULTS

4.1 *TCL1*-Tg:*p53*$^{-/-}$ mice develop aggressive CLL with early disease onset and short lifespan. P53 is one of the most frequently mutated genes in cancers (22). In human B-CLL, loss of p53 function has been associated with accelerated disease progression, poor prognosis, and resistance to antitumor agents (17, 113). Currently there is no CLL mouse model with loss of p53 for investigating the pathological process of this aggressive CLL. To create such a animal model, we used the well-characterized *TCL1* Transgenic CLL mice with p53wt to cross breed with *p53*$^{-/-}$ mice to generate progenies harboring *TCL1*-Tg:*p53*$^{-/-}$ genotype. Figure 2A shows the weights of spleens from 5 *TCL1*-

Tg:*p53*wt (*TCL1*-Tg) and 5 *TCL1*-Tg:*p53*$^{-/-}$ mice. 17 mice in total 20 *TCL1*-Tg:*p53*$^{-/-}$ mice developed CLL with early disease onset at the age of approximately 3 month, with severe splenomegaly by 4-5 month (Fig 4B). In contrast, the spleens of *TCL1*-Tg mice with wt p53 or *p53*$^{+/-}$ mice appeared relatively normal in size (Fig 2B). Histological examination of the spleen sections of 4-month old *TCL1*-Tg mice showed normal tissue architecture, whereas the histological sections of the spleen from 4-month *TCL1*-Tg:*p53*$^{-/-}$ mice showed that the lymphoid follicles were ill-defined (Fig 2C). The germinal centers of the *TCL1*-Tg:*p53*$^{-/-}$ spleen exhibited histological features reminiscent of the proliferation centers characteristic of CLL/small lymphocytic lymphoma because of the presence of large lymphocytes with abundant eosinophilic cytoplasm. The red pulps in between the lymphoid follicles contained lymphoid cells with more abundant cytoplasm, granulocytes, and megakaryocytes compared with red pulps seen in *TCL1*-Tg mouse spleen (Figure 2C). Furthermore, blood smears revealed significant expansion of the white blood cells (WBC) in *TCL1*-Tg:*p53*$^{-/-}$ mice compared to *TCL1*-Tg mice (Figure 2D). Most of *TCL1*-Tg:*p53*$^{-/-}$ mice died at the age of 3-5 months, while the most of the *TCL1*-Tg mice survive more than 12 months (Fig 2E). This was consistent with the clinical observations that CLL patients with 17p deletion have significantly shorter overall survival compared to the CLL patients without 17p deletion (56).

Figure 2A-2B

A



B

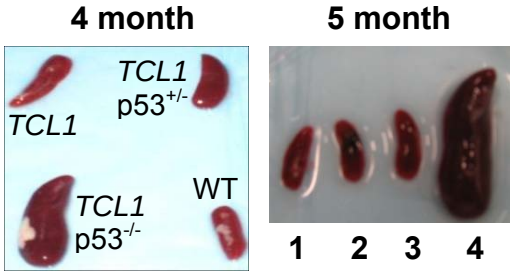


Figure 2. Characterization of CLL phenotype of *TCL1*-Tg:*p53*$^{-/-}$ mice. (A) Spleen weights shown for age- and sex-matched *TCL1*-Tg and *TCL1*-Tg:*p53*$^{-/-}$ mice at 4 months (n=5 per indicated genotype). **, $p<0.01$ between group. (B) Spleen size shown for age- and sex-matched WT, *TCL1*-Tg, *TCL1*-Tg:*p53*$^{+/-}$ and *TCL1*-Tg:p53$^{-/-}$ mice at 4 months and 5 months old of age (1. WT; 2. *TCL1*-Tg; 3. *TCL1*-Tg:*p53*$^{+/-}$; 4. *TCL1*-Tg:*p53*$^{-/-}$). Almost 100% *TCL1*-Tg:*p53*$^{-/-}$ mice older than 4 months had larger spleen compared to that of *TCL1*-Tg mice. (n=20 per indicated strain).

Figure 2C

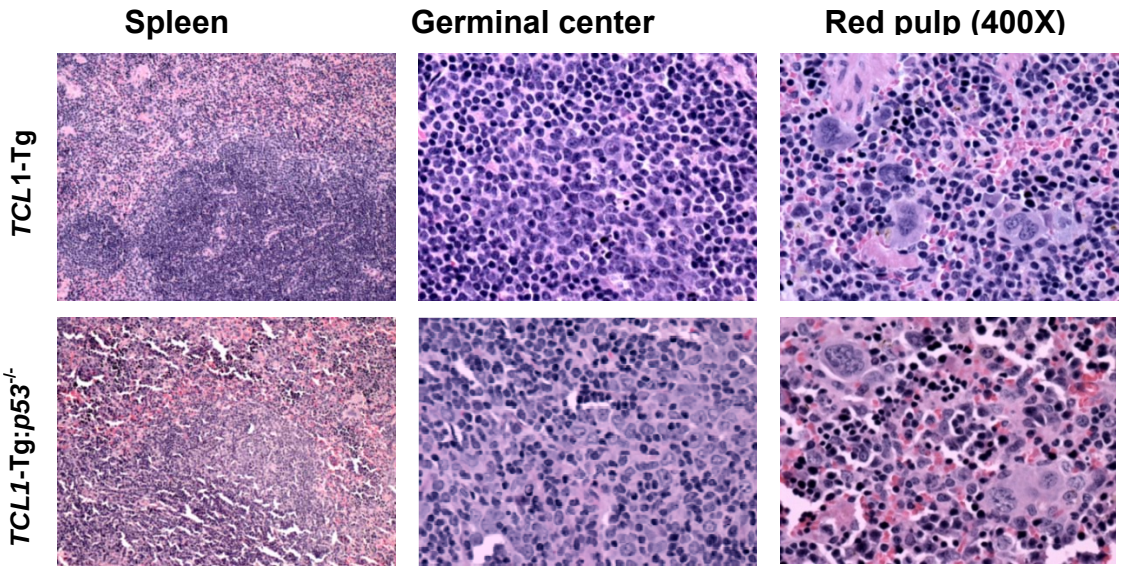


Figure 2C. Hematoxylin-eosin (H&E) staining. H&E staining of the spleen from *TCL1*-Tg and *TCL1*-Tg:*p53*$^{-/-}$ mice at 4-month of age. Mouse spleen tissues were fixed in 10% formalin buffered solution and embedded in paraffin. Tissue section from a representative 4-month *TCL1*-Tg mouse showed normal architecture (top panels), while the spleen from a representative *TCL1*-Tg:*p53*$^{-/-}$ mouse showed ill-defined lymphoid follicles (bottom panels) (n=6 per indicated strain).

Figure 2D

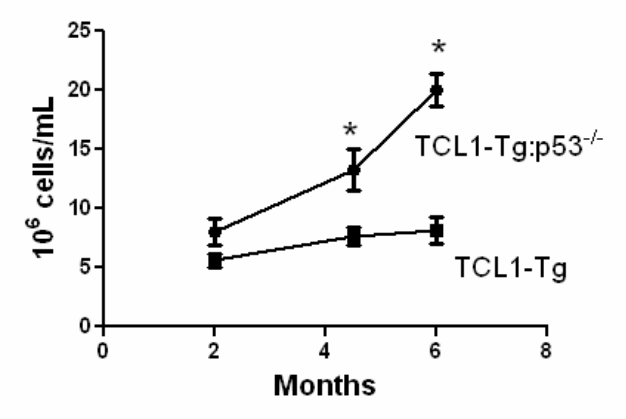


Figure 2D. White blood cells (WBC) count. Around 250 μL blood/mouse was collected from mouse tail of 5 mice for each genotype and put in an EDTA-coated tube. All blood samples were mixed well upon collection. Complete Blood Count (CBC) service and WBC differential count service were provided by Department of Veterinary Medicine & Surgery Section of Veterinary Laboratory Medicine in MD Anderson Cancer Center. WBC count in the indicated mouse strains at different ages (n=5 per indicated age). *, $p<0.05$ between groups.

Figure 2E

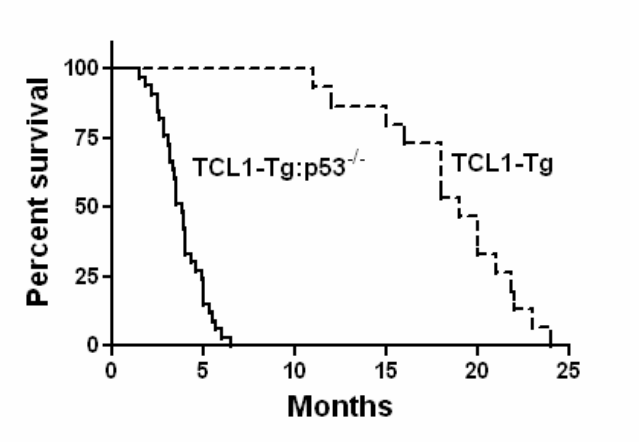

Percent survival
100
75
50
25
0
0
5
10
15
20
25
Months
TCL1-Tg:p53-/-
TCL1-Tg

Figure 2E. Survival curve (Kaplan-Meier) of *TCL1*-Tg (n=20) and *TCL1*-Tg:*p53*$^{-/-}$ mice (n=33). Median survival time for the *TCL1*-Tg:*p53*$^{-/-}$ mice was 3.8 months compared to 19 months for *TCL1*-Tg mice ($p<0.01$).

Since *TCL1*-Tg mice show features of human CLL with an increase in $CD5^+/IgM^+$ B cells in PC, spleen and bone marrow (60), we compared $CD5^+/IgM^+$ cells in the PC, spleen, and bone marrow of the wild-type mice, *TCL1*-Tg mice, *TCL1*-Tg:$p53^{+/-}$ mice, and *TCL1*-Tg:$p53^{-/-}$ mice. Flow cytometry analysis showed that at the age of 4 months, the wild-type mice showed 8% $CD5^+/IgM^+$ cells in the PC, the *TCL1*-Tg mice had 21% $CD5^+/IgM^+$ cells, and the *TCL1*-Tg:$p53^{+/-}$ and *TCL1*-Tg:$p53^{-/-}$ mice had 35% and 41% $CD5^+/IgM^+$ cells in the PC, respectively (Figure 3A). By 5 months, the $CD5^+/IgM^+$ cells in the PC of *TCL1*-Tg:$p53^{+/-}$ and *TCL1*-Tg:$p53^{-/-}$ mice increased to 58% and 79%, respectively (Figure 3B). In the spleens of a 5-month old mice, the $CD5^+/IgM^+$ cells were undetectable in the wild-type mice, 7% in *TCL1*-Tg mice, 15% in *TCL1*-Tg:$p53^{+/-}$ mice, and 20% in *TCL1*-Tg:$p53^{-/-}$ mice (Figure 3C). These data seemed consistent with the early onset of CLL in *TCL1*-Tg:$p53^{-/-}$ mice (Figure 2). At 3-5 months, no $CD5^+/IgM^+$ cells were detected in bone marrow of those mice. No such $CD5^+/IgM^+$ CLL population was observed in bone marrow of 7-month old *TCL1*-Tg:$p53^{+/-}$ mice (Figure 3D). In the spleens of 4-month old $p53^{-/-}$ mice, the $CD5^+/IgM^+$ cells were undetectable, and only about 10% $CD5^+/IgM^+$ cells were observed in the PC of those mice (Figure 3E).

Figure 3A-3C

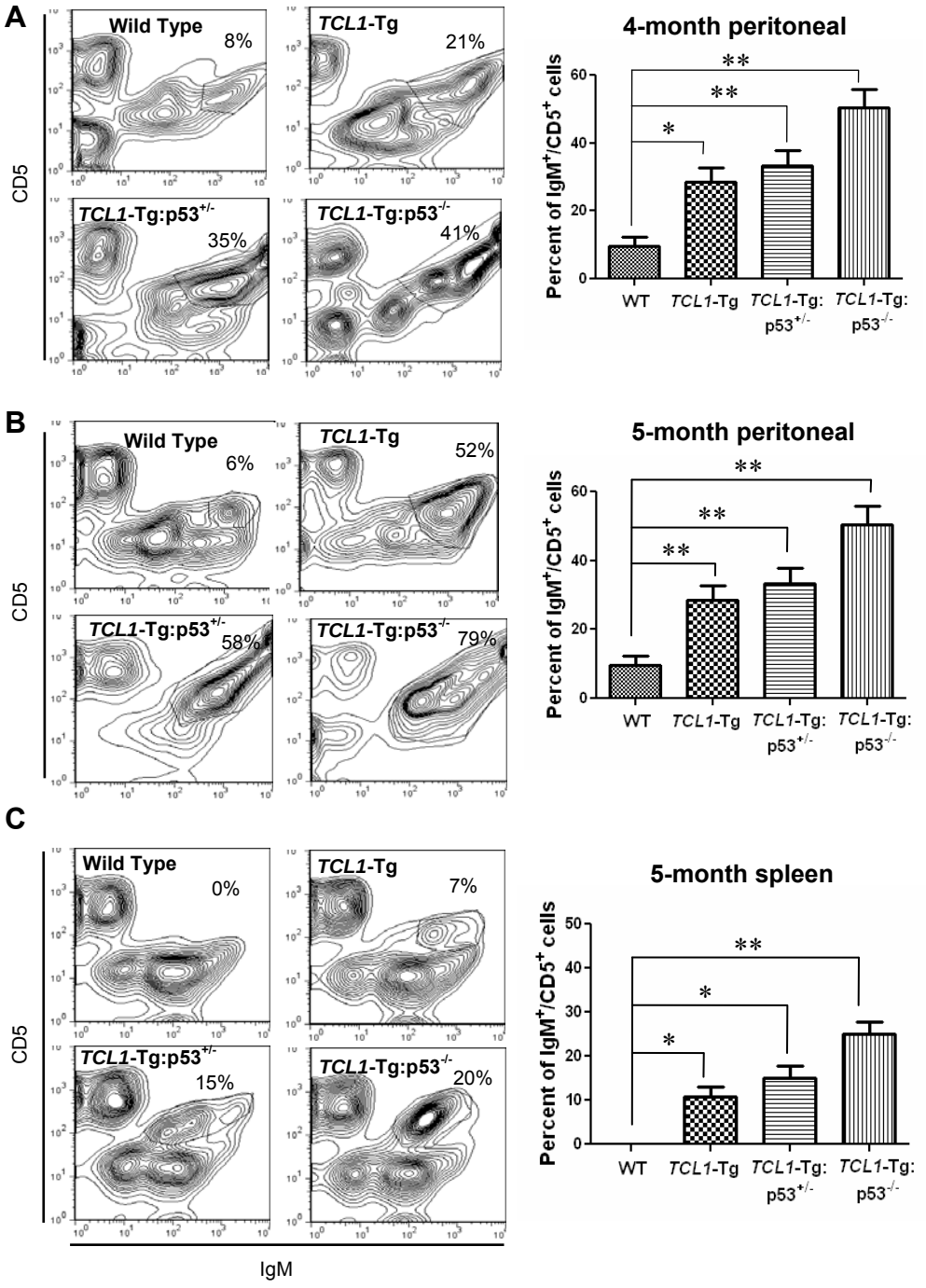

A
Wild Type
8%
TCL1-Tg
21%
TCL1-Tg:p53+/-
35%
TCL1-Tg:p53-/-
41%
CD5
4-month peritoneal
Percent of IgM+/CD5+ cells
WT
TCL1-Tg
TCL1-Tg: p53+/-
TCL1-Tg: p53-/-
B
Wild Type
6%
TCL1-Tg
52%
TCL1-Tg:p53+/-
58%
TCL1-Tg:p53-/-
79%
CD5
5-month peritoneal
Percent of IgM+/CD5+ cells
WT
TCL1-Tg
TCL1-Tg: p53+/-
TCL1-Tg: p53-/-
C
Wild Type
0%
TCL1-Tg
7%
TCL1-Tg:p53+/-
15%
TCL1-Tg:p53-/-
20%
CD5
IgM
5-month spleen
Percent of IgM+/CD5+ cells
WT
TCL1-Tg
TCL1-Tg: p53+/-
TCL1-Tg: p53-/-

