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The NOTCH1 and miR-34a signaling network is affected by *TP53* alterations in CLL

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ABSTRACT

In chronic lymphocytic leukemia (CLL), *TP53* mutations or deletions on chromosome 17p lead to adverse prognosis and reduced levels of miR-34a, which targets NOTCH1. Also, hyperactivated NOTCH1 signaling is crucial for CLL progression. Here we explored the interaction between p53, miR-34a, and NOTCH1 in CLL. We investigated the effect of p53 and miR-34a on NOTCH1 signaling and expression in CLL cells with altered *TP53*. Our results indicate that miR-34a reduces NOTCH1 3′ UTR activity but might not be a mediator between p53 signaling and NOTCH1. p53 activation increases miR-34a expression and NOTCH1 protein levels, correlating with decreased NOTCH1 and miR-34a levels in primary CLL cells with *TP53* alterations. Some samples with high NOTCH1 levels presented increased BCL-2, suggesting an anti-apoptotic mechanism of a potentially direct p53-NOTCH1 relation in CLL. This study deepens the understanding of the p53-miR-34a-NOTCH1 signaling network, providing insights that could guide future therapeutic strategies for CLL.

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CLL; NOTCH1; p53; miR-34a; signaling network

Introduction

The tumor suppressor gene *TP53* is the most frequently mutated gene in human cancers, with alterations occurring in approximately half of all cases [1–3]. Mutations in *TP53* lead to loss of its normal functions, enabling cancer cells to proliferate uncontrollably, evade apoptosis, and acquire invasive and metastatic properties, ultimately driving aggressive disease progression across multiple cancer types [1]. Consequently, the evaluation of *TP53* mutation status has immediate clinical relevance for cancer diagnosis, prognosis, and treatment selection [4]. Moreover, *TP53* mutations are associated with treatment resistance in various cancers [5].

The p53 protein, encoded by *TP53*, plays a crucial role in regulating apoptosis and maintaining genomic stability by activating the transcription of pro-apoptotic genes [6] and genes involved in cell cycle arrest and DNA repair in response to DNA damage [7–9]. In addition to its direct transcriptional targets, p53 also regulates gene expression post-transcriptionally through microRNAs (miRNAs). These p53-regulated miRNAs influence various cellular processes, including cell cycle progression, migration, epithelial-mesenchymal transition, stemness, metabolism, differentiation, and cell survival [10]. Disruption of the p53-miRNA axis is a common event in cancer, underscoring its importance in maintaining genomic integrity and preventing tumorigenesis [10–12]. Therefore, investigating different p53-miRNA networks in cancer is of great interest.

One such p53-regulated miRNA is miR-34a, a well-established tumor suppressor that is directly transactivated by p53 [13–18]. MiR-34a exerts its tumor-suppressive functions by targeting genes involved in cell cycle progression, apoptosis, invasion, and metastasis [19–21]. In chronic lymphocytic leukemia (CLL), both *TP53* and miR-34a have prognostic

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significance. Low miR-34a expression correlates with *TP53* mutations or deletions on chromosome 17p (del(17p)) and poor prognosis [22,23]. Consequently, dysregulation of miR-34a due to *TP53* alterations leads to the loss of regulation of miR-34a targeted pathways, such as apoptosis and cell proliferation.

One of the target genes of miR-34a is *NOTCH1*, which encodes a transmembrane receptor that regulates cell fate decisions, proliferation, and survival in various tissues [24]. Due to its role in regulating crucial cellular functions, NOTCH1 signaling is frequently dysregulated in different types of cancer [25]. In CLL, NOTCH1 is hyperactivated in approximately 50% of cases [26], either through mutations in the *NOTCH1* gene itself or in its regulators, such as *FBXW7*, *MED12*, and *SPEN* [27–31]. NOTCH1 hyperactivation contributes to CLL pathogenesis and progression by upregulating genes involved in cell cycle regulation and proliferation, such as *MYC*, and by interacting with other signaling pathways like NF-κB and B-cell receptor (BCR) signaling [26]. *NOTCH1* mutations are associated with poor prognostic features in CLL, including unmutated IGHV genes and *TP53* disruption, and patients with *NOTCH1* mutations have shorter time to disease progression and overall survival [32–34]. Moreover, NOTCH1 hyperactivation has been linked to the transformation of CLL to the more aggressive Richter's syndrome [27, 35]. Given its occurrence in 50% of CLL cases and its clinical impact, understanding the mechanisms behind NOTCH1 dysregulation is of high interest.

Our research aims to elucidate the interplay between *TP53* mutations, miR-34a expression, and NOTCH1 signaling in CLL. We hypothesize that miR-34a acts as a regulatory node, mediating the effects of *TP53* mutations on NOTCH1 pathway activation. To address this hypothesis, here we (1) quantified the impact of miR-34a downregulation on NOTCH1 signaling in CLL cells with and without *TP53* mutations, (2) assessed how NOTCH1 protein expression levels correlate with *TP53* mutation status and miR-34a levels and (3) investigated the potential of miR-34a to modulate the effects of NOTCH1 signaling in CLL.

This study not only fills the existing knowledge gap regarding the molecular dynamics involving *TP53*, miR-34a, and NOTCH1. Our study also explores the prognostic and therapeutic implications of these interactions in CLL. Studies to elucidate the regulatory network between p53, miR-34a, and NOTCH1 could lead to improved risk stratification of CLL patients and the development of novel targeted therapeutic approaches, especially for those harboring *TP53* mutations. Ultimately, a better understanding of the p53-miR-34a-NOTCH1 axis may pave the way for personalized medicine strategies that enhance prognosis and treatment outcomes for CLL patients with poor prognosis.

Material and methods

CLL Patient samples

Primary CLL cells were obtained from patients at the outpatient clinic of the university hospital Ulm after written informed consent (Ulm Ethics Committee, Votum 96/08) in accordance with the Declaration of Helsinki. CLL B-cells were isolated from heparinized blood of CLL patients using the B-CLL cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions, and viably preserved in liquid nitrogen. Patient characteristics are shown in supplementary Table 1.

Information about additional methods can be found in the supplementary information file.

Results

In this study, we aimed to elucidate the interplay between p53, miR-34a, and NOTCH1 in chronic lymphocytic leukemia (CLL) cells. We investigated the relationship between p53 and miR-34a expression in CLL cell lines, assessed the potential regulation of NOTCH1 by miR-34a using luciferase reporter assays, and explored the effects of p53 modulation on NOTCH1 expression and signaling activity. We also examined the direct impact of miR-34a on NOTCH1 and validated our findings in primary CLL cells from patients with wild-type or altered *TP53* status.

