SIRT3 overexpression and epigenetic silencing of catalase regulate ROS accumulation in CLL cells activating AXL signaling axis

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Abstract
Mitochondrial metabolism is the key source for abundant ROS in chronic lymphocytic leukemia (CLL) cells. Here, we detected significantly lower superoxide anion (O2−) levels with increased accumulation of hydrogen peroxide (H2O2) in CLL cells vs. normal B-cells. Further analysis indicated that mitochondrial superoxide dismutase (SOD)2, which converts O2− into H2O2 remained deacetylated in CLL cells due to SIRT3 overexpression resulting its constitutive activation. In addition, catalase expression was also reduced in CLL cells suggesting impairment of H2O2-conversion into water and O2 which may cause H2O2-accumulation. Importantly, we identified two CpG-islands in the catalase promoter and discovered that while the distal CpG-island (−3619 to −3765) remained methylated in both normal B-cells and CLL cells, variable degrees of methylation were discernible in the proximal CpG-island (−174 to −332) only in CLL cells. Finally, treatment of CLL cells with a demethylating agent increased catalase mRNA levels. Functionally, ROS accumulation in CLL cells activated the AXL survival axis while upregulated SIRT3, suggesting that CLL cells rapidly remove highly reactive O2− to avoid its cytotoxic effect but maintain increased H2O2-level to promote cell survival. Therefore, abrogation of aberrantly activated cell survival pathways using antioxidants can be an effective intervention in CLL therapy in combination with conventional agents.

Introduction
CLL is an adult B-cell malignancy with highly variable disease course1 and unpredictable response to therapeutic agents. Reactive oxygen species (ROS) have been identified as signaling molecules in various pathways regulating cell survival. Oxidants such as H2O2 are connected to lymphocyte activation while the molecular mechanisms are less clear2.

The main physiological function of mitochondria is the production of ATP by oxidative phosphorylation and the essential metabolites to accomplish the bioenergetics and biosynthetic demands of cells3. A fundamental observation in biology is that mitochondrial function, as measured by increased ROS, changes with age, suggesting a potential mechanistic link between the cellular processes governing longevity and mitochondrial metabolism homeostasis. Thus, mitochondrial dysfunction inevitably enhances ROS production resulting in oxidative stress, which are observed in multiple age-related illnesses including cancer4. Increased ROS levels promote genetic instability and development of drug resistance, harness cell signaling5,6, and altogether account for cancer cells’ aggressive behavior7. Thus, a challenge for novel therapeutic strategies will be the fine tuning of intracellular ROS signaling to effectively deprive cells from ROS-induced tumor promoting events, towards tipping the balance to ROS-induced apoptotic signaling or ROS inhibition using antioxidants.
PI3K/AKT activation via ligation of growth factors to their cellular receptors is associated with increased oxygen consumption as well as an increase in total cellular ATP derived from both glycolytic and oxidative sources in mitochondria resulting in accumulation of ROS while other factors may also play a role. Among the mitochondrial Sirtuins, SIRT3 with NAD+-dependent protein deacetylase activity regulates the production of ROS via the electron transport chain, as well as the detoxification of ROS through activation of antioxidant enzymes. SODs are a class of enzymes that catalyze the detoxification of O$_2^-$ into O$_2$ and H$_2$O$_2$, followed by conversion of H$_2$O$_2$ into O$_2$ and water by catalase. Mitochondrial SOD2 is an antioxidant enzyme and plays a crucial role in controlling ROS production. SIRT3 lowers ROS levels by deacetylating and increasing enzymatic activity of SOD2. Elevated ROS, on the other hand, activates SIRT3 expression to deacetylate SOD2 and thus clearing ROS. Furthermore, SIRT3 interacts with Forkhead box O (FOXO)3a transcription factor to activate SOD2 and catalase gene transcription, suggesting the existence of a functional interplay between SIRT3/SOD2/catalase and accumulation of ROS.