Figure 3. *TCL1*-Tg:*p53*$^{-/-}$ mice had early onset of leukemia and increased CD5^{+}/IgM^{+} B cells compared to *TCL1*-Tg mice. PC cells and splenocytes were isolated and treated with ACK cell lysis buffer for 2 minutes on ice to remove red blood cells. After lysis, RPMI medium with 10% FBS was added to the cells to stop the lysis. Afterwards, the cells were washed once by PBS and filtered through cell strainer with 40 μM nylon mesh for single cell preparation. (A) Flow cytometry analysis (FITC-IgM/APC-CD5 staining) of PC cells from sex-matched different mouse strains collected at 4-month old of age (n=4 per indicated genotype), and quantitative bar graph shown on the right panel. (B) Flow cytometry analysis (FITC-IgM/APC-CD5 staining) of PC cells from sex-matched different mouse strains collected at 5-month old of age (n=4 per indicated genotype), and quantitative bar graph shown on the right panel. (C) Flow cytometry analysis (FITC-IgM/APC-CD5 staining) of splenocytes from sex-matched different mouse strains collected at 5-month old of age (n=4 per indicated genotype), and quantitative bar graph shown on the right panel.

Figure 3D

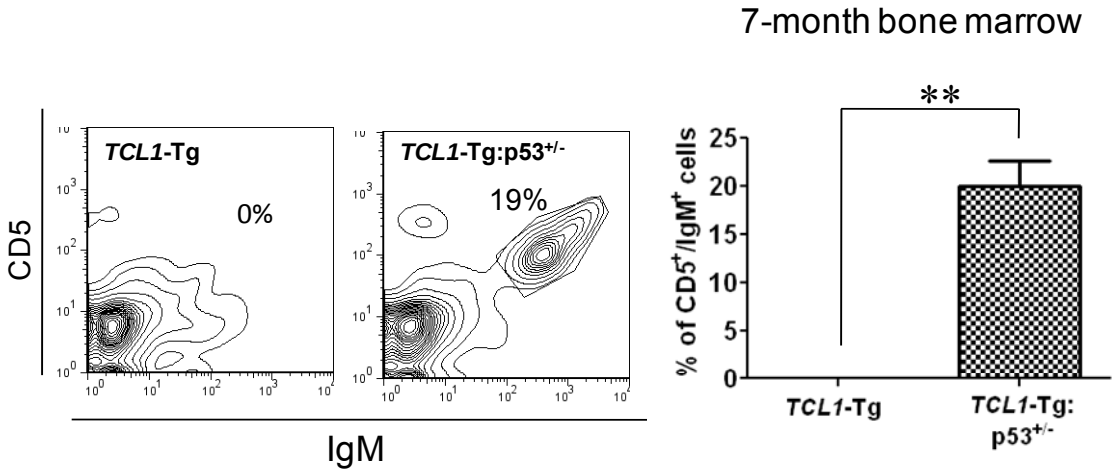

7-month bone marrow
TCL1-Tg
0%
TCL1-Tg:p53+/-
19%
CD5
IgM
**
% of CD5+/IgM+ cells
25
20
15
10
5
0
TCL1-Tg
TCL1-Tg:
p53+/-

Figure 3D. Flow cytometry analysis (FITC-IgM/APC-CD5 staining) of bone marrow cells from sex-matched different mouse strains collected at 7-month old of age (n=4 per indicated genotype), and quantitative bar graph shown on the right panel. **, p<0.01 between groups. Bone marrow cells were isolated from mouse legs and washed once by PBS and filtered through cell strainer with 40 μM nylon mesh for single cell preparation.

Figure 3E

4-month p53$^{-/-}$

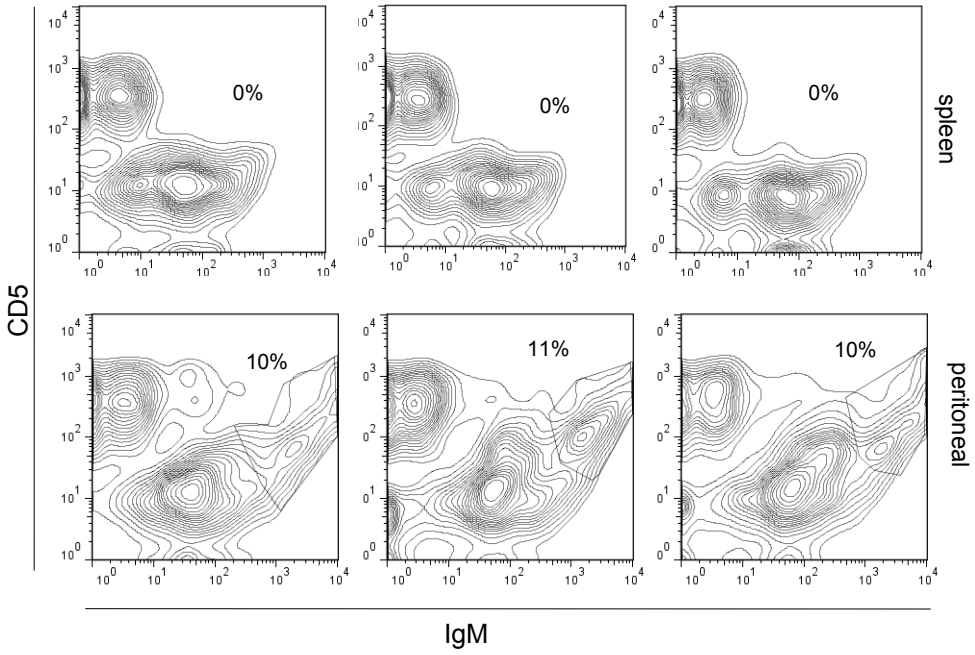


Figure 3E. Flow cytometry analysis (FITC-IgM/APC-CD5 staining) of splenocytes and PC cells from 3 *p53*$^{-/-}$ mice at 4-month old of age. PC cells and splenocytes were isolated and treated with ACK cell lysis buffer for 2 minutes on ice to remove red blood cells. After lysis, RPMI medium with 10% FBS was added to the cells to stop the lysis. Afterwards, the cells were washed once by PBS and filtered through cell strainer with 40 μM nylon mesh for single cell preparation.

4.2 Loss of p53 in CLL cells promotes proliferation and cell survival.

Recent study suggested that CLL cells in *TCL1*-Tg mice may undergo accelerated cell proliferation accompanied by elevated cell apoptosis, thereby displayed a low accumulation of CLL cells and slow disease progression (70). Since p53 plays a pivotal role in regulation of cell proliferation and apoptosis in response to various stimuli, we examined if a loss of p53 might affect CLL cells proliferation in *TCL1*-Tg mice. Immunostaining of spleen tissue slides with the proliferation marker Ki-67 revealed that Ki67-positive cells were significantly higher in the spleens of *TCL1*-Tg:*p53*$^{-/-}$ mice compared to that of *TCL1*-Tg mice (Figure 4A, 4C), suggesting an increase in proliferation of the splenocytes in *TCL1*-Tg:*p53*$^{-/-}$ mice. We then used TUNEL assay to compare *in vivo* apoptosis of splenocytes in *TCL1*-Tg and *TCL1*-Tg:*p53*$^{-/-}$ mice. TUNEL staining of the spleen tissue sections showed that *TCL1*-Tg:*p53*$^{-/-}$ mice had significantly less apoptotic cells in the spleen compared to that in the spleen of *TCL1*-Tg mice (Figure 4B, 4D). Consistently, annexin-V/PI double-staining of the splenocytes revealed that the isolated splenocytes from *TCL1*-Tg:*p53*$^{-/-}$ mice were less apoptotic when cultured *in vitro* for 24-72 h compared to the splenocytes isolated from *TCL1*-Tg mice culture under identical conditions (data not shown). Taken together, these data suggest that the loss of p53 in *TCL1*-Tg mice seems to promote cell proliferation and decrease apoptosis. This might account for the much higher accumulation of CLL cells and rapid disease progression in *TCL1*-Tg:*p53*$^{-/-}$ mice.

Figure 4A-4D

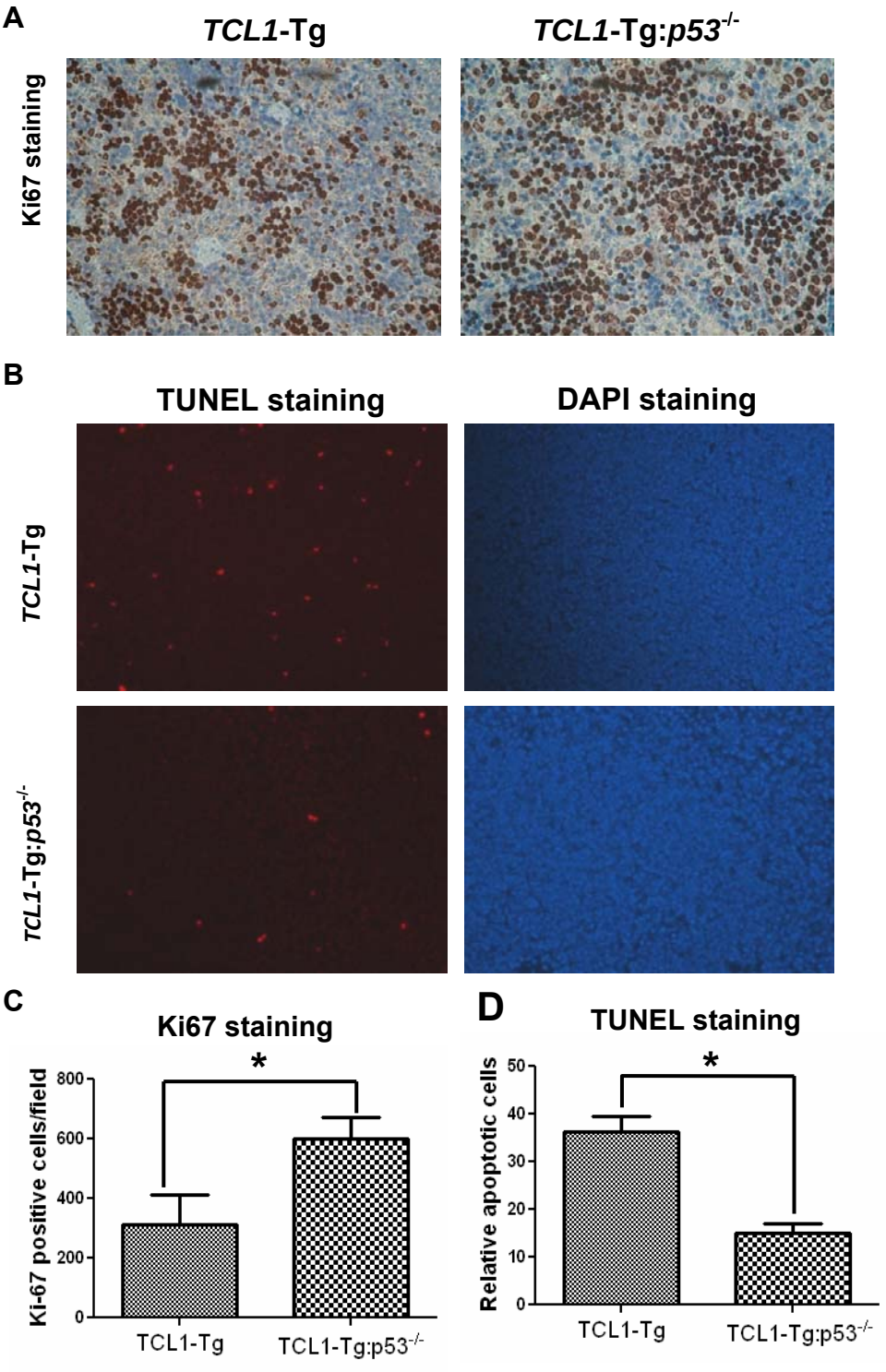

A
TCL1-Tg
TCL1-Tg:p53-/-
Ki67 staining
B
TUNEL staining
DAPI staining
TCL1-Tg
TCL1-Tg:p53-/-
C
Ki67 staining
Ki-67 positive cells/field
800
600
400
200
0
*
TCL1-Tg
TCL1-Tg:p53-/-
D
TUNEL staining
Relative apoptotic cells
50
40
30
20
10
0
*
TCL1-Tg
TCL1-Tg:p53-/-

Figure 4. P53 deficiency increased cell proliferation and elevated cell survival of leukemia cells from *TCL1*-Tg:*p53*$^{-/-}$ mice. (A) Mouse splenic sections were stained with the proliferation marker Ki-67 for age and sex-matched *TCL1*-Tg and *TCL1*-Tg:*p53*$^{-/-}$ mice. (B) Left panel, apoptosis in splenic sections shown by staining with TUNEL-TMR-Red. Right panel, DAPI staining indicates nuclear staining of total cells. (C) Statistic analysis of the results for A showed average of Ki67-positive cells/field from splenic sections, respectively (n=3 per group). *, $p<0.05$, **, $p<0.01$ between groups. (D) Statistic analysis of the results for B showed average of TUENL-positive cells/field from splenic sections, respectively (n=3 per group). *, $p<0.05$, **, $p<0.01$ between groups.