MiR-34a positively correlates with p53 activation and expression

To investigate the relationship between p53 and miR-34a in CLL, we first assessed miR-34a expression levels in CLL cell lines with wild-type (HG3, WA-OSEL, EHEB) or mutated (MEC-1, MEC-2) *TP53* upon activation of p53 with Nutlin-3a, a small-molecule inhibitor of the p53-MDM2 interaction [36]. Nutlin-3a treatment (5 µM) for 24 h caused a tendency of increased miR-34a expression in *TP53* wild-type cell lines but not in *TP53*-mutated cell lines (Figure 1(A)). To further investigate the kinetics of miR-34a upregulation in response to p53 activation, we performed time course experiments in EHEB and WA-OSEL cells. These cells were treated with Nutlin-3a (5 µM) for 24, 48, and 72 h, and miR-34a expression was assessed at each time point. We observed a significant upregulation of miR-34a after 48 h ($p=0.0266$ and $p=0.0761$, respectively)

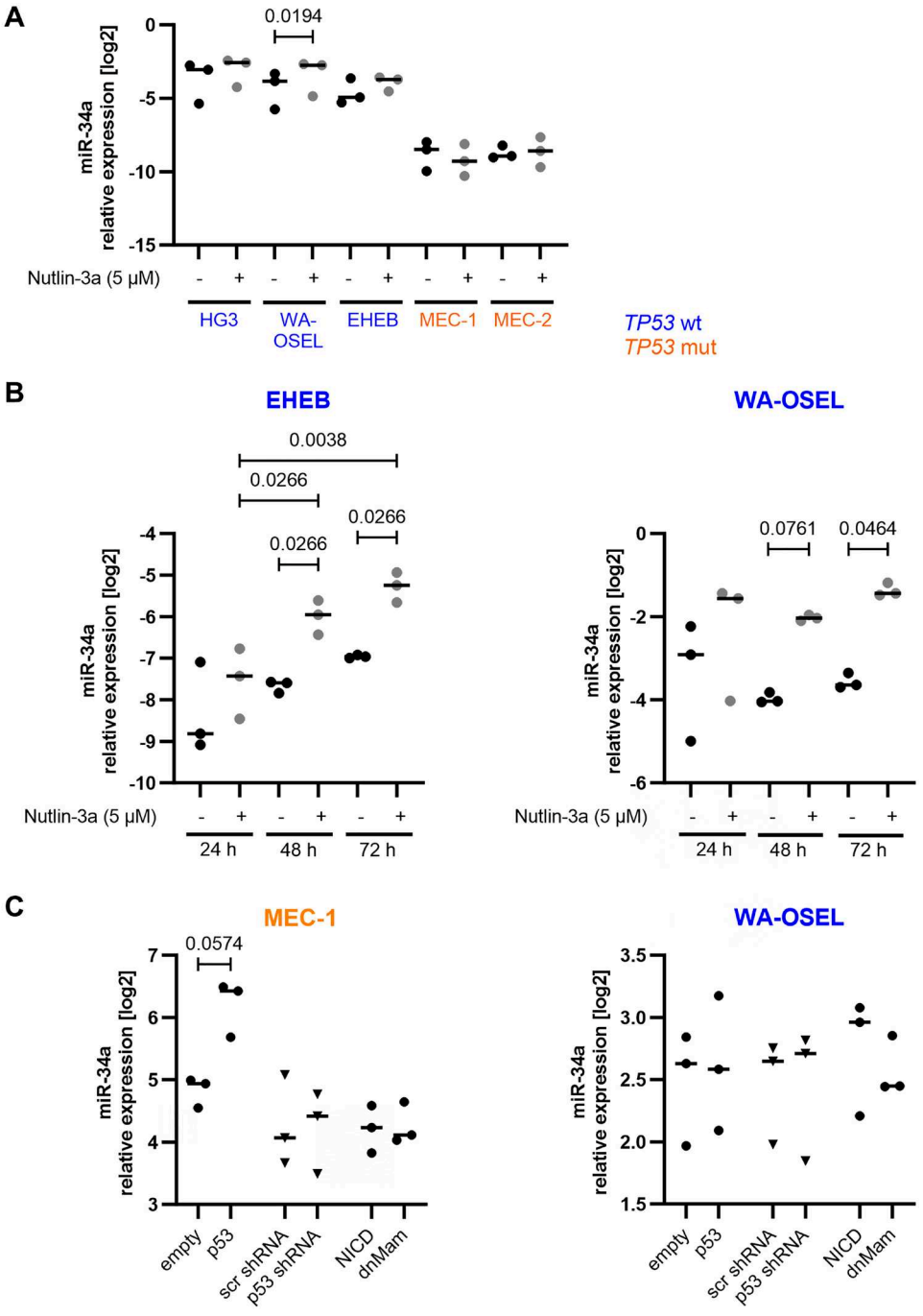


Figure 1. miR-34a positively correlates with p53 activation/expression. (A) Relative miR-34a expression in the CLL cell lines HG3, WA-OSEL, EHEB harboring wild type *TP53* (*TP53* wt; blue) and MEC-1 and MEC-2 harboring mutated *TP53* (*TP53* mut; orange). Data points represent three individual experiments in which cells were treated with DMSO (black) as control or 5 μM Nutlin-3a (gray) for 24 h to activate p53. MiR-34a expression was analyzed by qPCR and normalized to the expression of three housekeeping genes. Statistical testing was performed using paired t-test to compare between DMSO and Nutlin-3a treatment for each cell line. P-values are shown when significant or of particular interest. (B) Relative miR-34a expression in WA-OSEL and EHEB cell line cells (both *TP53* wt) upon Nutlin-3a treatment (5 μM) for 24, 48 and 72 h compared to DMSO control treatment to observe the kinetics of miR-34a expression in response to Nutlin-3a treatment. Data points represent three independent experiments. Statistical testing was performed using ordinary one-way ANOVA followed by Holm-Sidak's multiple comparisons test to compare between DMSO and Nutlin-3a treatment for each time point and between the three time points. P-values are shown when significant or of particular interest. (C) Relative miR-34a expression in WA-OSEL (*TP53* wt) and MEC-1 (*TP53* mut) cell line cells 24 h after transfection with a p53 overexpression plasmid, a shRNA targeting p53 and their respective controls. NICD and dominant negative MAM-L (dnMam) were transfected to hyperactivate or downregulate NOTCH1 signaling, respectively. Data points represent three independent experiments. Statistical testing was performed using ordinary one-way ANOVA followed by Holm-Sidak's multiple comparisons test in order to compare between the differently transfected samples. P-values are shown when significant or of particular interest.

and 72 h ($p=0.0266$ and $p=0.0464$, respectively) of Nutlin-3a treatment in both cell lines (Figure 1(B)). These results suggest that p53 activation leads to a time-dependent increase in miR-34a expression in CLL cells with wild-type *TP53*.