Cancer cells including leukemic cells live under oxidative stress, which may lead to cell proliferation and transformation. Several studies have identified mitochondrial metabolism as the key source for ROS accumulation in CLL which, on the other hand, is linked to CLL cell survival. However, the precise mechanism of ROS elevation and activation of survival pathway(s) in CLL remain largely undefined. In this study, we have shown that overexpression of SIRT3 and variable degrees of promoter methylation of the catalase gene may impair ROS clearance leading to accumulation of H$_2$O$_2$ in CLL cells which, on the other hand, activates the AXL/AKT/ERK and fibroblast growth factor receptor (FGFR) cell survival signaling axes. Of relevance, earlier, we have shown that CLL cells express AXL as a constitutively active receptor tyrosine kinase (RTK), albeit at differential levels, and acts as a docking site of multiple signal mediators including PI3K/AKT, Lyn, SYK/ZAP70 and phospholipase C (PLC)γ2, regulating CLL cell survival. In addition, AXL acts as an upstream regulator of FGFR signal in CLL cells.

Materials and methods

Reagents

Phospho-tyrosine antibody 4G10 (Millipore); Actin, AXL, β-tubulin, GAPDH antibodies (Santa Cruz Biotechnologies) and fluorescence-conjugated antibodies to CD5 and CD19 (BD Biosciences) were used in this study. All other antibodies were obtained from Cell Signaling Technologies. Dihydroethidium (DHE) and 2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Molecular Probes); EpiMark Bisulfite conversion Kit (New England BioLabs); IL-2/IL-15 (Peprotech); demethylating agent, 5′-aza-deoxycytidine (AZA), RNA isolation kit, H$_2$O$_2$, Trolox (Sigma); mitochondria isolation kit (ThermoScientific); AXL-inhibitor SGI-7079 (Selleckchem); catalase and GAPDH primers (SABiosciences) were also purchased. CpG oligodeoxynucleotides (ODN) were synthesized in the Mayo Clinic Genomic Core facility.

Sample size and isolation/purification of primary cells

Blood samples from a total of 67 previously untreated CLL patients and 30 age-matched, normal healthy subjects were obtained for this study. Samples from normal individuals (N) or CLL patients (P) were assigned arbitrary numbers and presented in Supplementary Table S1. The mean age for the 67 CLL patients was 67.7 years (standard deviation of 10.0, range 44–91 years). Among the CLL patients 73% (49/67) were male, 22% (14/65) were Rai Stage III or IV, 63% (29/46) had IGHV mutation, 30% (15/50) ZAP70 positive (≥20%), 27% (16/60) CD38 positive (≥20%), and 23% (14/61) normal FISH. CLL samples were randomly selected for each experiment based on cell availability but not based on any patient characteristics or experiment outcomes. All patients provided written informed consent according to the Declaration of Helsinki to the OUHSC and Mayo Clinic Institutional Review Boards, which approved these studies.

Primary CLL cells or normal B-cells were purified from blood of CLL patients or healthy individuals, respectively, using RosetteSep B-cell enrichment kit (STEMCELL). Typical purification range of CD5+/CD19+ CLL cells was ≥95–99% and >95% pure CD19+ B-cells were obtained from normal blood.

Cell culture

CLL cells were cultured for optimum viability in serum-free AIM-V (GIBCO) medium. A CLL-derived cell line (MEC1), diffuse large B-cell lymphoma cell lines (DHL2, DHL6, and LY3) and a mantle cell lymphoma cell line (Mino) were purchased from ATCC. Cell lines were tested for Mycoplasma negativity before use.

Measurement of H$_2$O$_2$ and O$_2^-$

1–2 × 10$^6$ purified, normal B-cells or CLL cells were incubated with CM-H$_2$DCFDA (10 μM) to detect H$_2$O$_2$ or DHE (5 μM) for O$_2^-$ at 37°C for 1 h, followed by flow cytometric analysis (BD Canto II).

Immunoprecipitation and western blot analysis

Tyrosine-phosphorylated proteins or AXL were immunoprecipitated from CLL cell lysates (0.2 mg) using 4G10 or an AXL-specific antibody, respectively, followed by
Results

Methylation study

Promoter methylation status of the catalase gene was analyzed by PCR-based methylation-specific PCR (MSP) after bisulfite modification of genomic DNA using a bisulfite conversion Kit. All the primers (Supplementary Table S2) were designed using ‘Meth Primer’ online primer designing tool23. Briefly, 1 µg of genomic DNA isolated from CLL cells or normal B-cells was modified by bisulfite reaction, followed by PCR using methylation/ unmethylation-specific (M/U) primer sets. PCR products were analyzed on agarose gels.