4.3 Leukemia cells from *TCL1*-Tg:*p53*$^{-/-}$ mice or from CLL patients with 17p deletion are resistant to chemotherapeutic drugs. The observations that the leukemia cells isolated from *TCL1*-Tg:*p53*$^{-/-}$ mice exhibited less spontaneous apoptosis (Fig 4B, 4D) prompted us to speculate that the leukemia cells with loss of p53 might be less sensitive to apoptotic induction and thus might be more resistant to chemotherapeutic agents. To test this possibility, we isolated splenocytes from the wild-type control mice and from *TCL1*-Tg, *TCL1*-Tg:*p53*$^{+/-}$ and *TCL1*-Tg:*p53*$^{-/-}$ mice, and then treated the cells with several standard anti-CLL chemotherapeutic agents in culture (114). As shown in Figure 5, the splenocytes from the control and *TCL1*-Tg mice exhibited similar sensitivity to F-ara-A (active form of fludarabine, 10 μM), chlorambucil (10 μM), and oxaliplatin (10 μM). The loss of one p53 allele (*TCL1*-Tg:*p53*$^{+/-}$) caused a moderate decrease in drug sensitivity, whereas the loss of both p53 alleles (*TCL1*-Tg:*p53*$^{-/-}$) led to a significant resistance to all three chemotherapeutic agents (Figure 5).

Figure 5

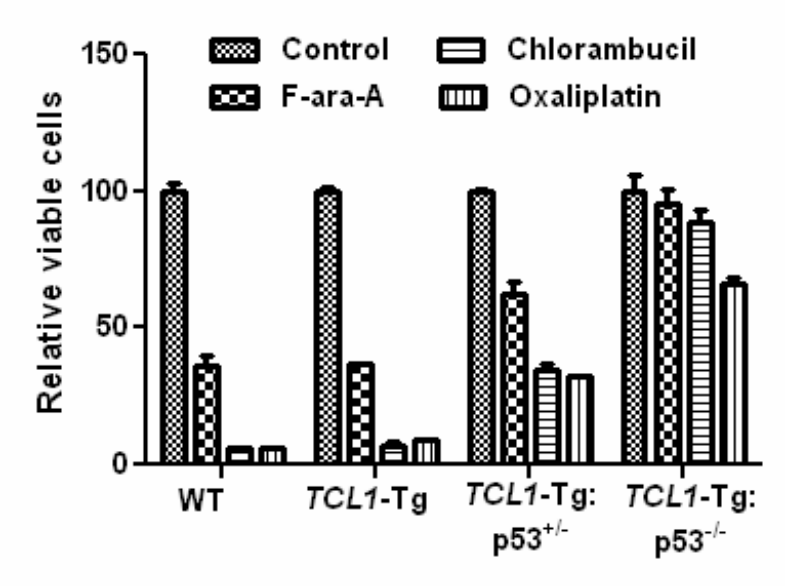

Control
Chlorambucil
F-ara-A
Oxaliplatin
Relative viable cells
150
100
50
0
WT
TCL1-Tg
TCL1-Tg: p53+/-
TCL1-Tg: p53-/-

Figure 5. P53 deletion makes mouse leukemia cells more resistant to standard CLL drug treatment. Splenic cells from indicated mouse strains were cultured in RPMI1640 medium with 10% FBS plus 1% PS and treated with 10uM F-ara-A, Chlorambucil and Oxaliplatin for 48h, and Annexin V-PI analysis by FACS was performed to detect apoptosis on those splenocytes (n=4 per group).

To further confirm the correlation between the loss of p53 and drug resistance in primary CLL cells from patients, we compared the drug sensitivity of primary leukemia cells from CLL patients with or without 17p deletion. Flow cytometry analysis showed that CLL cells isolated from patients without 17p deletion were sensitive to F-ara-A (10μM) and oxaliplatin (10μM), which caused a loss of 40-50% cell viability during the 48-h drug incubation (Figure 6). For instance, in patient #1 the control cell showed 78% viability, and drug treatment led to a substantial decrease of viable cells (37-41%). Similar results were observed in CLL cells from patient #2. In contrast, CLL cells with 17p deletion were highly resistant to F-ara-A and oxaliplatin (Figure 6, patients #3 & #4). For instance, in CLL sample #4, the control sample without drug treatment showed 87% viable cells. After treatment with F-ara-A or oxaliplatin, the cell viability remained at 85-86%. Similar drug resistance was observed in patient sample #3. Treatment resistance was also observed in other patient samples with 17p deletion (patients #8 to #10) as shown in Figure 7. The Figure 8 showed similar drug resistance of $17p^-$ B cells isolated from primary CLL cells. The results from experiments with leukemia cells from mice and from CLL patients consistently suggest that the loss of p53 leads to the drug resistance to standard chemotherapeutic drugs.

Figure 6

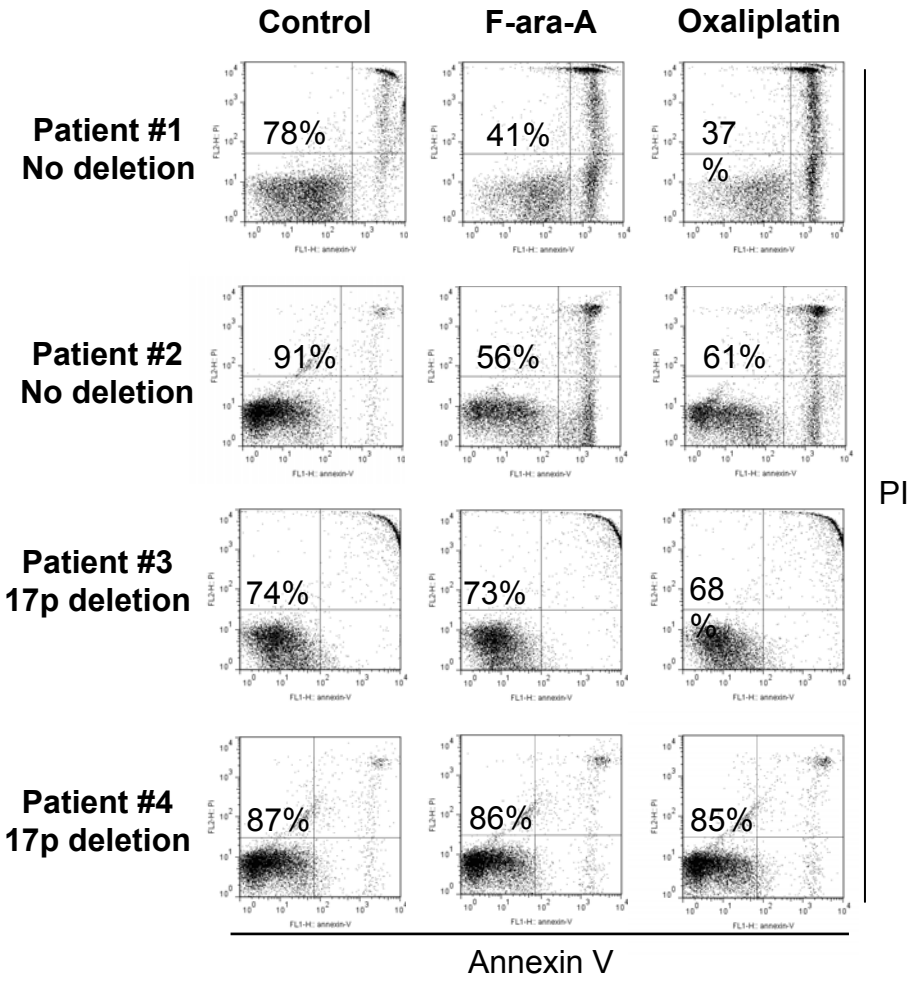

Control
F-ara-A
Oxaliplatin
Patient #1
No deletion
78%
41%
37
%
Patient #2
No deletion
91%
56%
61%
Patient #3
17p deletion
74%
73%
68
%
Patient #4
17p deletion
87%
86%
85%
PI
Annexin V

Figure 6. P53 deletion makes human leukemia cells more resistant to standard CLL drug treatment. White blood cells were isolated from CLL patients' blood and cultured in RPMI1640 medium containing 10% FBS and 1% PS. CLL patient samples with 17p WT (Pt #1 & Pt #2) or with >70% 17p deletion (Pt #3 & Pt #4) were treated with 10uM F-ara-A and 10uM Oxaliplatin for 48h, respectively. Cell apoptosis was analyzed by Annexin V-PI double staining.

Figure 7

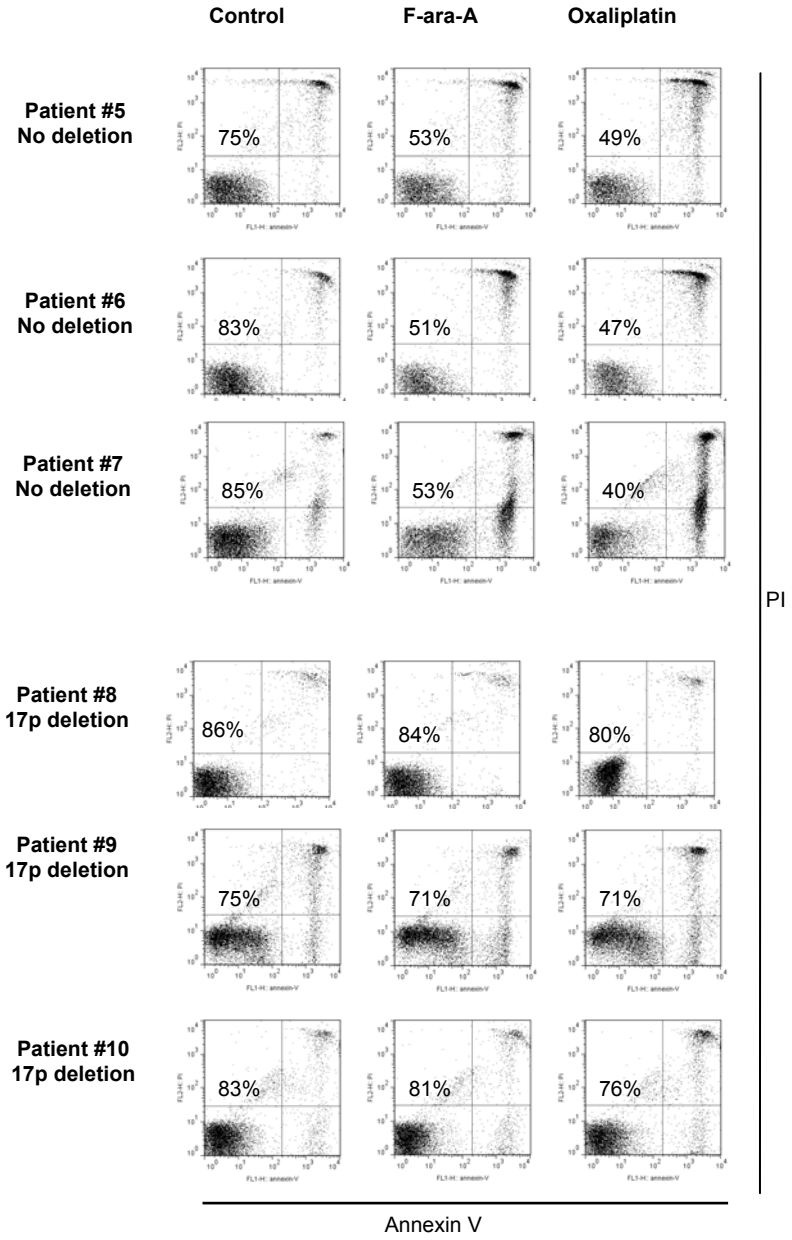

Control
F-ara-A
Oxaliplatin
Patient #5
No deletion
75%
53%
49%
Patient #6
No deletion
83%
51%
47%
Patient #7
No deletion
85%
53%
40%
Patient #8
17p deletion
86%
84%
80%
Patient #9
17p deletion
75%
71%
71%
Patient #10
17p deletion
83%
81%
76%
PI
Annexin V

Figure 7. For more patient samples, p53 deletion makes leukemia cells more resistant to standard CLL drug treatment. CLL patient samples with 17p WT (Pt #5-7) or with 17p deletion (Pt #8-10) were treated with 10uM F-ara-A and 10uM Oxaliplatin for 48h, respectively. Cell apoptosis was analyzed by Annexin V-PI double staining.