To further validate the direct regulation of miR-34a by p53, we transfected WA-OSEL (*TP53* wild-type) and MEC-1 (*TP53*-mutated) cells with a p53 overexpression plasmid or a p53-targeting shRNA. In addition, we overexpressed the intracellular domain of NOTCH1 (NICD) or a dominant-negative form of Mastermind-like (dnMam) to modulate NOTCH1 signaling and assess potential crosstalk with the p53-miR-34a axis. In MEC-1 cells, p53 overexpression led to a significant increase in miR-34a levels ($p=0.0574$), while p53 knockdown had no significant effect (Figure 1(C), left panel). Conversely, in WA-OSEL cells, p53 overexpression did not significantly alter miR-34a expression probably because of mechanisms regulating the existing endogenous p53 function, which is not necessary in *TP53*-mutated MEC-1 cells (Figure 1(C), right panel). Modulation of NOTCH1 signaling did not significantly impact miR-34a levels in either cell line, suggesting that the regulation of miR-34a by p53 is independent of NOTCH1 signaling. These results confirm that p53 directly regulates miR-34a transcription in CLL cells and that this regulation is dependent on the *TP53* mutation status.

MiR-34a decreases the activity of a NOTCH1 3′ UTR reporter construct

After we validated the direct regulation of miR-34a by p53, we next sought to investigate the potential regulation of NOTCH1 by miR-34a. To this end, we employed luciferase reporter constructs containing the wild-type *NOTCH1* 3′ UTR, a *NOTCH1* 3′ UTR with deleted miR-34a binding sites (delta), or no 3′ UTR (empty) (Figure 2(A)). Transfection of HEK293 cells with an hsa-miR-34a precursor (pre-miR-34a; Thermo Fisher AM17100 PM11030) significantly decreased the activity of the wild-type *NOTCH1* 3′ UTR reporter ($p<0.0001$) but not the delta or empty reporters (Figure 2(B), left panel). Interestingly, pre-miR-34a transfection also led to an increase in the activity of the empty reporter, possibly due to nonspecific effects on transcription. However, the decrease in activity observed with the wild-type *NOTCH1* 3′ UTR reporter was greater, indicating a specific targeting effect of miR-34a on the *NOTCH1* 3′ UTR. Conversely, transfection with an anti-miR-34a oligonucleotide (Thermo Fisher AM17000 AM11030) had no effect on the activity of all three reporters (Figure 2(B), right panel). These results demonstrate that miR-34a directly targets the *NOTCH1* 3′ UTR, potentially regulating *NOTCH1* expression at the post-transcriptional level.

P53 upregulation correlates with increased NOTCH1 protein levels and a trend of upregulating NOTCH1 signaling activity

Given the direct regulation of miR-34a by p53 and the targeting of *NOTCH1* by miR-34a, we next explored the relationship between p53 and NOTCH1 in CLL cells. We treated MEC-1 (*TP53*-mutated) and WA-OSEL (*TP53* wild-type) cells with Nutlin-3a (5 μM) for 24 h or transfected them with a p53 overexpression plasmid or a p53-targeting shRNA. Western blot analysis revealed that Nutlin-3a treatment increased p53 and NOTCH1 protein levels in WA-OSEL cells but not in MEC-1 cells (Figure 3(A)).

To assess the impact of p53 on NOTCH1 signaling activity, we transfected MEC-1 and WA-OSEL cells with a NOTCH1-responsive luciferase reporter construct and either a p53 overexpression plasmid or a p53-targeting shRNA. In WA-OSEL cells, p53 overexpression resulted in an increase in NOTCH1 reporter activity ($p=0.0517$), while p53 knockdown showed a trend toward decreased activity (Figure 3(B), right panel). No significant changes in NOTCH1 reporter activity were observed in MEC-1 cells upon p53 manipulation (Figure 3(B), left panel).

Similarly, p53 overexpression led to increased NOTCH1 protein levels in both cell lines, while p53 knockdown decreased NOTCH1 levels in WA-OSEL cells (Figure 3(C)). These results suggest that p53 not only upregulates NOTCH1 expression but also tends to increase NOTCH1 signaling activity in CLL cells with wild-type *TP53*. The observed increase in NOTCH1 levels upon p53 activation or overexpression is in line with previous reports indicating that *NOTCH1* is a direct transcriptional target of p53 [37–39].

However, it is important to note that the effects on NOTCH1 signaling activity were less pronounced than those on NOTCH1 protein levels, possibly due to the involvement of additional regulatory mechanisms or the limited sensitivity of the luciferase reporter assay. Additional limitations include the low transfection efficiency of the CLL cells. As a marker of successful p53 modulation we therefore tested for induction of p21, which is a direct target of p53 [7]. Samples showing induction or downregulation of p21 due to p53 overexpression or knockdown by shRNA compared to control samples were considered as successful transfection. In WA-OSEL cells we couldn't meet these quality criteria in one single representative experiment. Therefore, the western blot of WA-OSEL shows the results of two independent experiments, with lanes 1–4 from experiment 1 and lanes 5–6 from experiment 2 (Figure 3(C)). The remaining samples of WA-OSEL experiments 1 and 2 are shown in Supplementary Figure 1.