CG-methylation status of the catalase promoter was further analyzed by sequencing the PCR products of the bisulfite-converted genomic DNA from normal B-cells (n = 5), CLL cells (n = 32), and B-cell lymphoma cell lines. Briefly, PCR products were purified from gels, sequenced (OUHSC DNA Sequencing facility) and scored for methylation in each 'CG' site as one allele or both allele or no methylation at both the alleles depending on the presence of both C/T peak or only C peak or only T peak in chromatogram, respectively. Catalase promoter methylation status of individual samples was calculated as percent (%) of methylated CG sites.

Treatment of cells with 5'-azacitidine and BCR stimulation

MEC1/DHL6 cells (0.5 × 10⁶ cells/mL) were treated with 1 µM AZA for 72 h, followed by release of treatment for 24 h or left untreated and total RNA was extracted. Similarly, primary CLL cells were treated with 2.5 µg/mL CpG ODN, 100 U/mL IL-2, and 10 ng/mL IL-15 with 1 µM AZA for 72 h or left untreated24 for RNA extraction. Catalase mRNA levels were determined by qRT-PCR.

For BCR stimulation, purified CLL cells (5.0 × 10⁶/mL) from treatment naïve CLL patients (P65 and P68) were treated in vitro with 10 µg/mL goat anti-human IgM antibody for 0, 15, 30, and 60 min or left untreated. Cell lysates were analyzed for the phosphorylation of BTK and expression of SIRT3 in western blots using specific antibodies.

Treatment of CLL cells with H₂O₂ and inhibitors of ROS, AXL, or BTK

CLL cells (5.0 × 10⁶/mL) pretreated with DMSO or sublethal dose of a high-affinity AXL inhibitor, SGI-707925 or a BTK inhibitor, ibrutinib (0.75 µM for 1 h) were exposed to H₂O₂ (0.6 mM) for 5 min26 or left untreated/unexposed. Cell lysates were prepared as described earlier20–22 to analyze the activation status of AXL, BTK, AKT, ERK1/2, and expression of SIRT3/SOD2 in immunoprecipitation/western blots using specific antibodies.

In few experiments, CLL cells were also treated with 50–100 µM of H₂O₂ or a ROS-inhibitor, Trolox (50 µM) for 4 h, and cell lysates were analyzed for P-AKT/P-ERK1/2 and SIRT3 expression in western blots.

Statistical analysis

A Wilcoxon test was calculated to compare the values of O₂⁻ levels (MFI) between normal B-cells and CLL cells since the data was not normally distributed. A two-tailed independent t-test for unequal variances (Satterthwaite) was calculated to compare the H₂O₂ accumulation between normal B-cells and CLL cells. The Spearman’s correlation test was calculated to evaluate the degree of CpG methylation in Island-I of the catalase promoter and its protein levels in CLL cells. PRIZM GraphPad software was used to create the statistical figures and for statistical analysis with the exception of the Spearman correlation coefficient that was calculated using SAS 9.4 (Cary, NC).

With 12 normal individuals and 27 CLL patients in ROS measurement group (Fig. 1, panels A, B), an independent t-test will have at least 80% power at the 5% significance level to detect an effect size of 1.0. Experiments were repeated for three times and presented the representative results, as appropriate.

Results

CLL cells generate increased levels of H₂O₂ with reduced O₂⁻ levels

Two of the most important ROS components generated in cells are O₂⁻ and H₂O₂. Thus, in an effort to assess the levels of O₂⁻ and H₂O₂, freshly isolated CLL cells (n = 27) and normal B-cells (n = 12) were treated with DHE or DCFDA to detect O₂⁻ and H₂O₂, respectively, by flow cytometry. While we detected significantly lower O₂⁻ levels (p < 0.0001) (Fig. 1A), increased accumulation of H₂O₂ (p = 0.0035) was discernible, albeit in variable...
Fig. 1 (See legend on next page.)
levels, in CLL cells (Fig. 1B) from the majority of CLL patients compared to normal B-cells under similar experimental conditions. Further analysis indicated that the levels of H₂O₂ accumulated in CLL cells were not associated with the known CLL prognostic factors including IGHV mutational status or ZAP70 positivity, although the sample size was small. Together, these findings suggest that compared to normal B-cells, (i) mitochondrial SOD2 may be highly active in CLL cells; while (ii) conversion of H₂O₂ into O₂ and water is likely impaired.