Figure 8

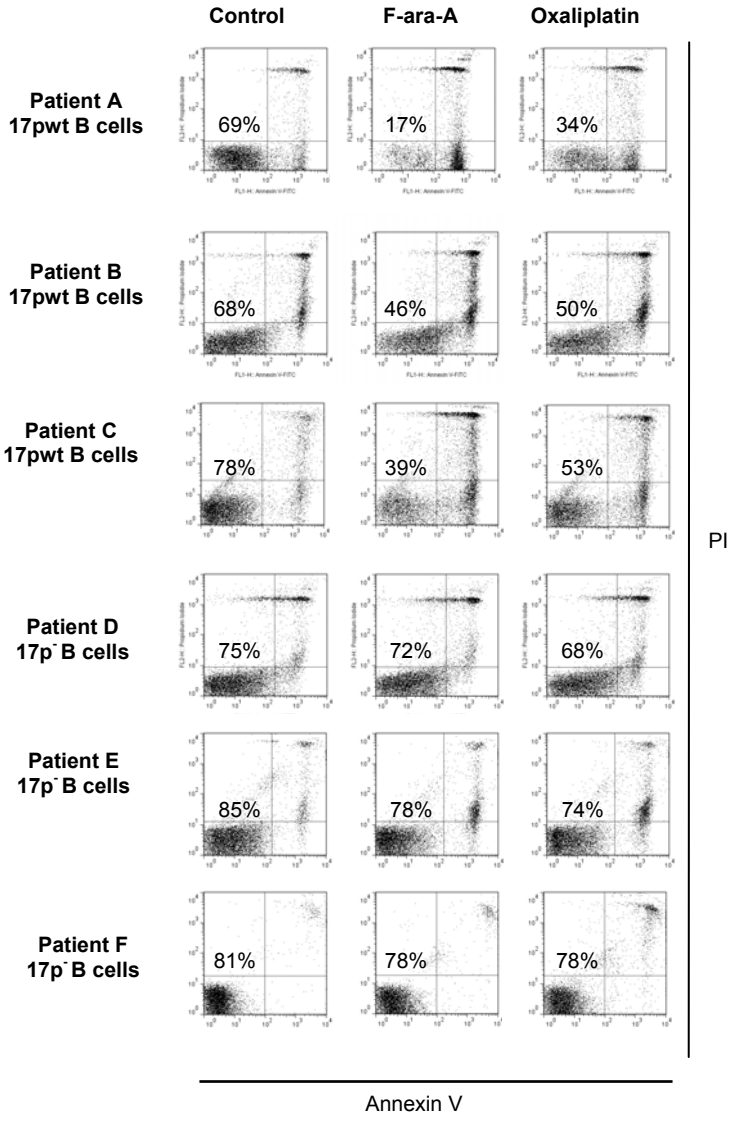

Control
F-ara-A
Oxaliplatin
Patient A
17pwt B cells
69%
17%
34%
Patient B
17pwt B cells
68%
46%
50%
Patient C
17pwt B cells
78%
39%
53%
Patient D
17p- B cells
75%
72%
68%
Patient E
17p- B cells
85%
78%
74%
Patient F
17p- B cells
81%
78%
78%
PI
Annexin V

Figure 8. P53 deletion makes human leukemia B cells more resistant to standard CLL drug treatment. White blood cells were isolated from CLL patients' blood and cultured in RPMI1640 medium containing 10% FBS and 1% PS. CD19 positive B cells purified from CLL patient samples with 17p WT (Pt A-C) or with 17p deletion (Pt D-F). After purification, B cells were treated with 10uM F-ara-A and 10uM Oxaliplatin for 48h, respectively. Cell apoptosis was analyzed by Annexin V-PI double staining.

4.4 Loss of p53 in CLL cells promotes Mcl-1 expression associated with down-regulation of miR-15a and miR-16-1. To investigate the mechanisms that contribute to the drug resistant phenotype in CLL cells lacking p53, we first compared the expression of the anti-apoptotic Bcl-2 family members including Bcl-2, Mcl-1, and Bcl-XL in CLL cells isolated from the spleens and PCs of the *TCL1*-Tg and *TCL1*-Tg:*p53*$^{-/-}$ mice. Western blot analysis showed that the expression of these anti-apoptotic molecules increased to various degrees in the p53-null cells (Figure 9A), with the elevation of Mcl-1 protein being the most prominent event, which was detected in leukemia cells isolated from spleen and PC. Bcl-XL protein levels were also increased in both the splenocytes and PC cells from *TCL1*-Tg:*p53*$^{-/-}$ mice compared to *TCL1*-Tg mice. Interestingly, the increased Bcl-2 was observed in the peritoneal leukemia cells but not in splenocytes (Figure 9A). Real-time RT-PCR analysis showed a significant increase in mRNA expression of Mcl-1, Bcl-XL, and Bcl-2 in the splenocytes and PC cells from *TCL1*-Tg:*p53*$^{-/-}$ mice compared to those from *TCL1*-Tg mice (Figures 9B-9D). Importantly, the increase in Mcl-1, Bcl-XL, and Bcl-2 protein expression was also observed in primary CLL cells isolated from patients with 17p deletion (Figure 10A). Such increase was consistently observed in multiple patient samples. Real-time RT-PCR analysis showed that the mRNA expression of these 3 molecules was also increased in CLL cells with 17p deletion (Fig 10B). These data together suggest that the increased expression of Bcl-2 family members occurred mainly at transcriptional level. Consistently, both protein and mRNA levels of Mcl-1 and

Bcl-XL were increased in $17p^-$ B cells isolated from patient CLL cells (Figure 11 & Figures 12A-12B). Interestingly, the increased Bcl-2 expression was observed in $17p^-$ B cells from part of patient samples (Fig 11 & Fig 12C).

Figure 9A

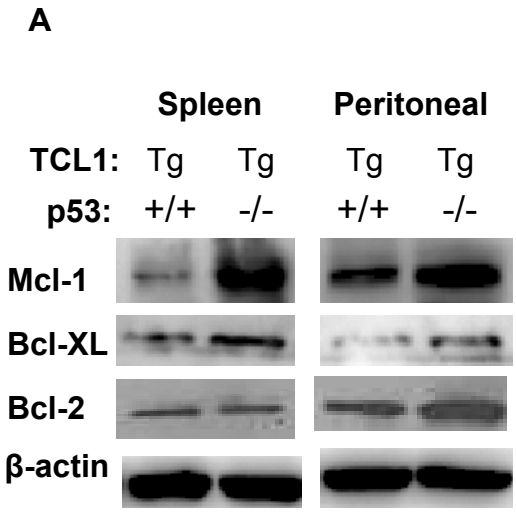
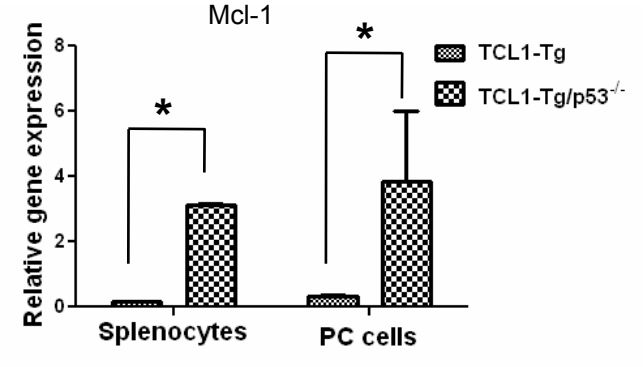

A
Spleen
Peritoneal
TCL1: Tg Tg Tg Tg
p53: +/+ -/- +/+ -/-
Mcl-1
Bcl-XL
Bcl-2
β-actin

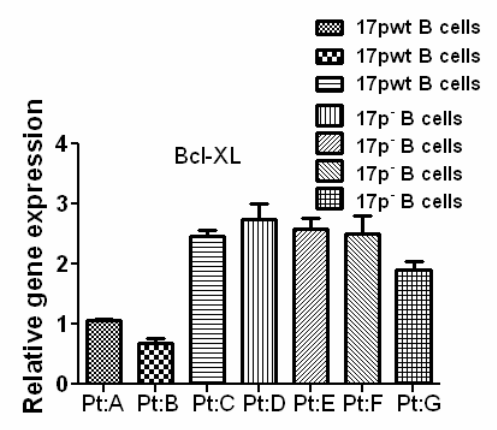
Figure 9. Bcl-2 survival family gene expression in mouse splenocytes and PC cells isolated from *TCL1*-Tg and *TCL1*-Tg:*p53*$^{-/-}$ mice. (A) Mcl-1, Bcl-XL and Bcl-2 protein expression shown by Western blot analysis. (splenocytes were isolated from 4-month old mice; n=4 per group).

Figure 9B-9D

B



C



D

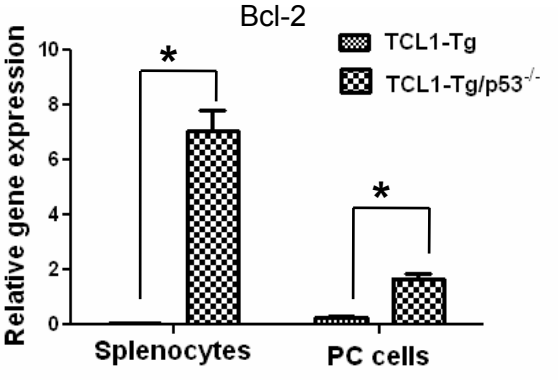


Figure 9. Bcl-2 survival gene expression in cells from *TCL1*-Tg and *TCL1*-Tg:*p53*$^{-/-}$ mice. (B) Mcl-1 mRNA levels in mouse splenocytes and PC cells shown by RT-PCR (n=4 per indicated strain). *, $p<0.05$, **, $p<0.01$ between groups. (C) Bcl-XL mRNA levels in mouse splenocytes and PC cells shown by RT-PCR (n=4 per indicated strain). *, $p<0.05$, **, $p<0.01$ between groups. (D) Bcl-2 mRNA levels in mouse splenocytes and PC cells shown by RT-PCR (n=4 per indicated strain). *, $p<0.05$, **, $p<0.01$ between groups.

Figure 10A-10B

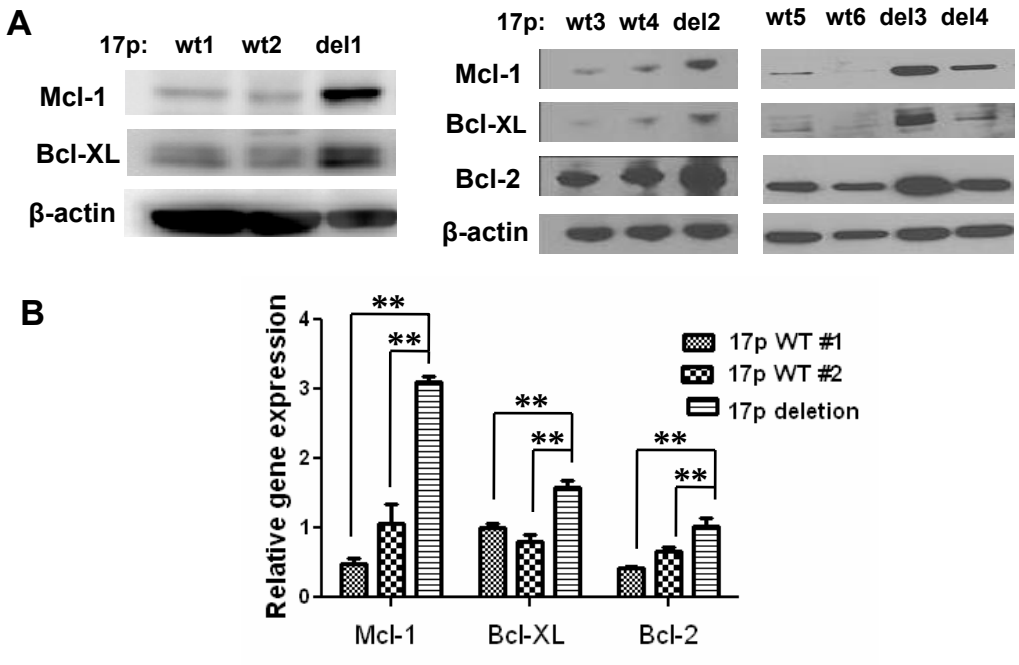


Figure 10. Bcl-2 survival family gene expression in CLL cells from human patient samples with 17p deletion or with 17p wt. (A) Western blot analysis showed Mcl-1, Bcl-XL and Bcl-2 protein levels in CLL cells from human CLL patient samples with or without 17p deletion (4 >70% 17p deletion samples: del1-4; 6 17p WT samples: WT1-6). (B) Mcl-1, Bcl-XL and Bcl-2 mRNA levels in CLL cells from human patients with or without 17p deletion shown by RT-PCR. *, $p<0.05$, **, $p<0.01$ between groups.

Figure 11

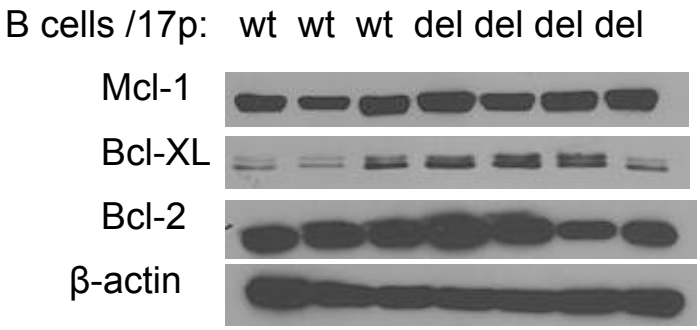
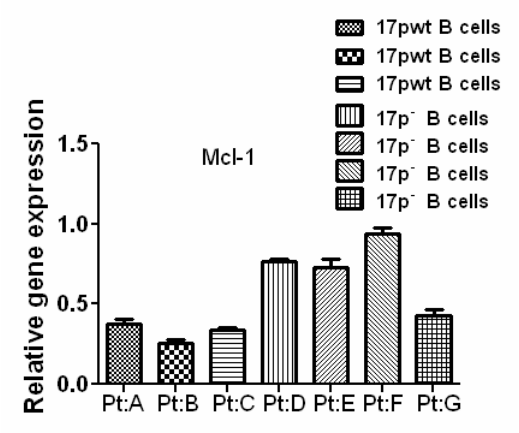


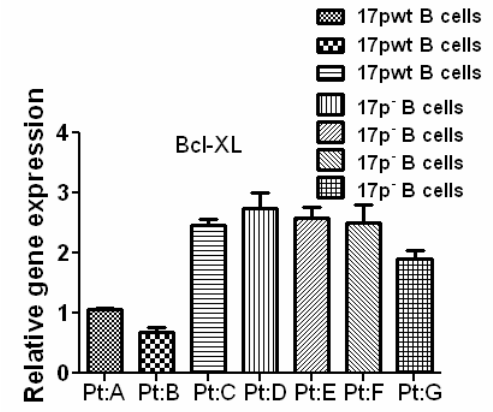
Figure 11. Bcl-2 survival family gene expression in CLL-B cells from patient samples with 17p deletion or with 17p wt. Western blot analysis showed Mcl-1, Bcl-XL and Bcl-2 protein levels in CD19 positive B cells from human CLL patient samples with or without 17p deletion (3 17p WT samples and 4 $17p^-$ samples).