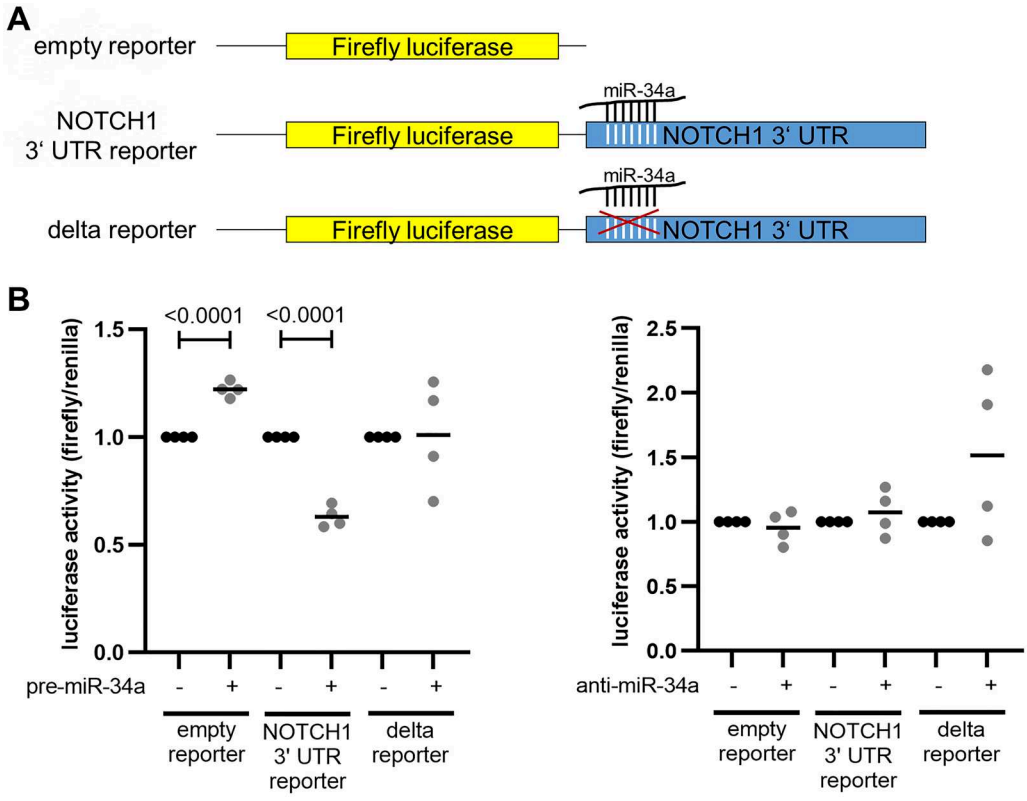


Figure 2. miR-34a decreases the activity of a *NOTCH1* 3′ UTR reporter construct. (A) Schematic of the luciferase reporter constructs encoding the firefly luciferase followed by no 3′ UTR sequence (empty reporter), the 3′ UTR sequence of human wild type *NOTCH1* (*NOTCH1* 3′ UTR reporter) or the *NOTCH1* 3′ UTR sequence in which the binding sites for miR-34a were deleted (Delta reporter). (B) HEK293 cells were transfected with empty reporter, *NOTCH1* 3′ UTR reporter or Delta reporter and an hsa-miR-34a-precursor construct (pre-miR-34a; gray; left panel) or an anti-hsa-miR-34a (anti-miR-34a; gray; right panel) with the corresponding negative controls (black). Firefly luciferase activity was measured 24 h after transfection together with activity of the co-transfected renilla luciferase for internal signal normalization. Data points represent the firefly/renilla ratios of four individual experiments that are shown normalized to the negative controls for pre-miR-34a (left panel) and anti-miR-34a (right panel), respectively. Statistical testing was performed using unpaired t-test. P-values are shown when significant or of particular interest.

MiR-34a overexpression or inhibition does not significantly affect NOTCH1 protein levels or signaling activity

Although our results demonstrate that miR-34a directly targets the *NOTCH1* 3′ UTR and that p53 positively regulates NOTCH1 expression, the direct impact of miR-34a on NOTCH1 in CLL cells remained unclear. To address this, we transfected MEC-1 and WA-OSEL cells with pre-miR-34a or anti-miR-34a oligonucleotides and assessed miR-34a expression, NOTCH1 protein levels, and NOTCH1 signaling activity. Transfection with pre-miR-34a significantly increased miR-34a levels in both cell lines ($p < 0.0001$), while anti-miR-34a significantly decreased miR-34a levels ($p = 0.0380$ for MEC-1; $p = 0.0171$ for WA-OSEL) (Figure 4(A)).

However, neither miR-34a overexpression nor inhibition significantly affected NOTCH1 protein levels (Figure 4(B)) or NOTCH1 reporter activity (Figure 4(C)) in either cell line. Due to high variability in this experimental series there was no significant difference, but we cannot exclude that a larger number of experimental repetitions could disclose an effect. Therefore, we cannot exclude that miR-34 has an effect on NOTCH1 signaling activity in CLL cell lines. Since we observed before a positive regulation of NOTCH1 signaling by p53 upregulation (Figure 3), we can already conclude that miR-34a might not be a mediator between p53 and NOTCH1 signaling in CLL. In addition, these results might suggest that, despite directly targeting the *NOTCH1* 3′ UTR, miR-34a alone may not be sufficient to significantly modulate NOTCH1 expression and signaling in CLL cells. This raises the possibility that other factors, such as the overall cellular context or additional regulatory mechanisms, may influence the impact of miR-34a on NOTCH1 in CLL. For instance,