Mitochondrial SOD2 remains highly active in CLL cells

SOD2 is reported to have a crucial role in controlling the level of ROS, as mitochondria consume over 90% of intracellular oxygen and generate a large flux of ROS. Thus, to delineate the mechanism of why CLL cells generate lower levels of O₂⁻, we first assessed the mitochondrial SOD2 activation status. While oxidative stress-induced SOD2 expression is believed to be an important cellular defense mechanism, acetylation/deacetylation at the lysine-68 residue (K68) of SOD2 regulates its activity. Therefore, to define the acetylation status as a readout of SOD2 activation in CLL cells, mitochondrial fractions were isolated from purified CLL cells and normal B-cells. First, the expression of voltage-dependent anion channel (VDAC) protein, ubiquitously expressed and located in the outer mitochondrial membrane, was analyzed to assess the purity of the cytoplasmic/mitochondrial fractions isolated from purified normal B-cells (N1–N4) or CLL cells (P6, P10, and P11) in western blots using a specific antibody. Results suggested that the mitochondrial fractions used in these experiments were highly purified based on the presence of VDAC, while it was not detectable in the cytoplasmic fractions (Supplementary Fig. S1). Further analysis detected that the levels of acetylated-SOD2 at K68 were substantially reduced in the mitochondrial fractions of CLL cells as compared to that in normal B-cells (Fig. 1C), suggesting that SOD2 in CLL cells is likely enzymatically highly active converting O₂⁻ into H₂O₂ more efficiently. Importantly, we did not find any significant alteration of SOD2 levels between CLL cells and normal B-cells (Fig. 1C). Together, these findings may explain, at least in part, why CLL cells exhibiting lower levels of O₂⁻ compared to normal B-cells (Fig. 1A).

CLL cells overexpress SIRT3

As SIRT3 is known to deacetylate and activate SOD2, we next assessed SIRT3 expression status in CLL cells by western blots. Indeed, compared to normal B-cells, we detected CLL cells overexpressed SIRT3, albeit at variable levels (Fig. 1D). Of relevance, earlier studies suggested that pharmacological augmentation of mitochondrial ROS increases SIRT3 mRNA and protein levels in in vitro cell culture systems. Thus, we examined if ROS regulates SIRT3 levels in CLL cells. Indeed, inhibition of endogenous ROS in CLL cells reduced SIRT3 expression (Fig. 1E) while, in vitro treatment of leukemic B-cells with exogenous H₂O₂, which generates ROS, upregulated SIRT3 (Fig. 1F). Together, these findings indicate the existence of a positive feedback loop between ROS accumulation and SIRT3 expression in CLL cells.

Engagement of the B-cell receptor (BCR) in B-lymphocytes initiates a series of tightly controlled signaling events including activation of AKT, ERK1/2, and NF-κB pathways leading to transcription of genes responsible for B-cell development, survival and proliferation. As BCR signaling pathway has emerged as a key driver for the expansion of neoplastic B-cell clones and pathogenesis in several B-cell malignancies including CLL, we explored if in vitro activation of BCR signal in primary CLL cells could also induce SIRT3 expression. Indeed, we detected, enforced BCR-activation-induced SIRT3 expression in CLL cells (as early as in 15 min) in a time-dependent manner (Fig. 1G). In total, our findings...
suggest that while BCR signal upregulates SIRT3, elevation of ROS may maintain its constitutive level in CLL cells.

**CLL cells express reduced levels of catalase**

While we detected lower levels of O$_2^-$, an increased accumulation of H$_2$O$_2$ was also detected in CLL cells (Fig. 1B); suggesting that conversion of H$_2$O$_2$ into water and O$_2$ is likely to be impaired. As catalase enzyme catalyzes the conversion process of H$_2$O$_2$, its expression level is a critical factor for ROS clearance from the cells. Therefore, to define why CLL cells from some patients show increased H$_2$O$_2$ accumulation, catalase protein levels were assessed in CLL cells vs. normal B-cells in western blots. Indeed, reduced expression of catalase was detectable in CLL cells from majority of patients as compared to normal B-cells (Fig. 2A).