Figure 12A-12C

A



B



C

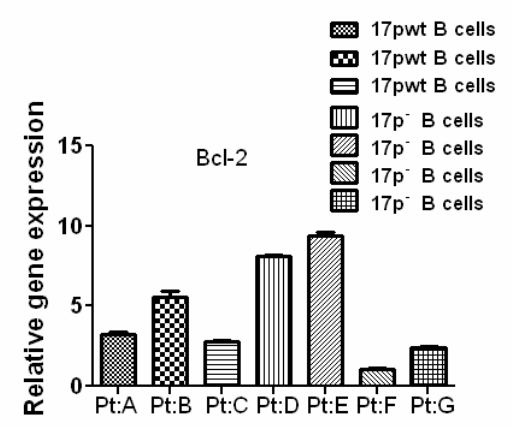
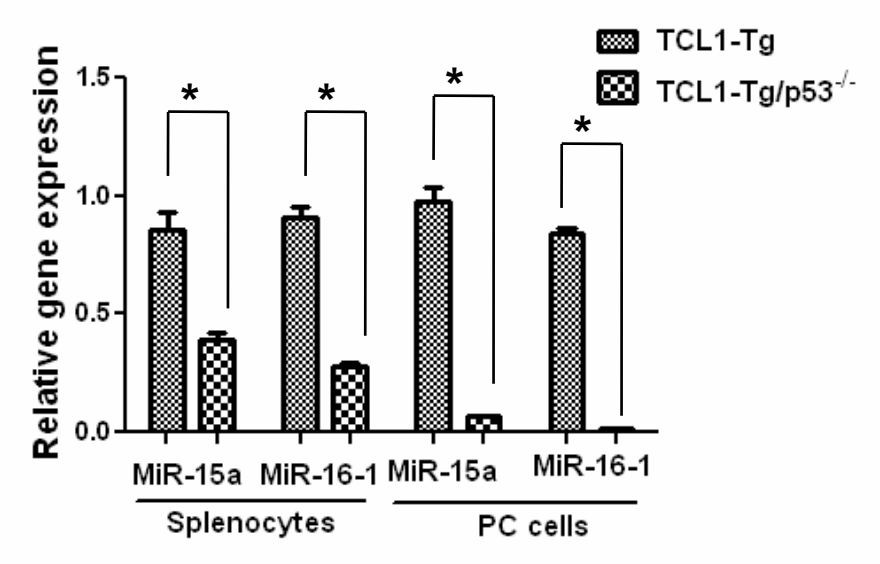


Figure 12. mRNA levels of Bcl-2 survival family in CLL-B cells from patient samples with 17p deletion or with 17p wt. (A) Mcl-1 mRNA levels in CD19 positive B cells from human CLL patient samples with or without 17p deletion (3 17p WT samples and 4 $17p^-$ samples). (B) Bcl-XL mRNA levels in CD19 positive B cells from human CLL patients with or without 17p deletion (3 17p WT samples and 4 $17p^-$ samples). (C) Bcl-2 mRNA levels in CD19 positive B cells from human CLL patients with or without 17p deletion (3 17p WT samples and 4 $17p^-$ samples).

To further investigate the possible mechanisms by which loss of p53 led to increased expression of multiple Bcl-2 family members, we speculated that since the expression of Bcl-2 multiple family members is known to be regulated by certain microRNAs (miR15a, miR-16) (115), it is possible that the loss of p53 might cause a decrease expression of some specific microRNAs, leading to over-expression of Mcl-1, Bcl-XL, and Bcl-2. To test this possibility, we first isolated total RNA from the splenocytes of *TCL1*-Tg and *TCL1*-Tg:*p53*$^{-/-}$ mice, and explored the expression profiles of microRNAs. Among the >300 microRNAs detected, miR-15a and miR-16-1 were markedly decreased in *TCL1*-Tg:*p53*$^{-/-}$ mice compared to that of *TCL1*-Tg mice. The decrease of miR-15a and miR-16-1 in cells from *TCL1*-Tg:*p53*$^{-/-}$ mice was further confirmed by real-time RT-PCR using the leukemia cells isolated from the spleens and PCs of *TCL1*-Tg and *TCL1*-Tg:*p53*$^{-/-}$ mice. As shown in Figure 13A, there was a significant decrease in the expression of miR-15a and miR-16-1 in both splenocytes and PC cells from mice without p53. Importantly, the decrease in miR-15a/16 expression was further confirmed in primary CLL cells isolated from patients with 17p deletion (Figure 13B). Consistently, similar results were observed in B cells isolated from primary CLL cells (Figure 14). These data together suggest that suppression of miR15a/miR16-1 expression may be an important mechanism by which the loss of p53 enhances the expression of Mcl-1, Bcl-2 and Bcl-XL, leading to increased cell viability and drug resistance.

Figure 13A-13B

A



B

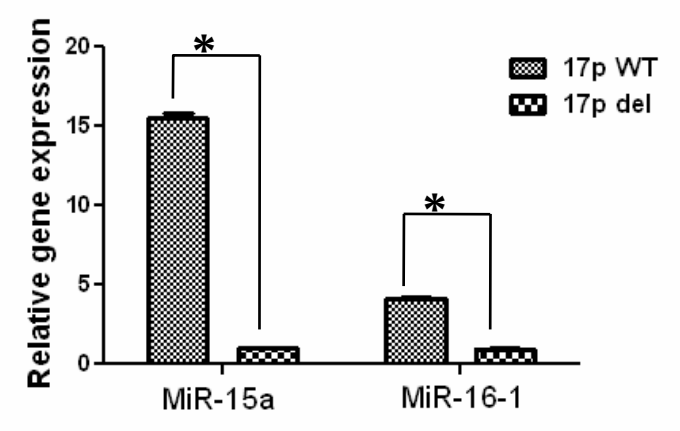
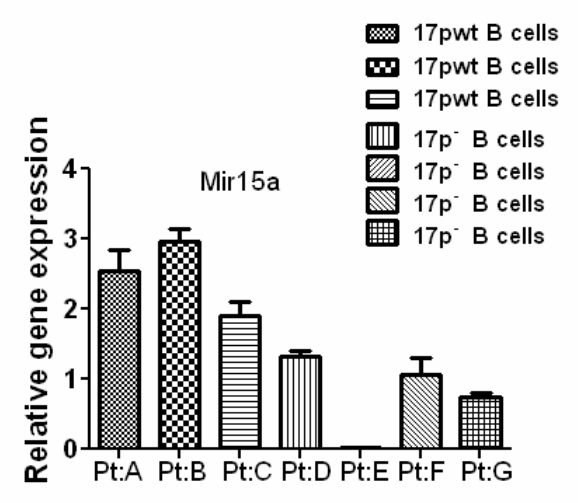


Figure 13. Expression of tumor suppressor miR-15a/16-1 in mouse and human leukemia cells. (A) MiR-15a/16-1 expression in mouse splenocytes and PC cells shown by RT-PCR. *, $p<0.05$, **, $p<0.01$ between groups. (B) MiR-15a/16-1 expression in CLL cells from patient samples with or without 17p deletion shown by RT-PCR. *, $p<0.05$, **, $p<0.01$ between groups.

Figure 14A-14B

A



B

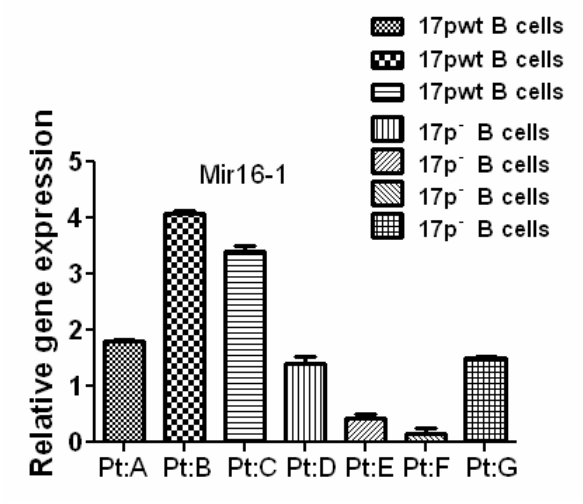


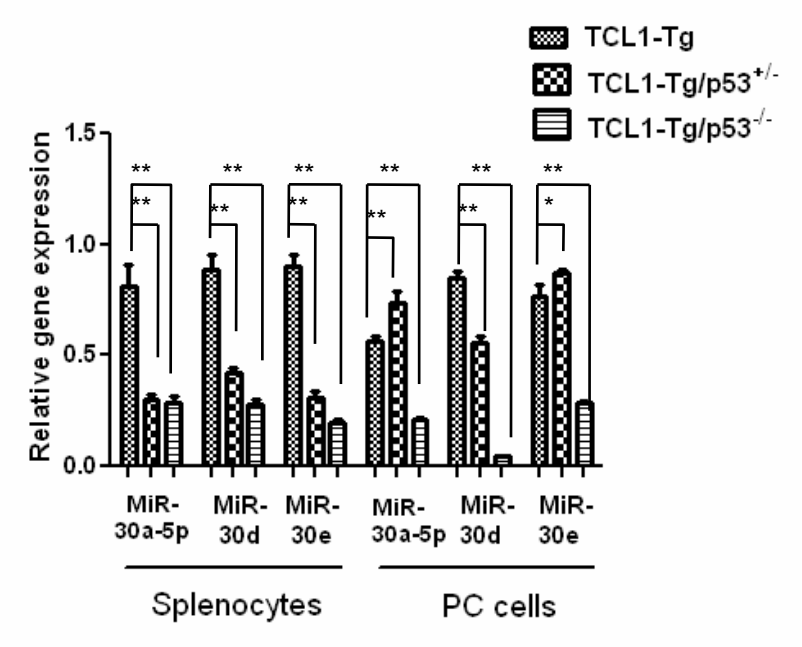
Figure 14. MiR15a/16-1 expression in CLL-B cells. (A) MiR-15a expression in CD19 positive B cells purified from patient samples with or without 17p deletion shown by RT-PCR (3 17p WT samples and 4 $17p^-$ samples). (B) MiR-16-1 expression in CD19 positive B cells purified from patient samples with or without 17p deletion shown by RT-PCR (3 17p WT samples and 4 $17p^-$ samples).

4.5 Loss of p53 in CLL cells causes down-regulation of miR-30 family, leading to upregulation of downstream oncogenes EZH2 and B-Myb. In addition, among the >300 microRNAs detected, miR-30a, miR-30e, and particularly miR-30d were markedly decreased in *TCL1*-Tg:*p53*$^{-/-}$ mice compared to that of *TCL1*-Tg mice. The downregulation of miR-30 family in cells from *TCL1*-Tg:*p53*$^{-/-}$ mice was further confirmed by real-time RT-PCR using the leukemia cells isolated from the spleens and PCs of *TCL1*-Tg and *TCL1*-Tg:*p53*$^{-/-}$ mice. As shown in Figure 15A, there was a significant decrease in the expression of miR-30 family in both splenocytes and PC cells from mice without p53. Importantly, the decrease in miR-30 family expression was further confirmed in primary CLL cells isolated from patients with 17p deletion (Figure 15B). It has been reported that miR-30d targets EZH2 which is involved in repressing gene expression through methylation of histone H3 on lysine 27 and upregulated in anaplastic thyroid carcinomas (90). Over years, many studies have established that EZH2 is overexpressed in various cancers including some hematologic malignancies, and such overexpression is associated with aggressive disease progression (91, 92). The role of EZH2 in CLL has yet to be examined *in vitro* and *in vivo*. Another target of miR-30 is B-Myb, which expression can be regulated by miR-30 and miR-29 during cellular senescence (95). B-Myb, a transcription factor, is involved in cell proliferation and transcription and carcinogenesis. B-Myb overexpression presents in various cancers and is associated with aggressive tumor growth and poor outcomes (96-98). However, the role of B-Myb in CLL is still unknown. Both EZH2 and B-

Myb are upregulated in mouse CLL cells with p53 deletion as shown in Figure 16. The EZH2 inhibitor 3-deazaneplanocin (DZNep) led to induction of more apoptosis in splenocytes isolated from *TCL1*-Tg:*p53*$^{-/-}$ mice compared to that of *TCL1*-Tg mice (Figure 17). Taking together, this study provides evidence to support the important role of p53→miR30d→EZH2 & B-Myb axis in the development of aggressive CLL.