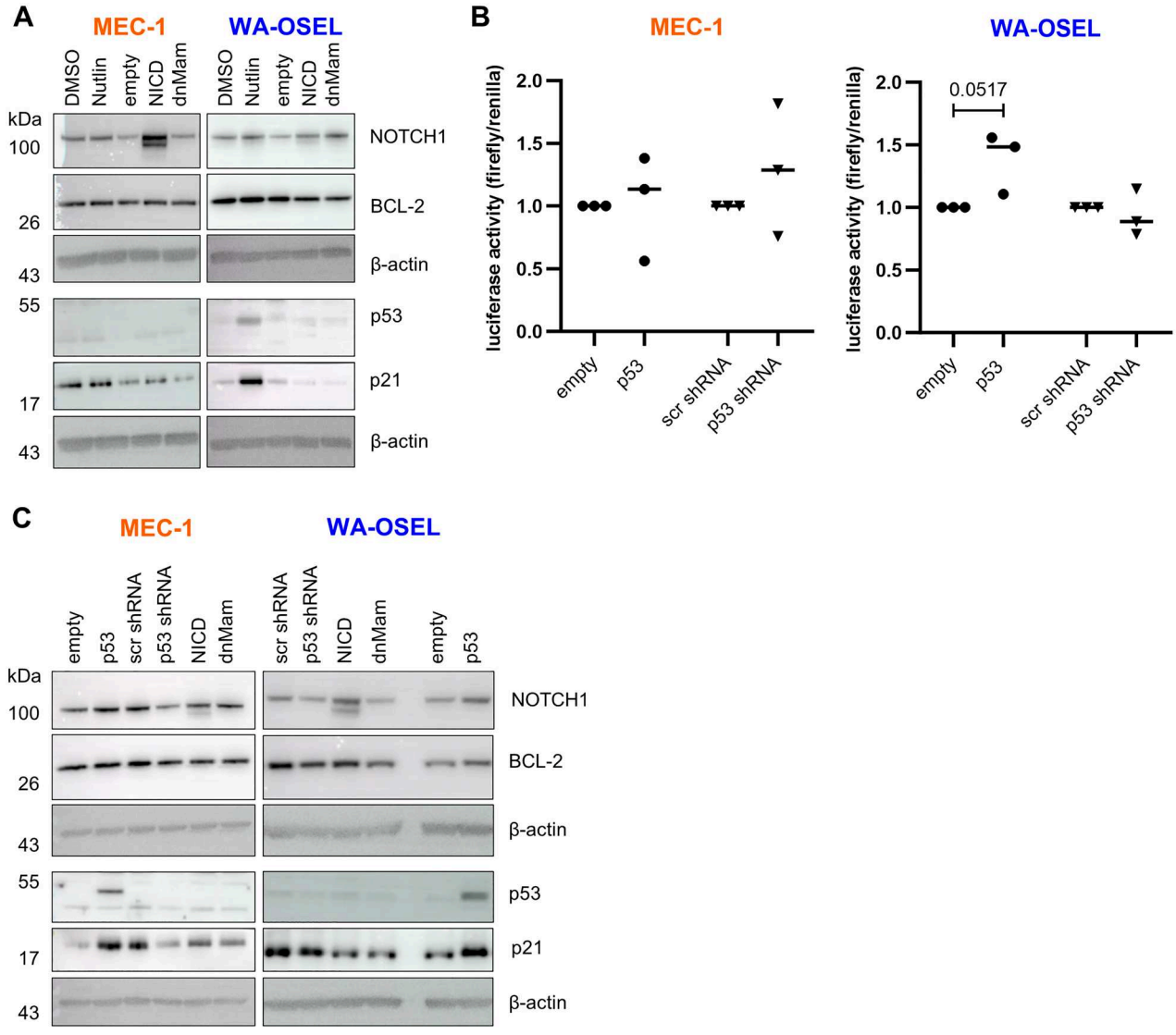


Figure 3. p53 Upregulation correlates with increased NOTCH1 protein levels and a trend of upregulating NOTCH1 signaling activity. (A) NOTCH1, BCL-2, p53, and p21 protein levels in MEC-1 (*TP53* mut) and WA-OSEL (*TP53* wt) cell line cells upon treatment with DMSO or 5 µM Nutlin-3a to activate p53 or after overexpression of NICD or dominant negative MAM-L (dnMam) to activate or inhibit NOTCH1 signaling, respectively. β-Actin was used as loading control. (B) NOTCH1 signaling activity measured *via* activity of a firefly luciferase NOTCH1-reporter and a constitutively expressed renilla luciferase. Data points represent firefly/renilla ratios of three independent experiments in which MEC-1 (*TP53* mut) and WA-OSEL (*TP53* wt) cell line cells were transfected with p53, a p53-targeting shRNA, and the respective negative controls (empty and scr shRNA). Luciferase ratios are shown normalized to the negative controls. Statistical testing was performed using unpaired t-test. P-values are shown when significant or of particular interest. (C) NOTCH1, BCL-2, p53 and p21 protein levels in MEC-1 (*TP53* mut) and WA-OSEL (*TP53* wt) cell line cells transfected with p53, a p53-targeting shRNA and the respective negative controls. NICD or dominant negative MAM-L (dnMam) were overexpressed to activate or inhibit NOTCH1 signaling, respectively. β-Actin was used as loading control. The western blot of WA-OSEL shows the results of two independent experiments. Lanes 1-4 are from experiment 1, lanes 5–6 are from experiment 2. Due to variable transfection efficiency of CLL cell lines, knockdown or overexpression of p53 was not equally successful in each experiment. Here we show only the results of the experiments where transfection was considered successful. The remaining samples from WA-OSEL experiments 1 and 2 are shown in Supplementary Figure 1.

the presence of competing endogenous RNAs (ceRNAs) or RNA-binding proteins could potentially interfere with miR-34a-mediated regulation of NOTCH1 [40,41]. Additionally, the relatively modest changes in miR-34a levels achieved by transient transfection at lower efficiencies may not be sufficient to elicit a significant response in NOTCH1 expression and signaling. Future studies employing stable miR-34a overexpression or knockout systems could provide further insights into the long-term effects of miR-34a modulation on NOTCH1 in CLL cells.