Next, to elucidate if reduced protein levels of catalase in CLL cells was a result of its reduced mRNA expression, we quantified catalase transcript levels in CLL cells from randomly chosen patients ($n = 10$) by qRT-PCR. Consistent with the catalase protein levels, we also detected reduced levels of catalase transcripts in CLL cells from majority of CLL patients (8 of 10) as compared to the mean value of catalase mRNA levels in normal B-cells ($n = 5$) (Fig. 2B); suggesting that catalase expression in CLL cells is likely regulated at the level of transcription.

Catalase is epigenetically regulated in CLL cells

To delineate the mechanism of reduced catalase mRNA levels in CLL cells, we first explored if catalase gene transcription was regulated epigenetically, for example, via methylation of the catalase promoter. Thus, we analyzed the entire catalase promoter for CpG-island prediction (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi)$^{23}$. Two predicted “CpG-islands” were identified$^{32}$: (i) Proximal (Island-I; nt −174 to −332) and (ii) Distal (Island-II; nt −3619 to −3765) containing 15 and 8 putative CpG methylation sites, respectively, in the catalase promoter (Fig. 3A–C). To find if these predicted methylation sites in the catalase promoter are methylated, genomic DNA from normal B-cells ($n = 5$) or CLL cells ($n = 32$) were bisulfite converted, PCR amplified and sequenced. Our results demonstrated methylation in the CpG-Island-II in both normal B-cells and CLL cells, while the CpG-Island-I of the catalase promoter exhibited variable degrees of methylation only in CLL cells, but not in normal B-cells (Fig. 4A–D).

Further analysis finds a significant ($p = 0.0162$) inverse relationship (Spearman correlation coefficient is $-0.85$) between the degree of CpG methylation in Island-I of the catalase promoter and its protein levels in CLL cells (Fig. 4E). In addition, our results also suggested variable levels of methylation in the CpG-Island-I in DHL2, LY3, or Mino cells (Fig. 4F). In total, these findings suggest that methylation of the “CpG-Island-I” may regulate catalase expression in CLL cells.
Finally, to interrogate if the catalase promoter methylation plays a functional role in regulating its transcription, cells were treated in vitro with 5′-azacytidine (AZA) either alone (MEC1 and DHL6) or together with IL-2/IL-15/CpG ODN (CLL cells; *n* = 4)\(^24\), and catalase mRNA level was determined by qRT-PCR. Indeed, we detected increase of catalase transcript levels in both MEC1/DHL6 cells (Fig. 4G) as well as in primary CLL cells (Fig. 4H) treated with AZA. Thus, findings from these series of experiments suggest that catalase expression in CLL cells is regulated epigenetically, at least in part, via promoter methylation specifically of the “CpG-Island-I”.

**Enforced induction of ROS activates AXL signaling axis**

Accumulation of ROS has been shown to activate several RTKs in a ligand-independent manner resulting in cell growth\(^33^–^35\). It is now well established that H\(_2\)O\(_2\) acts as a second messenger for signal transduction and signal amplification. Thus, we interrogated if increased production of H\(_2\)O\(_2\) could activate the AXL signaling axis, which is constitutively active and critical for CLL cell survival\(^24\). For this, CLL cells were treated with H\(_2\)O\(_2\) for 5 min and generation of ROS was determined by flow cytometry. Results showed a subtle increase of ROS in H\(_2\)O\(_2\)-treated vs. untreated CLL cells from basal levels (Fig. 5A). Next, we determined if enforced increase of ROS had any impact on tyrosine-phosphorylated proteins in H\(_2\)O\(_2\)-treated vs. untreated CLL cells. Indeed, we detected substantial increase of tyrosine phosphorylation levels on several proteins in H\(_2\)O\(_2\)-treated CLL cells (Fig. 5B) including AXL (Fig. 5C) and FGFR (Fig. 5D), but not on Tyro3 (data not shown) or other RTKs like c-MET or IGF1R\(^\beta\) (Fig. 5D). Given our finding that AXL positively regulates FGFR signal\(^22\), increase of FGFR phosphorylation levels in H\(_2\)O\(_2\)-treated CLL cells (Fig. 5D) was likely as a result of AXL activation by ROS (Fig. 5C).
**Fig. 4** (See legend on next page.)
tyrosine-phosphorylated protein bands (<100 kDa) appeared on the blot (Fig. 5B) may be the downstream targets of AXL including LYN (56 kDa), SYK (72 kDa), and/or ZAP70.