Figure 15A-15B

A



B

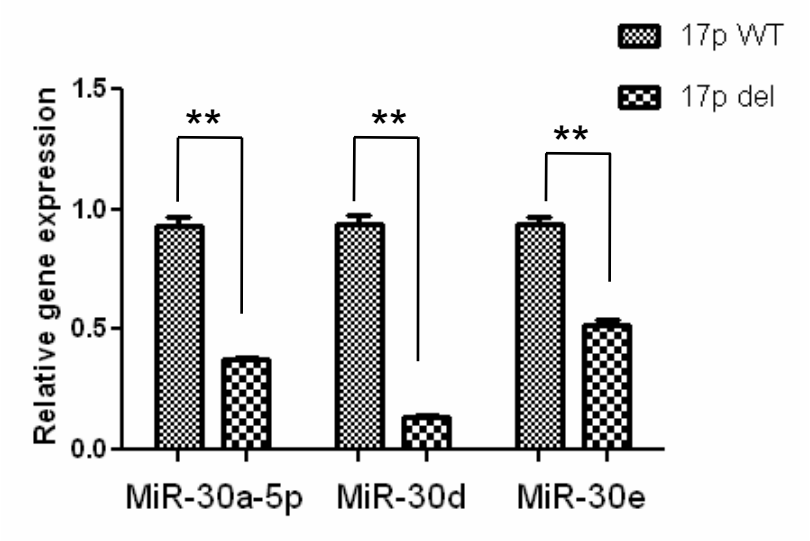
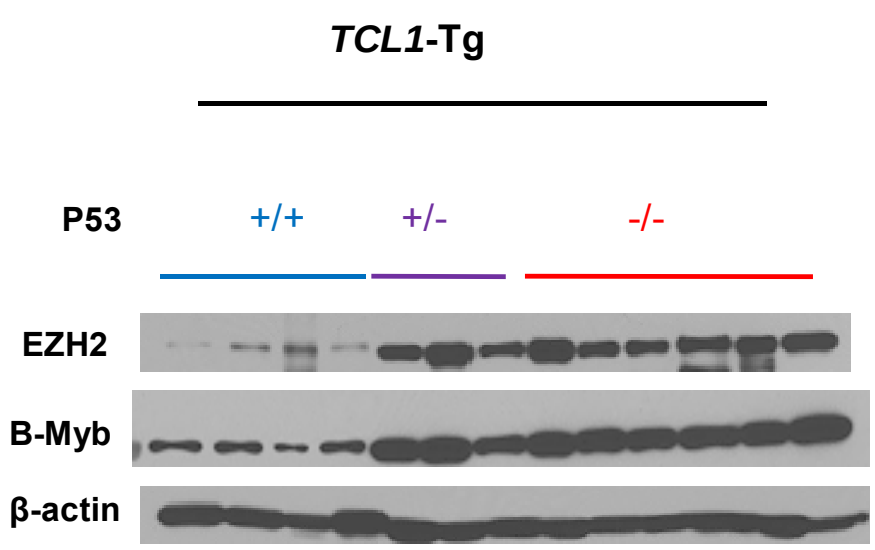


Figure 15. Expression of tumor suppressor miR-30 family in mouse and human leukemia cells. (A) MiR-30a, miR-30d and miR-30e expression in mouse splenocytes and PC cells shown by RT-PCR. *, $p<0.05$, **, $p<0.01$ between groups. (B) MiR-30a, miR-30d and miR-30e expression in CLL cells from human patient samples with or without 17p deletion shown by RT-PCR. *, $p<0.05$, **, $p<0.01$ between groups.

Figure 16A-16B

A



B

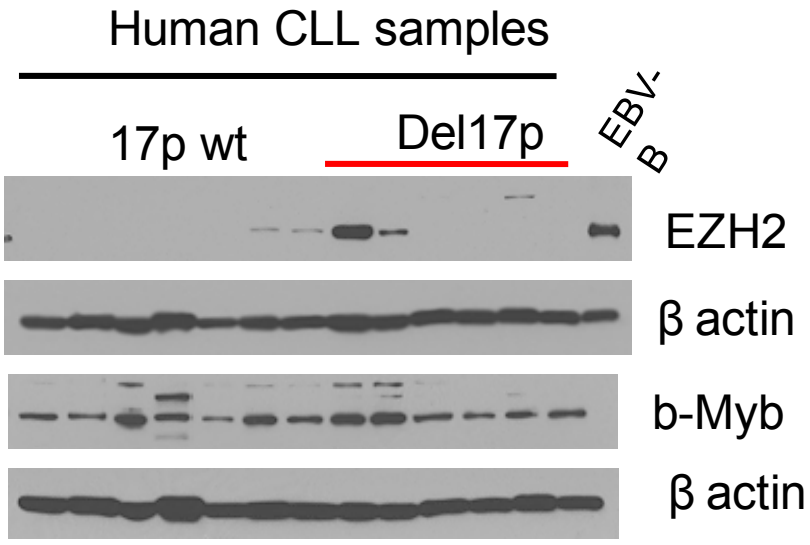


Figure 16. Expression of EZH2 and B-Myb in mouse and human CLL cells. (A) Expression of EZH2 and B-Myb in splenocytes from 3 *TCL1*-Tg mice, 3 *TCL1*-Tg:*p53*$^{+/-}$ mice, and 6 *TCL1*-Tg:*p53*$^{-/-}$ mice. (B) Expression of EZH2 and B-Myb in 7 human CLL samples with 17p wt and 6 CLL samples with 17p deletion.

Figure 17

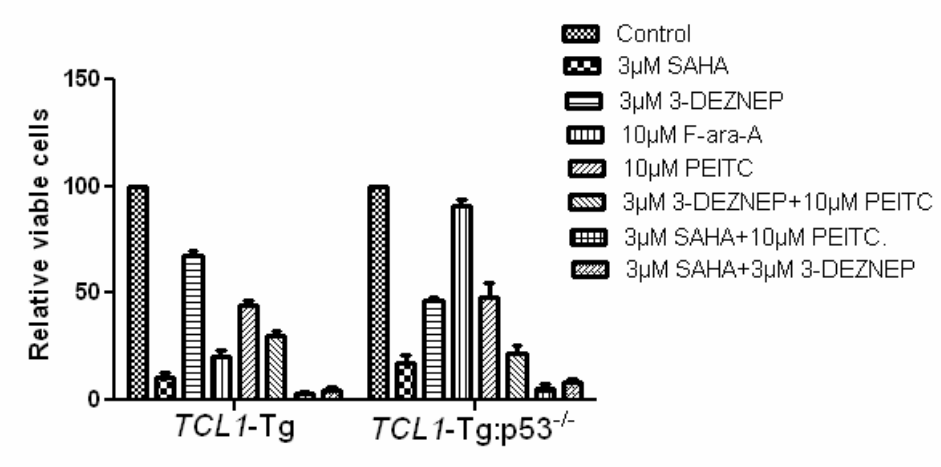

Control
3μM SAHA
3μM 3-DEZNEP
10μM F-ara-A
10μM PEITC
3μM 3-DEZNEP+10μM PEITC
3μM SAHA+10μM PEITC.
3μM SAHA+3μM 3-DEZNEP
Relative viable cells
150
100
50
0
TCL1-Tg
TCL1-Tg:p53-/-

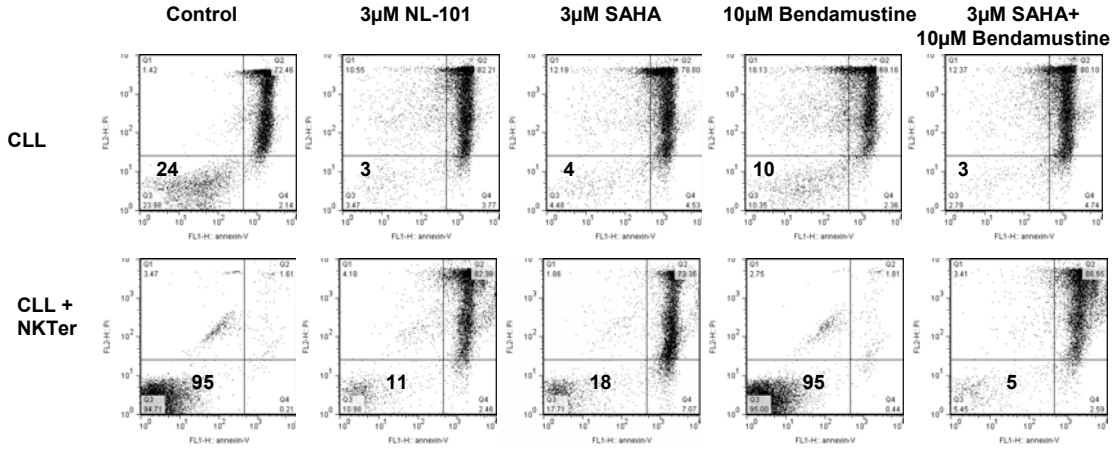
Figure 17. DZNep treatment in mouse splenocytes. Splenic cells from 3 *TCL1*-Tg and 3 *TCL1*-Tg:*p53*$^{-/-}$ mice were cultured in RPMI1640 medium with 10% FBS plus 1% PS and treated with 3uM SAHA, 3uM 3-DEZNP, 10uM F-ara-A or their combination for 48h. PEITC treatment was only 4 h. Annexin V-PI analysis by FACS was performed to detect apoptosis for those splenocytes.

4.6 New compound NL-101 induces significant apoptosis in CLL cells with or without p53 deficiency. Despite of the fact that various advanced therapeutic strategies have improved the CLL treatment in clinic, the disease is not cured yet. As shown in Figures 5-8, CLL cells with p53 deficiency were resistant to conventional CLL chemotherapies such as F-ara-A. Therefore it is urgent to study new therapeutic strategies for the treatment of the subtype of CLL with p53 deficiency. It was observed that new compound NL-101 induced significant apoptosis in CLL cell with 17p wt (Figure 18). NL-101 contains both the HDACI-SAHA and the alkylating agent-Bendamustine moieties in 1:1 ratio. 48h treatment of 3μM NL-101 induced around 85% apoptosis in CLL cells cocultured with NKTert cells. 48h treatment of 3μM SAHA only induced about 75% apoptosis in the CLL cells when cocultured with stromal cells NKTert. However, 10μM Bendamustine didn't induce any apoptosis in the CLL cells cocultured with NKTert cells. The combination of 3μM SAHA + 10μM Bendamustine almost killed all CLL cells even cocultured with NKTert cells. Without coculture, those compounds only or their combination efficiently induced apoptosis in the CLL cells (Figure 18A). The bar graph in Figure 18B showed efficient killing effect of NL101 or SAHA combined with alkylating agents in CLL cells with or without coculture with stromal cells. Interestingly, NL-101 and PEITC both can induce significant apoptosis in CLL cells with 17p deletion (Figure 19). As mentioned before, a subtype of CLL with chromosome 17p deletion is associated with accelerated disease progression, drug resistance, and poor prognosis due to loss of p53 gene (100). In our studies, we observed

that 4h 10μM PEITC treatment or 48h 6μM NL-101 treatment only induced significant apoptosis in the subtype of CLL cells with 17p deletion with or without coculture with stromal NKTert cells. Similarly SAHA combined with Chlorambucil or Bendamustine also induced significant apoptosis in this subtype of CLL with or without coculture with stromal NKTert cells. The NL-101 compound combined with PEITC almost killed all of this subtype of CLL cells as shown in Figure 21. Consistently the compound NL-101 also induced significant apoptosis in mouse CLL cells when cocultured with stromal cells. Interestingly not only NL-101 treatment but also SAHA treatment only induced significant apoptosis in mouse splenocytes isolated from *TCL1*-Tg and *TCL1*-Tg:*p53*$^{-/-}$ mice (Figure 20).

Figure 18A-18B

A



B

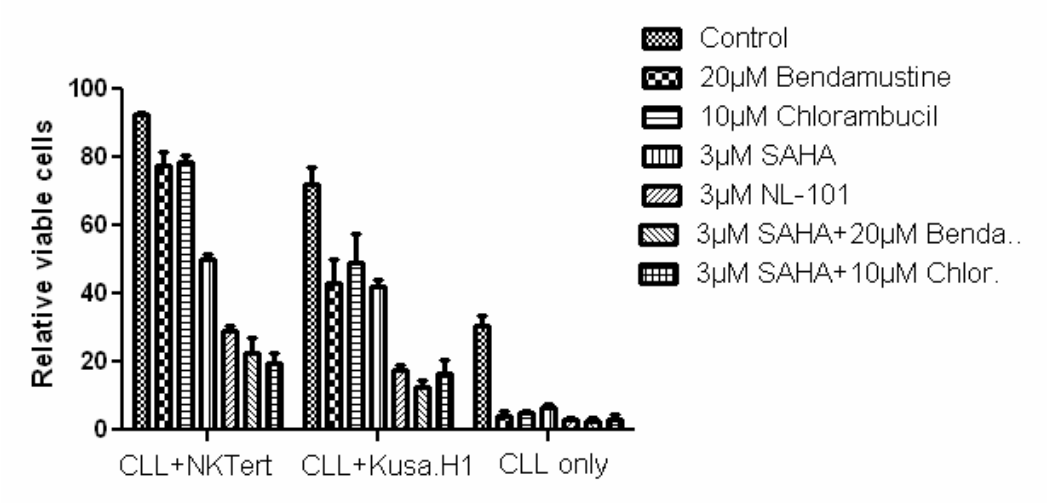


Figure 18. NL-101 treatment in human CLL cells with 17p wt. White blood cells were isolated from CLL patients' blood and cultured in RPMI1640 medium containing 10% FBS and 1% PS. (A) CLL patient samples with 17p wt were treated with 3uM NL-101, 3uM SAHA, 10uM Bendamustine and 3uM SAHA+10uM Bendamustine for 48h when cocultured with human stromal cells NKTert or without coculture. Cell apoptosis was analyzed by Annexin V-PI staining. (B) The quantitative bar graph showed the treatment results analyzed by Annexin V-PI staining in human CLL cells cocultured with stromal cells NKTert or Kusa.H1 or without coculture.

Figure 19

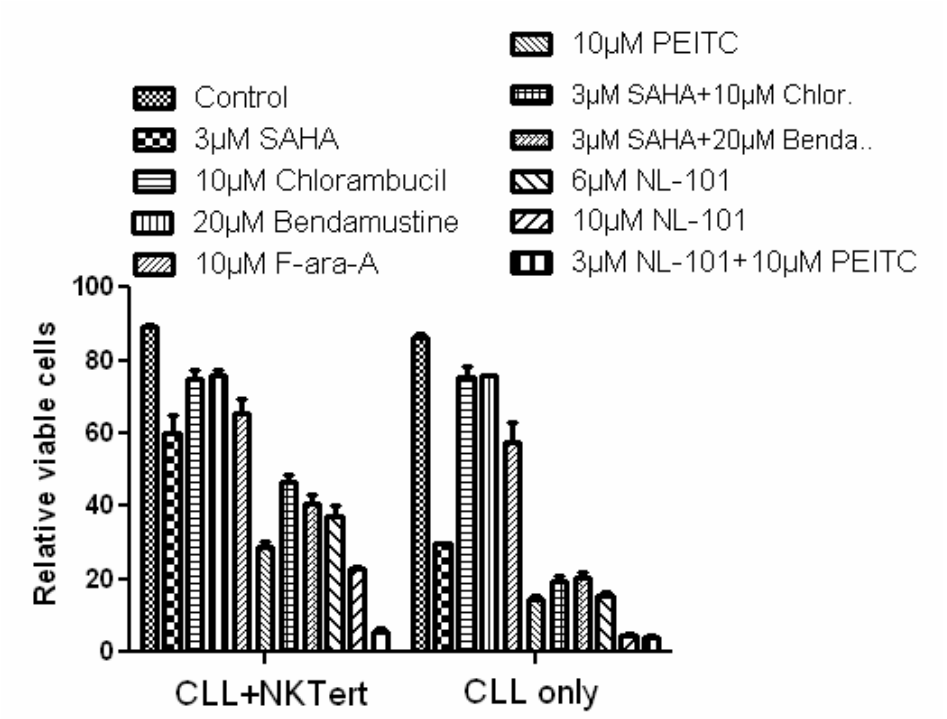

Control
3μM SAHA
10μM Chlorambucil
20μM Bendamustine
10μM F-ara-A
10μM PEITC
3μM SAHA+10μM Chlor.
3μM SAHA+20μM Benda..
6μM NL-101
10μM NL-101
3μM NL-101+10μM PEITC
Relative viable cells
100
80
60
40
20
0
CLL+NKTert
CLL only

Figure 19. NL-101, PEITC and other treatments in human CLL cells with 17p deletion 7. White blood cells were isolated from CLL patients' blood and cultured in RPMI1640 medium containing 10% FBS and 1% PS. CLL patient samples with 17p deletion were treated with 3uM SAHA, 10uM Chlorambucil, 20uM Bendamustine, 10uM F-ara-A, 6uM or 10uM NL-101 and their combinations for 48h with or without coculture with stromal NKTert cells. 10uM PEITC treatment was only 4h. Cell apoptosis was analyzed by Annexin V-PI staining.