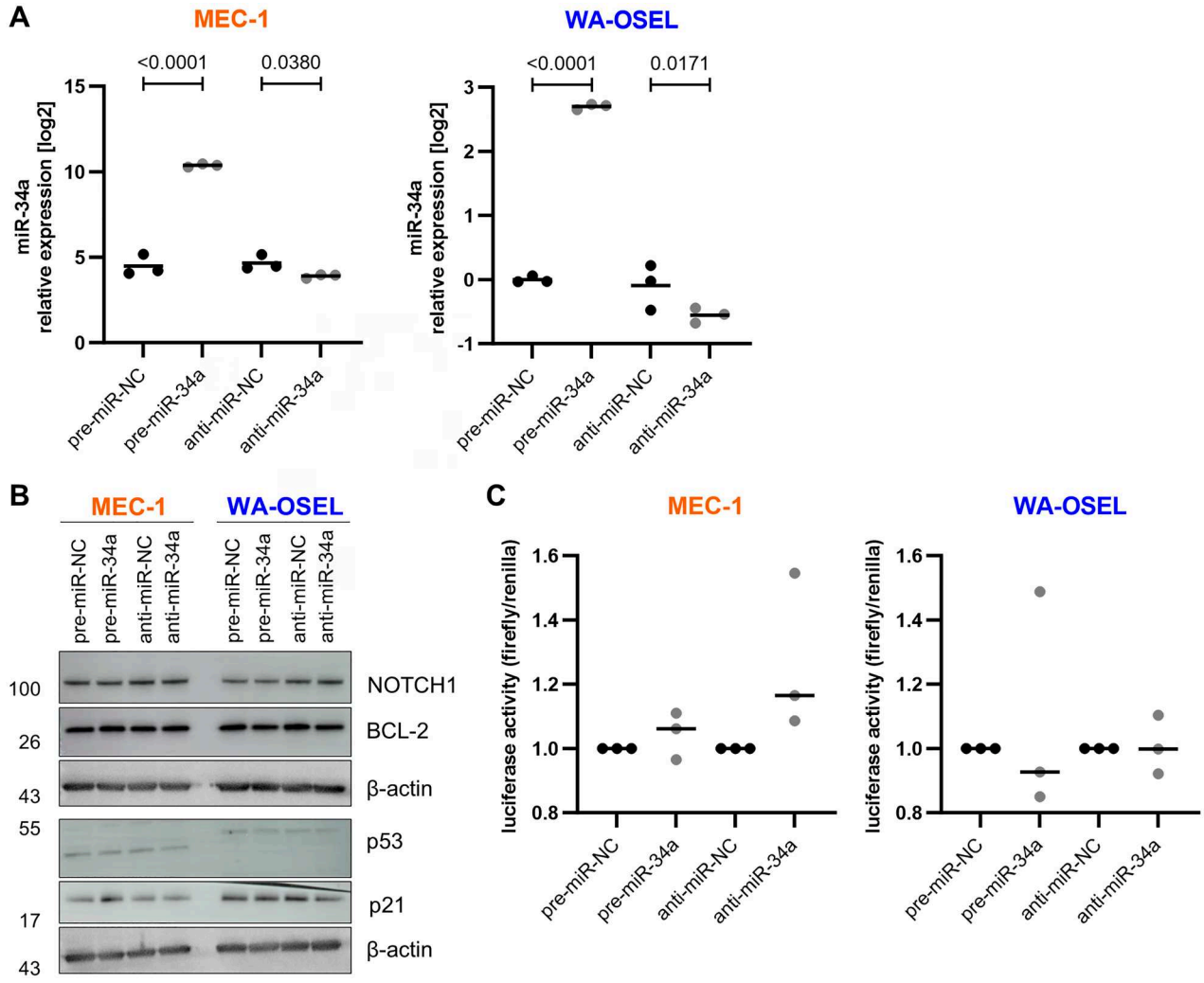


Figure 4. miR-34a overexpression/inhibition does not significantly affect NOTCH1 protein levels or signaling activity. (A) Relative miR-34a expression levels in the CLL cell lines MEC-1 (*TP53* mut) and WA-OSEL (*TP53* wt) transfected with an hsa-miR-34a-precursor construct (pre-miR-34a) or an anti-hsa-miR-34a construct (anti-miR-34a) and their respective negative controls (NC). MiR-34a expression was analyzed by qPCR and normalized to the expression of three housekeeping genes. Data points represent three independent experiments. Statistical testing was performed using ordinary one-way ANOVA followed by Holm-Sidak's multiple comparisons test. P-values are shown when significant or of particular interest. (B) NOTCH1, BCL-2, p53 and p21 protein levels in MEC-1 (*TP53* mut) and WA-OSEL (*TP53* wt) transfected with pre-miR-34a or anti-miR-34a and their respective negative controls (NC). β-Actin was used as loading control. (C) NOTCH1 signaling activity measured *via* activity of a firefly luciferase NOTCH1-reporter and a constitutively expressed renilla luciferase. Data points represent firefly/renilla ratios of three independent experiments in which MEC-1 (*TP53* mut) and WA-OSEL (*TP53* wt) cell line cells were transfected with pre-miR-34a or anti-miR-34a and their respective negative controls (NC). Ratios are shown normalized to the negative controls. Statistical testing was performed using unpaired t-test.

CLL patients with altered TP53 status have decreased NOTCH1 protein levels and miR-34a expression

To validate our *in vitro* findings in primary samples, we assessed NOTCH1 protein levels and miR-34a expression in primary CLL cells from patients with wild-type ($n=10$) or altered (mutated or deleted; $n=10$) *TP53*. Western blot analysis revealed significantly lower NOTCH1 protein levels in CLL cells from patients with altered *TP53* compared to those with wild-type *TP53* ($p=0.0044$) (Figure 5(A,B)). Interestingly, p21 protein levels which we assessed in previous experiments as marker for p53 activity were more prominent in cells from *TP53*-altered patients where p53 is expected to be dysfunctional. It was reported that some p53 mutants in CLL retain their transactivation ability and can still induce p21 expression [42], but also other factors might regulate p21 expression in CLL or compensate for the loss of p53 [43]. In addition, patients with the highest NOTCH1 levels also exhibited increased BCL-2 levels, suggesting a potential anti-apoptotic mechanism associated with