As ROS can also activate SIRT3 expression and our findings that long-term exposure (4 h) of CLL cells to H2O2 may result in SIRT3 upregulation (Fig. 1F), we further analyzed the H2O2-exposed CLL cell lysates used above (P1–P5) to assess the expression status of SIRT3 and SOD2 in western blots. A low to moderate increase (~1.5–2-fold) of SIRT3 expression was discernible in CLL cells (4 of 5) within a 5-min of exposure to H2O2, while no significant alteration of SOD2 expression was detected (Fig. 5E). Whether this elevation of SIRT3 expression in H2O2-exposed CLL cells is linked to AXL activation remains to be elucidated.

Given that BCR pathway is critical for CLL cell survival/proliferation and that, several of its downstream signaling components are the targets for therapy, we investigated the impact of enforced ROS accumulation on BCR signal. Thus, purified CLL cells used in panels B–D (P2–P5) were pretreated with ibrutinib for 1 h prior exposing the cells to H2O2 for 5 min or left untreated/unexposed. Cell lysates were analyzed to detect phosphorylation status of BTK (as an indicator of BCR activation) in western blot. While H2O2-treatment could not activate BTK from its basal level (upper panel, Fig. 5F), it activated AXL (lower panel, Fig. 5F), consistent with the findings in Fig. 5C, which remained unaffected prior ibrutinib treatment. In total, these results suggest that accumulation of ROS may activate AXL but not the BCR signal in CLL cells and that, ibrutinib treatment may not have any impact on ROS-induced AXL activation.

Further analysis of the H2O2-exposed CLL cell lysates used in panels B–E demonstrated a robust activation of AKT and ERK1/2, downstream signal mediators of AXL (Fig. 5G). Indeed, H2O2-mediated activation of AKT/ERK in CLL cells was completely inhibited upon pretreatment of the cells with a high-affinity AXL inhibitor (Fig. 5H). As most kinase inhibitors show off-target effects, we pretreated purified CLL cells (n = 2) with an AXL inhibitor, a BCR-ABL inhibitor asiminh or a JAK2 inhibitor fedratinib for 2 h prior exposing to H2O2. Cell lysates were analyzed for P-AKT and P-ERK1/2 in western blots. Interestingly, we detected a patient-specific effects of the inhibitors on AKT/ERK activation (Supplementary Fig. S2). While pretreatment of CLL cells with an AXL inhibitor reduced H2O2-induced AKT/ERK activation levels in both the samples, asiminh completely inhibited P-AKT level in CLL cells from one patient but not in other (Supplementary Fig. S2). Fedratinib pretreatment, on the other hand, did not show any inhibitory effect on H2O2-induced AKT activation in CLL cells obtained from both the patients. Further analysis suggests that asiminh and fedratinib are able to target ERK1/2 activation (Supplementary Fig. S2). A recent in vivo study demonstrated that cardiotoxicity induced by Ponatinib, another BCR-ABL inhibitor, was a result of direct inhibition of AKT/ERK signaling axes by the targeted-agent in cardiomyocytes. Although this study suggested asiminh as a potentially much less cardiotoxic, we believe that asiminh may exert patient-specific inhibitory effects on AKT/ERK in CLL cells. However, treatment of CLL cells with a ROS-inhibitor inhibited both P-AKT and P-ERK1/2 (Fig. 5I). Taken together, these results suggest that CLL cells may escape the detrimental cytotoxic effects of increased ROS accumulation
Fig. 5 (See legend on next page.)
by activating key cell survival signaling pathways including AXL.

Discussion

Compared with normal cells, cancer cells have higher levels of ROS, which seem to be required for malignant initiation and progression\(^\text{39}\)\(^\text{40}\), and the unchecked ROS accumulation is thought to play a part in the conversion from normal hematopoietic stem cells to leukemic cells\(^\text{39}\)\(^\text{40}\). The major endogenous source of cellular ROS is the mitochondrial electron transport chain, where continuous electron leakage to O\(_2^−\) occurs during aerobic respiration, generating O\(_2^−\)\(^\text{41}\). Although mitochondrial activities are higher in CLL cells\(^\text{19}\), we have detected significantly lower levels of O\(_2^−\) in CLL cells as compared to normal B-cells, suggesting a rapid conversion of O\(_2^−\) into H\(_2\)O\(_2\) by the mitochondrial superoxide detoxification enzyme SOD2, which likely remains as a highly active enzyme in CLL cells.