Figure 20

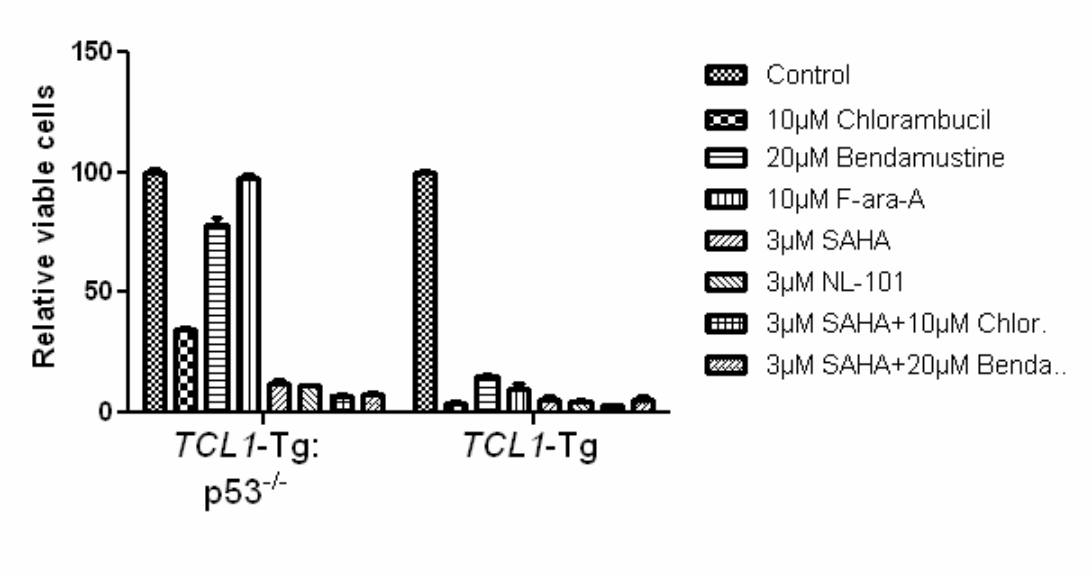

Relative viable cells
150
100
50
0
TCL1-Tg: p53-/-
TCL1-Tg
Control
10μM Chlorambucil
20μM Bendamustine
10μM F-ara-A
3μM SAHA
3μM NL-101
3μM SAHA+10μM Chlor.
3μM SAHA+20μM Benda..

Figure 20. NL-101 treatment in mouse CLL cells with or without p53 deficiency. Splenic cells from *TCL1*-Tg and *TCL1*-Tg:*p53*$^{-/-}$ mice were cultured in RPMI1640 medium with 10% FBS plus 1% PS and treated with 10uM F-ara-A or Chlorambucil, 20uM Bendamustine, 3uM SAHA or SAHA combined with the alkylating agents and 3uM NL-101 for 48h when cocultured with stromal Kusa.H1 cells. Annexin V-PI analysis by FACS was performed to detect apoptosis for those splenocytes.

4.7 NL-101 and PEITC treatment prolonged the survival time for *TCL1*-Tg:*p53*$^{-/-}$ mice. Since we observed efficient killing effects of NL-101 and PEITC in CLL cells with p53 deficiency *in vitro*, the NL-101 and PEITC treatment *in vivo* were performed using intravenous injection (i.v. injection). Tail inflammation was observed after 6 times of iv injection for some *TCL1*-Tg:*p53*$^{-/-}$ mice. As shown in Figure 21, only 6 times i.v. injection of NL-101 prolonged the survival time of *TCL1*-Tg:*p53*$^{-/-}$ mice. PEITC treatment significantly prolonged the survival time for the *TCL1*-Tg:*p53*$^{-/-}$ mice (Figure 21).

Figure 21

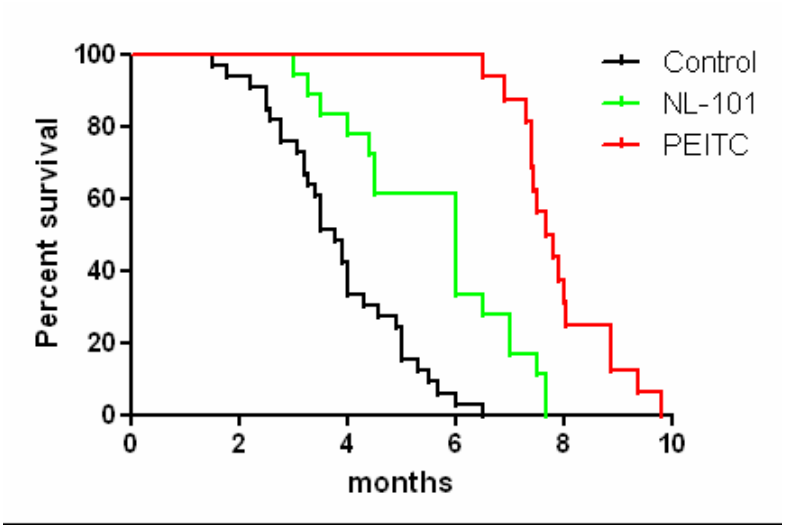

Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
months
Control
NL-101
PEITC

Figure 21. Survival curve (Kaplan-Meier) of *TCL1*-Tg:*p53*$^{-/-}$ mice treated with NL-101 or PEITC (n=20 per group). NL-101 treatment plan: 2 times/month i.v. injection of 6mg/ml for 3 months; PEITC treatment plan: i.v. injection of Nano-PEITC weekly by 10mg/ml (1mg/10g weight) concentration.

4.8 Underlying mechanisms of NL-101 induced apoptosis in CLL cells.

After observing significant killing effect of NL-101 in CLL cells, we hypothesized that the HDACI-moiety of NL-101 can overcome resistance of the alkylating agent-moiety in CLL treatment. More DNA strand breaks were observed in CLL cells treated with NL-101 only or SAHA combined with Bendamustine than Bendamustine treatment only analyzed by comet assay (Figure 22). In addition, HDACI such as SAHA can overcome resistance of alkylating agents in CLL treatment by reducing CXCL12 secreted from stromal cells. RT-PCR experiments showed reduced levels of CXCL12 in stromal NKTert cells treated with HDACI or NL-101 (Figure 23). In Figure 24, ELISA assay showed significant decrease of CXCL12 levels in Kusa.H1 and NKTert cells treated by HDACI such as SAHA and LBH. Interestingly the CXCL12 level was very low in stromal HS5 cells.

Figure 22

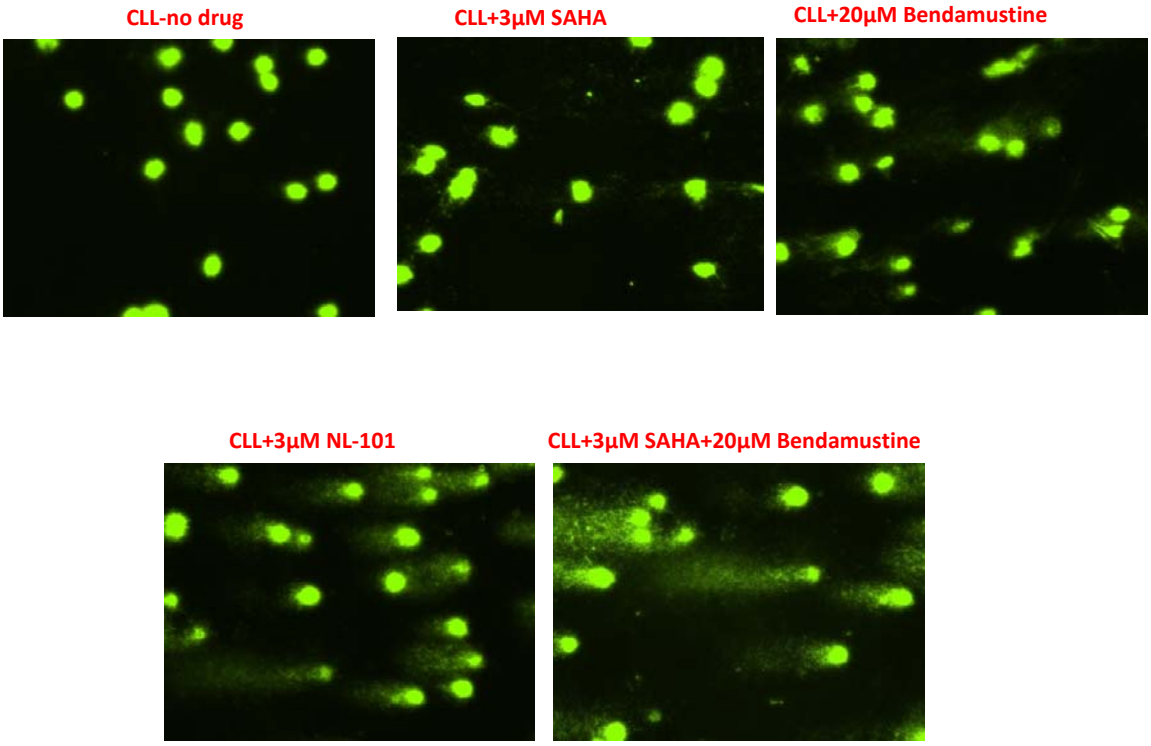
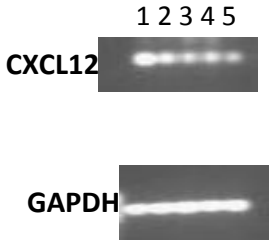

CLL-no drug
CLL+3µM SAHA
CLL+20µM Bendamustine
CLL+3µM NL-101
CLL+3µM SAHA+20µM Bendamustine

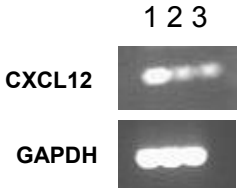
Figure 22. DNA strand breaks in CLL cells analyzed by Comet assay. CLL cells were treated with 3μM SAHA, 3μM NL-101, 20μM Bendamustine or 3μM SAHA+20μM Bendamustine for 24h. The comet assay was performed as described above.

Figure 23

RT-PCR:



1. NKTert
2. NKTert+3μM SAHA
3. NKTert+6μM SAHA
4. NKTert+3μM LBH
5. NKTert+6μM LBH



1. NKTert
2. NKTert+3μM SAHA+10μM Chlorambucil
3. NKTert +3μM NL-101

Figure 23. CXCL12 mRNA levels in NKTert cells treated with HDACI, HDACI combined with alkylating agents or NL-101 analyzed by RT-PCR. $1X10^5$ NKTert cells were seeded in a 6-well plate. The HDACI or NL-101 treatment was performed when the cells were 70% confluent. 48h later, the cells were collected and prepared for RNA isolation, cDNA synthesis and RT-PCR.