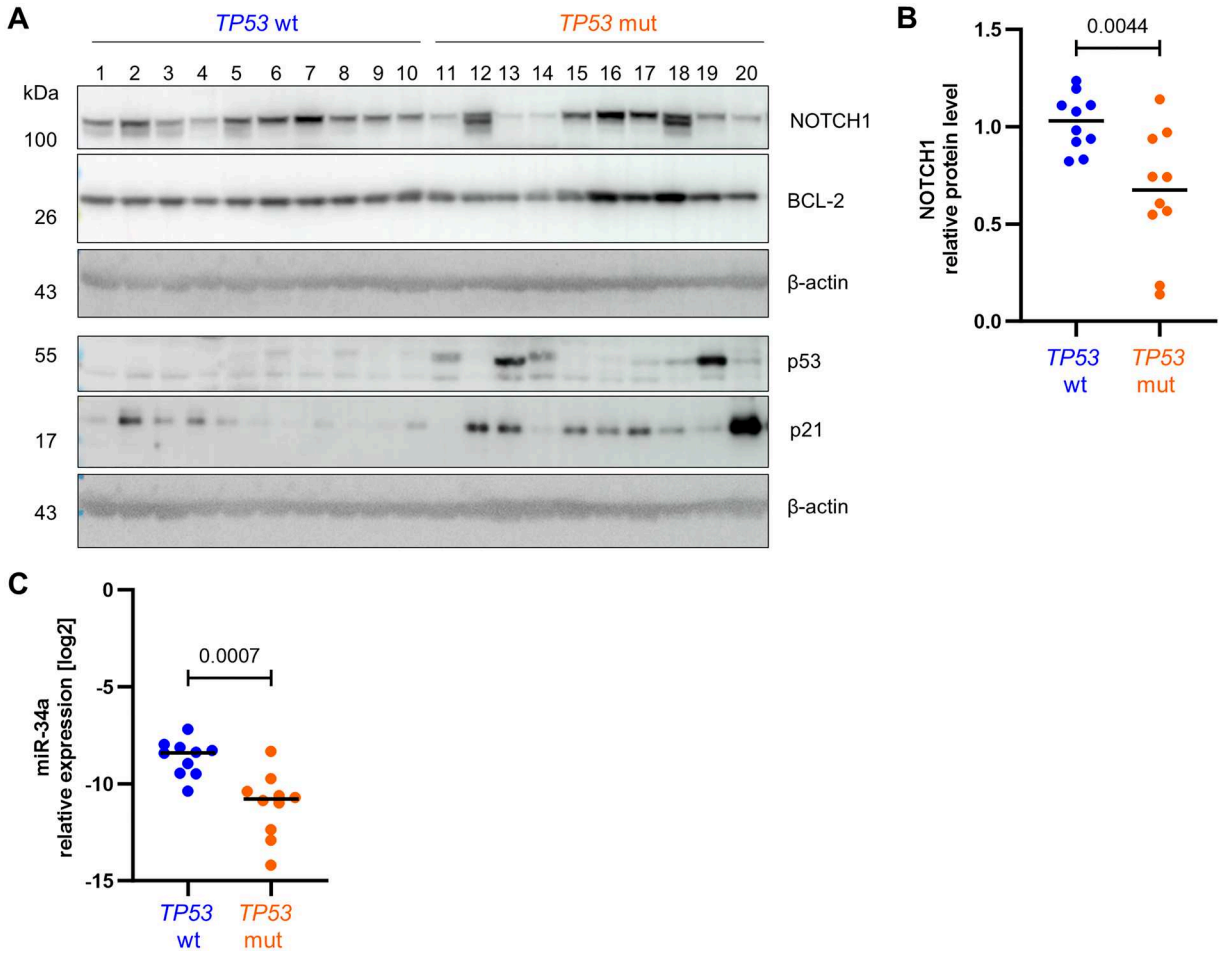


Figure 5. CLL patients with altered *TP53* status have decreased NOTCH1 protein levels and miR-34a expression levels. (A) NOTCH1, BCL-2, p53 and p21 protein levels in CLL patients with wild type *TP53* (*TP53* wt; $n = 10$) or *TP53* altered by mutation or deletion of chromosome 17p (*TP53* mut; $n = 10$). β-Actin was used as loading control. (B) NOTCH1 protein levels from the western blot in (A) relative to β-actin as assessed by densitometric quantification. A data point represents one protein band on the western blot. Statistical testing was performed using unpaired *t*-test. (C) Relative miR-34a expression levels in CLL patients with with wild type *TP53* (*TP53* wt; $n = 10$) or *TP53* altered by mutation or del(17p) (*TP53* mut; $n = 10$). MiR-34a expression was analyzed by qPCR and normalized to the expression of three housekeeping genes. The data points represent expression levels in individual patients. Statistical testing was performed using unpaired *t*-test.

NOTCH1 expression in CLL cells. This observation is particularly relevant, as BCL-2 is a key regulator of apoptosis and a therapeutic target in CLL [44]. The co-occurrence of high NOTCH1 and BCL-2 levels in a subset of CLL patients raises the possibility that NOTCH1 signaling may contribute to the survival and drug resistance of CLL cells by upregulating BCL-2 expression. Recent studies have already shown that NOTCH1 signaling is able to modulate expression of BCL-2 and other BCL-2 family members [45,46]. However, further research is needed to confirm the functional relationship between NOTCH1 and BCL-2 in CLL and to explore the therapeutic implications of targeting this axis.

In line with previous reports [22], miR-34a expression was significantly lower in CLL patients with altered *TP53* compared to those with wild-type *TP53* ($p = 0.0007$) (Figure 5(C)). These results corroborate our *in vitro* findings, indicating that p53 positively regulates NOTCH1 expression in CLL cells and that this regulation may be independent of miR-34a. The decreased NOTCH1 levels in patients with altered *TP53* status suggest that the loss of p53 function may contribute to reduced NOTCH1 expression in these cases, potentially impacting disease progression and treatment response. However, it is important to note that the relationship between p53 and NOTCH1 in CLL is likely to be complex and context-dependent, as evidenced by the variable NOTCH1 levels observed among patients with wild-type *TP53*. Additional factors, such as the presence of other genetic alterations or differences in the tumor microenvironment, may modulate the p53-NOTCH1 axis in individual patients.

In conclusion, our study demonstrates a direct positive correlation between p53 and NOTCH1 in CLL cells,

which appears to be independent of miR-34a-mediated regulation. While miR-34a directly targets the *NOTCH1* 3′ UTR, its modulation does not significantly impact NOTCH1 protein levels or signaling activity in CLL cell lines. These findings suggest that the p53-miR-34a axis may fine-tune NOTCH1 signaling under physiological conditions but may be overridden by other regulatory mechanisms in the context of CLL pathogenesis. The observed association between high NOTCH1 levels and increased BCL-2 expression in CLL patients highlights a potential anti-apoptotic role for NOTCH1 in this malignancy, which warrants further investigation.

Discussion

Our study provides novel insights into the complex interplay between p53, miR-34a, and NOTCH1 in CLL and underscores the importance of considering the cellular context when investigating the regulatory networks governing cancer cell behavior.

Our key findings demonstrate that p53 directly regulates NOTCH1 activity and protein expression. Unexpectedly, when miR-34a levels were altered through transfection or p53 manipulation this did not significantly influence NOTCH1 in CLL cells. In CLL cell lines and patient cells, we did not see a negative regulatory effect of the p53-miR-34a axis on NOTCH1 signaling, suggesting that miR-34a may fine-tune cellular processes within a more complex, context-dependent network.

In different contexts, p53 and NOTCH1 pathways can regulate each other positively or negatively. Either directly by interaction, where p53 binds the *NOTCH1* promoter to enhance *NOTCH1* expression [37,38] or NOTCH1 inhibits p53 phosphorylation and DNA binding [47] or indirectly *via* processes modulating downstream mediators like: (1) p53 inhibits expression of Presenilin, which is part of the γ-secretase complex necessary for NOTCH1 activation [48]; (2) suppression of MAML-p300 dependent transcriptional activity of p53 by competition with NOTCH1 [48,49]; (3) miR-34a, which is positively regulated by p53, increases p53 *via* inhibition of p53-repressing factors like NOTCH1 and Sirtuin1 (SIRT1) [50,51]. In nonmalignant cells, it is important that the relation between p53 and NOTCH1 is well balanced and the pro-apoptotic function of p53 is sustained, e.g. *via* the p53-miR-34a feedback loop. In contrast, in CLL, Secchiero et al. showed that p53 directly induced NOTCH1, suggesting an anti-apoptotic mechanism of p53 [39].