While the known primary regulation of SOD2 is through transcriptional activation, Chen et al.\(^\text{15}\) demonstrated that acetylation of SOD2 at K68 residue significantly reduces its activity while SIRT3 binds to, deacetylates and activates SOD2. In consistent with this report, we detected a marked reduction of acetylated-SOD2 levels in CLL cells indicating its constitutive enzymatic activity and that, the class-III histone deacetylase SIRT3 remained overexpressed, although at variable levels. While the precise mechanism of SIRT3 overexpression in CLL cells remains to be elucidated, we found that BCR stimulation could induce rapid upregulation of SIRT3 in CLL cells. However, we believe that increased ROS accumulation may also activate and maintain constitutive levels of SIRT3 in CLL cells as indicated in earlier studies\(^\text{15}\)\(^\text{29}\). Whether ROS-induced SIRT3 upregulation in CLL cells is linked to AXL activation remains to be elucidated.

Despite significant reduction of O\(_2^−\) levels, we found increased accumulation of H\(_2\)O\(_2\) in CLL cells. To delineate the mechanism of aberrant H\(_2\)O\(_2\) levels in CLL cells, we examined the expression status of catalase, which protects the cells by removing H\(_2\)O\(_2\). Our analysis finds reduced levels of mRNA and protein of catalase in CLL cells from majority of patients tested, suggesting that reduced expression of catalase may be responsible for H\(_2\)O\(_2\) accumulation in these leukemic cells. In an effort to decipher the mechanism of catalase downregulation, we identified two putative CpG-islands upon analysis of its promoter. Our results indicated that the CpG-Island-I may be critical to regulate catalase transcription as variable degrees of allele-specific methylation in this region were detectable only in CLL cells but, not in normal
response to H2O2 is likely to be a consequence of AXL elevation and that, activation of FGFR in CLL cells in dose suggesting that AXL may be highly sensitive to ROS with exogenous H2O2 induced activation of AXL, inde-

Interestingly, treatment of vascular smooth muscle cells mediated oxidation, leading to elevated phosphorylation dephosphorylation RTKs, can be inactivated by ROS-tyrosine phosphatases, which are responsible for

Previously untreated CLL patients (n = 10) (Supplementary Fig. S3); suggesting that SIRT3 mediated regulation of SOD2 or catalase gene transcription may be context-dependent and/or cell-type specific.

Oxidative stress, even at low levels, can activate RTKs including receptors for epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and FGF in a ligand-independent manner resulting in cell growth. Interestingly, treatment of vascular smooth muscle cells with exogenous H2O2 induced activation of AXL, independent of its ligand Gase. Thus, elevation of ROS in CLL cells certainly provides them with some advantages over normal B-cells. Indeed, we detected that enforced elevation of ROS in CLL cells activated AXL and its downstream targets FGFR, AKT and ERK1/2, but not IGF1R or c-MET. Earlier studies also demonstrated that H2O2 activated EGFR, PDGF and FGFR at concentrations ranging from 5–20 mM in vitro. In contrast, we detected a robust activation of AXL upon treatment of CLL cells with H2O2 at 8–30-fold lower dose suggesting that AXL may be highly sensitive to ROS elevation and that, activation of FGFR in CLL cells in response to H2O2 is likely to be a consequence of AXL activation; not a direct effect of H2O2 on FGFR. Protein tyrosine phosphatases, which are responsible for dephosphorylation of the target proteins. However, only a subtle reduction of total phosphatase levels was discernible in CLL cells in response to in vitro H2O2 treatment (Supplementary Fig. S4). Further study is needed to elucidate the mechanism of targeted activation of AXL in CLL cells in response to increased ROS.