Figure 24

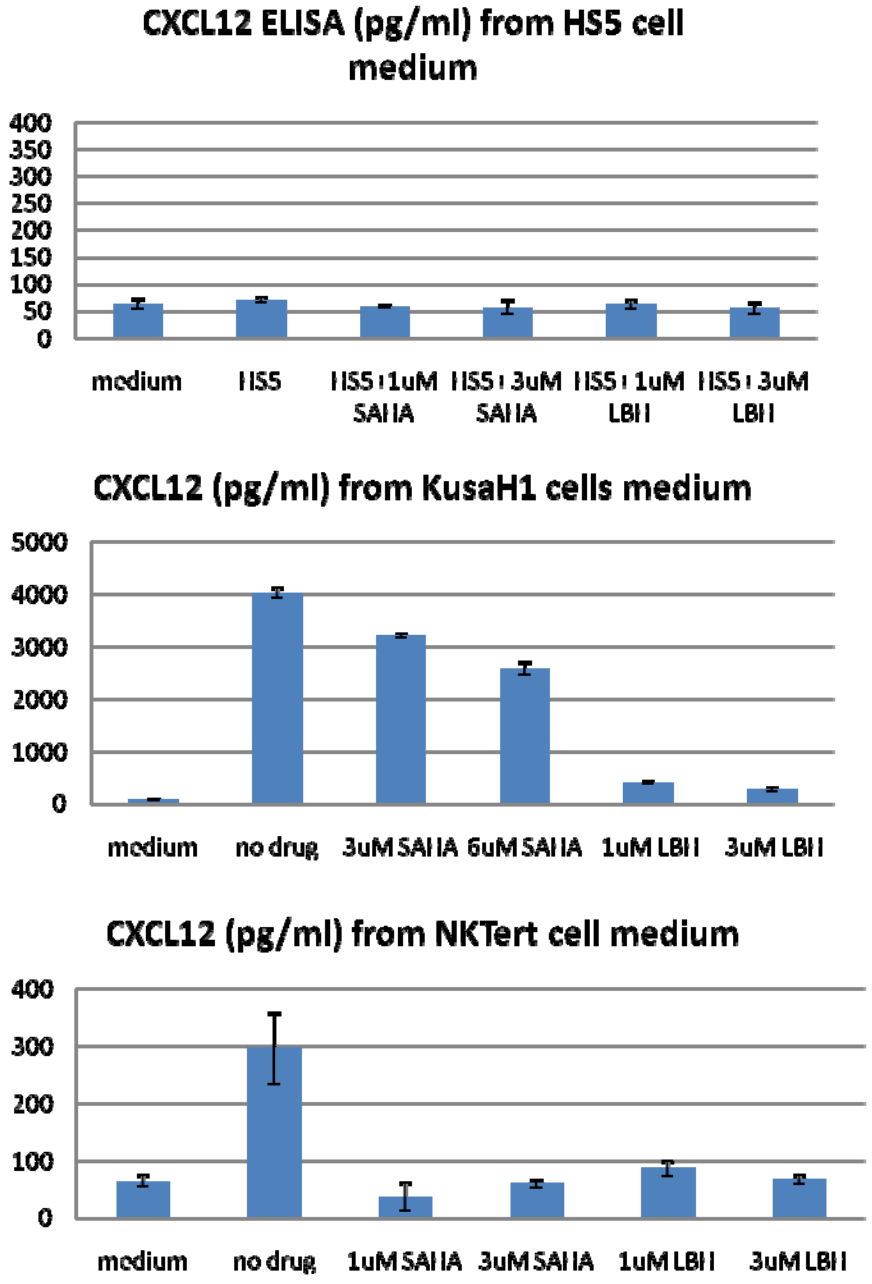

CXCL12 ELISA (pg/ml) from HS5 cell medium
400
350
300
250
200
150
100
50
0
medium
HS5
HS5 + 1uM SAHA
HS5 + 3uM SAHA
HS5 + 1uM LBH
HS5 + 3uM LBH
CXCL12 (pg/ml) from KusaH1 cells medium
5000
4000
3000
2000
1000
0
medium
no drug
3uM SAHA
6uM SAHA
1uM LBH
3uM LBH
CXCL12 (pg/ml) from NKTert cell medium
400
300
200
100
0
medium
no drug
1uM SAHA
3uM SAHA
1uM LBH
3uM LBH

Figure 24. CXCL12 levels in stromal HS5, Kusa.H1 and NKTert cells treated with SAHA or LBH determined by ELISA assay. $6X10^5$ HS5 cells, $1X10^4$ Kusa.H1 cells and $4X10^4$ NKTert cells were seeded in 24-well plates with RPMI1640 medium at day 1. At day 3, the medium was removed and 500μL fresh medium was added for each well. HDACI treatment was performed at day 3. ELISA assay was performed at day 4.

5. DISCUSSION

Recent progress in investigation of CLL biology and the development of new therapies such as F-ara-A-based regimens has led to significant improvements of therapeutic outcomes. However, many CLL patients, particularly those with loss of p53 function due to chromosome 17p deletion and p53 mutations, are refractory to the current therapeutic regimens with poor prognosis (56). Loss of p53 function can be due to deletion or mutations of the gene that encodes for p53, epigenetic silencing, and functional inactivation. In CLL, chromosome 17p deletion and p53 mutations are well-documented mechanisms that lead to loss of p53 function associated with poor prognosis (56, 116). The exact mechanisms by which loss of p53 may lead to aggressive disease progression and poor clinical outcomes in CLL remain illusive. Based on the important role of p53 in cell cycle control and regulation of apoptosis (117), it is generally speculated that loss of p53 function may result in impairment of cycle-cycle checkpoints and compromised apoptotic response, leading to disease progression and drug resistance. However, there is no conclusive evidence *in vivo* to support this notion, perhaps in part due to the lack of proper CLL animal model with loss of p53.

5.1 Rapid disease progression and treatment resistance occurred in *TCL1*-Tg:*p53*$^{-/-}$ mice. In the present study, we generated a mouse colony with *TCL1*-Tg:*p53*$^{-/-}$ genotype, and demonstrated that these mice developed leukemia that resembles aggressive human CLL. The *TCL1*-Tg:*p53*$^{-/-}$ mice exhibited signs of CLL disease around 3 months, with early appearance

of $CD5^+/IgM^+$ cells in the PC and spleen. Most *TCL1*-Tg:$p53^{-/-}$ mice showed highly abnormal accumulation of WBC in the blood and developed severe splenomegaly at 3-4 month, and died before 6 months. This is in contrast with the *TCL1*-Tg mice, which develop CLL approximately at the age of 1 year, and the disease progresses slowly (60). In the *TCL1*-Tg:$p53^{-/-}$ mice, we observed a significant increase in lymphoid cell proliferation in the spleen and a decrease in apoptosis. This may explain why these mice had severe accumulation of leukemia cells and enlargement of the spleen at early age. The control $p53^{-/-}$ mice didn't accumulate much leukemia cells at 4-month old of age, however, our mouse model exhibited significant higher number of leukemia cells, suggesting that *TCL1*-Tg:$p53^{-/-}$ mice had specific CLL features rather than lymphoma occurring in $p53^{-/-}$ mice. Since p53 plays a key role in enhancing the expression of apoptotic molecules such as Bax and PUMA (118), loss of p53 function would significantly compromise this apoptotic pathway, leading to resistance to chemotherapeutic agents. In fact, we observed that the leukemia cells isolated from *TCL1*-Tg:$p53^{-/-}$ mice or from CLL patients with 17p deletion were highly resistance to standard anti-CLL drugs F-ara-A and oxaliplatin. Furthermore, CD19 positive B cells purified from CLL patients with 17p deletion were highly resistance to F-ara-A and oxaliplatin. To overcome treatment resistance, new therapeutic strategies need to be applied in clinic such as PEITC and NL-101 compound.

5.2 Up-regulated Mcl-1 in leukemia cells with p53 deletion. An important observation in this study was the significant up-regulation of Mcl-1

expression in the leukemia cells lacking p53. This was seen both in the leukemia cells from the *TCL1*-Tg:*p53*$^{-/-}$ mice and in primary CLL cells from patients with 17p deletion (Figure 9 & 10). Mcl-1 is a key anti-apoptotic protein in the Bcl-2 family, and this molecule is known to be particularly important for the survival of CLL cells (85). Thus, upregulation of Mcl-1 may play a major role in apoptosis resistance in CLL cells lacking *p53*, and the moderate increase in Bcl-XL and Bcl-2 expression may also contribute to the increased viability of leukemia cells in *TCL1*-Tg:*p53*$^{-/-}$ mice. Since the increase in Mcl-1 expression was observed at mRNA and protein levels, it is likely that loss of p53 may promote Mcl-1 expression mainly at the transcriptional level. This is consistent with the observation that p53 transcriptionally represses Mcl-1 (119-122).

5.3 Down-regulated miR-15a/miR-16-1 in leukemia cells with p53 deletion. Interestingly, the miRNA expression levels of miR15a and miR-16-1 were significantly decreased in CLL cells with loss of p53. This was observed both in the *TCL1*-Tg:*p53*$^{-/-}$ mouse model and in primary CLL cells isolated from patients with 17p deletion. Because Mcl-1 is a target of miR-15a and miR-16-1 (123, 124), the decrease in these miRNAs would release their suppression on Mcl-1 and thus lead to its elevated expression. Thus, it is possible that p53 might regulate Mcl-1 expression through modulating miR15a/16. It has been shown previously that miRNAs may be involved in CLL pathogenesis and prognosis due to their function as oncogenes or tumor suppressors (102). For example, miR-15a/miR-16-1 is located in chromosome 13q14.3, a region that is

frequently mutated or deleted in CLL patients and may affect CLL cell survival and drug resistance (115). Intriguingly, p53 may regulate the expression of multiple miRNAs, many of which are closely involved in cell cycle regulation, proliferation and apoptosis (104). Our initial study using miRNA array to examine the effect of loss of *p53* on miRNA expression in the *TCL1*-Tg:*p53*$^{-/-}$ mice had identified miR-15a and miR-16-1 being significantly down-regulated, which was further validated by real-time RT-PCR. Clearly, miR-15a/16-1 expression is significantly lower in splenocytes and PC cells from *TCL1*-Tg:*p53*$^{-/-}$ mice than that of *TCL1*-Tg mice. These observations together suggest that p53→miR-15a/16-1→Mcl-1 axis may be an important pathway in regulating CLL cell apoptosis and drug resistance. The important role of the p53→miR-15a/16-1→Mcl-1 axis in the development of aggressive CLL merits further investigation in clinical setting.

5.4 Down-regulated miR-30d in leukemia cells with p53 deletion. Our initial study using miRNA array to examine the effect of loss of p53 on miRNA expression in the *TCL1*-Tg:*p53*$^{-/-}$ mice had identified miR-30a, miR-30e and particularly miR-30d being significantly down-regulated, which was further validated by real-time RT-PCR. Clearly, miR-30a/miR-30d/miR-30e levels were significantly lower in splenocytes and PC cells from *TCL1*-Tg:*p53*$^{-/-}$ mice than that of *TCL1*-Tg mice. It has been reported that EZH2 and B-Myb are targets of miR-30 family (90, 95). Since we observed significant up-regulation of EZH2 and B-Myb in leukemia cells with p53 deletion, p53→miR-

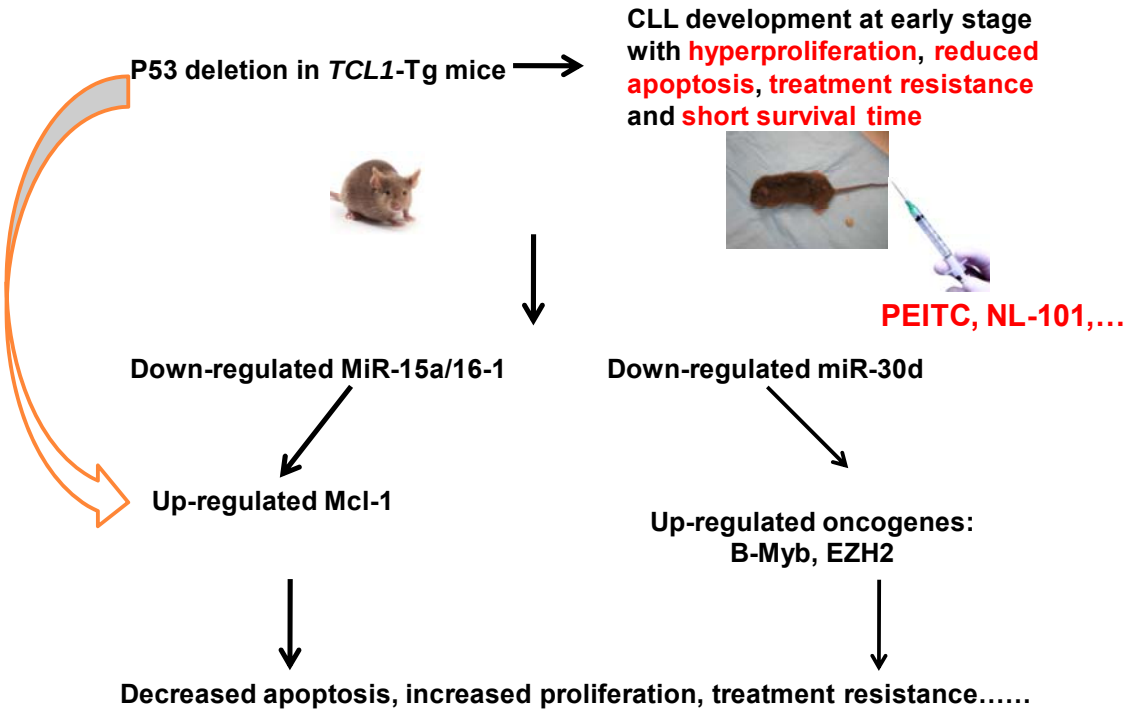
miR30→EZH2 & B-Myb axis may be another important pathway in regulating CLL cell apoptosis and drug resistance.

6. SUMMARY AND CONCLUSIONS

The whole study can be summarized in the following diagram. I have generated *TCL1*-Tg:*p53*$^{-/-}$ mice, which develop aggressive CLL with an early disease onset, likely due to increase proliferation of the leukemia cells and decrease in apoptosis. The CLL cells lacking p53 exhibited low sensitivity to standard anti-CLL drugs. In our study, we tested new compound NL-101 and PEITC in our mouse model. It has been reported that PEITC effectively eliminates F-ara-A-resistant CLL cells through ROS accumulation and glutathione depletion (45). We observed that PEITC was very efficient to eliminate CLL cells both *in vitro* and *in vivo*. The underlying mechanism for efficient killing effect of NL-101 in CLL is possible through HDAC inhibitor-overcoming the treatment resistance of alkylating agents by inhibiting CXCL12 and inducing more DNA damage to CLL cells. Our study also provided *in vivo* evidence that loss of p53 led to upregulation of Mcl-1 expression, probably though the down regulation of miR-15a and miR-16-1 and thus released their suppression on Mcl-1 expression. Another possibility is that p53 transcriptionally represses Mcl-1, leading to decrease in apoptosis and drug resistance in CLL cells from *TCL1*-Tg:*p53*$^{-/-}$ mice. Furthermore, loss of p53 cause down-regulation of miR-30 family especially miR-30d which regulates up-regulation of EZH2 & B-Myb, probably leading to rapid disease progression

and treatment resistance in leukemia cells lacking p53. The *TCL1*-Tg:*p53*$^{-/-}$ mouse colony may serve as a valuable mouse model to further investigate the pathogenesis of aggressive CLL due to loss of p53 function. In addition, since these mice develop leukemia at early age and die within 6 months, this animal model may be useful in testing new drugs for their in *vivo* therapeutic activity against aggressive CLL with loss of p53 function.

Summary


P53 deletion in *TCL1*-Tg mice
CLL development at early stage with hyperproliferation, reduced apoptosis, treatment resistance and short survival time
PEITC, NL-101,…
Down-regulated MiR-15a/16-1
Down-regulated miR-30d
Up-regulated Mcl-1
Up-regulated oncogenes: B-Myb, EZH2
Decreased apoptosis, increased proliferation, treatment resistance……

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Vita

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