In summary, we detected elevated levels of H2O2 but significantly lower levels of O2− in CLL cells, which could be attributable to reduced catalase levels and over-expression of SIRT3, respectively. In contrast to the superoxide anion and hydroxyl radical, the less reactive H2O2 is not only involved in many physiological processes, such as hypoxic signal transduction, cell differentiation, and proliferation but also plays a role in mediating immune responses. However, it exerts its effects depending on the cellular context, its local concentration as well as its exposure time. Thus H2O2 is no more considered as an unwanted rather toxic byproduct, but plays an important role in the control of vital cellular processes. Thus, we believe that generation of high level of superoxide anion may be detrimental to the cells while low level accumulation of H2O2 may play a vital role to prolong cancer cells’ survival in toxic environment. Given our findings, we also believe that CLL cells have adopted a process to rapidly neutralize superoxide anion accumulation (via SIRT3/SOD2) but utilize H2O2 to prolong their survival by activating cell survival pathways.

Efficient removal of O2− from the cellular environment is usually a beneficial outcome; increased accumulation of H2O2 due to reduced catalase expression while detrimental to normal cells but may promote genetic instability and cell signaling in malignant cells including CLL cells. It is, however, tempting to speculate about the formation of a ROS gradient from the source of production, where proteins under the influence of such a gradient would be oxidized, while others out of reach would be immune to increases in the level of ROS. Of relevance, prolonged production of ROS in response to BCR stimulation has been shown to promote B-cell activation and proliferation and that, activated cells also generate ROS as a byproduct of normal mitochondrial respiration. In addition, activation of the PI3K/mTOR pathway by BCR-ABL contributes to increased production of mitochondrial ROS in BCR-ABL-transfected murine pre-B-cells and human megakaryocytes. In agreement with these studies and given our findings, we speculate that constitutive activation of the BCR signal in CLL cells may upregulate SIRT3 and maintain its levels by generating ROS which, on the other hand, promotes cell survival through activation of RTKs like AXL.

In conclusion, we propose that constitutively active known cell survival signaling pathways (BCR/AXL) in CLL cells may activate and maintain elevated levels of ROS via PI3K/AKT/mTOR axis (Fig. 6), a key regulator of mitochondrial oxygen consumption and oxidative capacity. On the other hand, to escape from the detrimental effect of overproduced O2−, CLL cells adopted a mechanism to eliminate O2− more efficiently by converting inactive SOD2 into a constitutively active enzyme via inducing SIRT3 expression, while maintaining low levels of catalase through variable degrees of promoter methylation for H2O2 accumulation to promote cell survival (Fig. 6). Thus, increased ROS in CLL cells may not only activate AXL signaling axis but also upregulate SIRT3 to maintain this positive feedback loop. Therefore, one potential therapeutic intervention can be to increase ROS-scavenging capacity using antioxidants, thereby abrogating ROS signaling and suppressing tumor growth in combination with current CLL therapies. However, this hypothesis warrants further investigation.
Fig. 6 Proposed model of ROS generation and regulation of survival signals in CLL cells. Data presented in this study demonstrate that CLL cells contain elevated levels of H$_2$O$_2$ with significantly reduced O$_2^-$ levels compared to normal B-cells. This is primarily due to the presence of highly active mitochondrial SOD2 enzyme in CLL cells converting O$_2^-$ into H$_2$O$_2$ more rapidly. Overexpression of SIRT3 in CLL cells deacetylates SOD2 and likely maintains its constitutive enzymatic activity. Moreover, we have also detected reduced expression of catalase in CLL cells causing H$_2$O$_2$-accumulation. Although we have identified two distinct CpG-Islands (I and II) for methylation in the human catalase promoter, our results suggest that it is the Island-I, which primarily regulates catalase levels in CLL cells. Indeed, we have detected variable degrees of methylation in CpG-Island-I of the catalase promoter only in CLL cells and not in normal B-cells and that, catalase expression is reduced in CLL cells both at mRNA and protein levels. While released from mitochondria, H$_2$O$_2$ may activate the AXL/FGFR/ATK/ERK signaling axis and/or the BCR pathway, independent of ligand, and maintain constitutive activation levels of these cell survival pathways through a positive feedforward loop. For example, activation of ATK/mTOR pathway leads to increase of oxygen consumption and mitochondrial metabolic activities and thus, generation of ROS. Of note, we found that ROS generation or BCR activation induces SIRT3 expression in CLL cells. Therefore, one potential therapeutic intervention can be to increase ROS-scavenging capacity using antioxidants, thereby abrogating ROS signaling and suppressing tumor growth in combination with current CLL therapies.

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