Putting our results into this context, the direct positive effect of p53 on NOTCH1 might be preferably induced in CLL to sustain malignant anti-apoptotic functions. This anti-apoptotic function could also include upregulation of BCL-2, possibly *via* NOTCH1. This fits to our observation of increased BCL-2 in some CLL patients with high NOTCH1 levels. Interestingly, BCL-2 is also a target of miR-34a [52]. To maintain the anti-apoptotic status of CLL cells, the pro-apoptotic functions of the p53-miR-34a axis might be suppressed by competing endogenous RNAs (ceRNAs), RNA-binding proteins could potentially interfere with miR-34a function [40,41] or additional mechanisms that were already shown in other cancers. In hepatocellular carcinoma TGF-β inhibits miR-34a expression [53], and in DLBCL, MYC-mediated repression of miR-34a transcription causes upregulation of the miR-34a target Forkhead Box P1 (FOXP1) [54]. Even if the tumor-suppressive miR-34a function is similarly repressed in CLL, it is unexpected that we do not see a significant effect of experimental miR-34a overexpression on NOTCH1 signaling. It has been reported previously that *NOTCH1* is targeted by miR-34a [55], and we also show that miR-34a targets the *NOTCH1* 3′ UTR. This might be caused by technical issues and high variance of our data which might be solved by a larger number of experimental repetitions but could also be addressed by stable expression of a pre-miR-34a construct or miR-34a manipulation in primary CLL cells to have a more realistic context or even in co-culture with stromal cells since miR-34a was shown upregulated in lymph nodes of CLL patients [56].

Interestingly, miR-34a overexpression is associated with high levels of its target SIRT1 in smooth muscle cell differentiation from pluripotent stem cells [57]. It is possible that, in the context of CLL, miR-34a is not a negative but a positive regulator of NOTCH1 to sustain anti-apoptotic effects. This would be in contrast with findings in other cancers, such as colorectal cancer, glioblastoma and breast cancer, where miR-34a directly regulates NOTCH1 at the post-transcriptional level, affecting cell proliferation and apoptosis [58–60].

Other cancers have different tumor microenvironments, genetic backgrounds, and survival strategies that might explain contrasting roles of miR-34a. Nevertheless, a broader context of the complex miR-34a regulatory network including a variety of miR-34a targets in other cancers could guide potential future research directions in CLL.

Regarding other miR-34a targets in CLL, recent studies identified that murine double minute 4 (MDM4) is directly targeted and inhibited by miR-34a, leading to upregulation of p53 and increased apoptosis of CLL cells [61]. In addition, activation of miR-34a upon DNA damage repair, leads to interaction of miR-34a with the FOXP1 3′ UTR to downregulate FOXP1 levels. Consequently, low FOXP1 levels limit BCR signaling

partially by derepressing the BCR-inhibitory molecule CD22 [62]. Moreover, also the well-known miR-34a target and p53 regulator SIRT1 and b-MYB have been reported to be regulated by miR-34a in CLL [63,64]. Although not specifically stated for b-MYB, other MYB family members play a role in CLL pathogenesis by regulation of miR-155 [65]. Further research is necessary to validate these other miR-34a targets in CLL to better understand the overall role of miR-34a and the regulatory network around it. After understanding miR-34a's regulatory roles, we also need to consider how targeting these pathways could be clinically relevant, particularly in CLL, where prognostic and therapeutic strategies hinge on such molecular insights to improve clinical outcomes for CLL patients and beyond.

The clinical relevance of p53 status, miR-34a expression and NOTCH1 signaling in CLL is well established. *TP53* alterations predict poor survival and treatment resistance, including subclonal mutations that can be selected under treatment pressure and drive resistance or development of Richter's transformation. *NOTCH1* mutations and the presence of cleaved/activated NICD in CLL cells have predictive and prognostic impact on disease development and survival [32–35,66]. In addition, low miR-34a levels are a prognostic marker for adverse outcomes, independent of *TP53* status [22]. Therefore, miR-34a expression levels besides comprehensive *TP53* and *NOTCH1* mutation analysis *via* next-generation sequencing in CLL patients are important for prognostic assessments. Knowing the *TP53* and *NOTCH1* mutation status of CLL patients is crucial for choosing suitable and tailored treatment strategies. However, there are no effective treatment strategies specifically targeting p53, NOTCH1, or the regulatory network of miR-34a in CLL.

In contrast, in multiple myeloma, miR-34a was shown to enhance the effect of three anticancer agents: γ-secretase inhibitor, sirtinol, and zoledronic acid [67]. Moreover, in AML and breast cancer, miR-34a was reported to target the immune checkpoint PD-L1, which is frequently targeted to enhance anti-tumor immune responses [68,69]. This role of miR-34a in modulating immune checkpoint pathways suggests its potential in the development of new immunotherapies, especially for *TP53*-mutated cancers, and could inform new CLL treatment strategies.

Potential useful treatment strategies for CLL could include miR-34a restoration therapy. So far, the delivery mechanisms might be the highest hurdle. A phase I study to deliver a miR-34a mimetic *via* liposomal nanoparticles (MRX34) was terminated due to many adverse events but different delivery systems are in development [55]. However, restoration or overexpression of miR-34a in CLL could improve patient outcomes mainly by downregulating p53-inhibiting miR-34a targets like SIRT1 or MDM4, leading to upregulation of p53 function and thereby repressing CLL cell survival. Another effect of miR-34a restoration could be enhancement of the p53-miR-34a-PD-L1 axis to enhance anti-tumor immune response to overcome immune evasion and improve treatment outcomes.

Direct targeting of NOTCH1 signaling is still a potential treatment strategy for CLL. Although γ-secretase inhibitors have not been successfully tested due to severe side effects in CLL, other direct and indirect NOTCH1 inhibitors are in development [70]. Learning from other cancers, there could also be therapeutic implications from targeting other miR-34a targets described above. FOXP1 is upregulated in more aggressive disease and associated with shorter OS [71]; targeting FOXP1/BCR signaling could induce apoptosis in CLL cells. Targeting the miR-34a targets MDM4 or SIRT1 could help enhance anti-tumorigenic p53 function, but only in p53 wild-type patients. In addition, investigation of combination therapies that include γ-secretase/other NOTCH1 inhibitors, miR-34a mimetics, and PD-L1 or other miR-34a target inhibitors could help develop new treatment strategies exploiting potential synergistic effects of inhibiting several players of the p53-miR-34a network. Hints that miR-34a is downregulated upon ibrutinib treatment [56] raise questions about the impact of current treatments on the p53 regulatory network and how adjustments in therapy might be needed for optimal treatment outcomes, considering the broad role of miR-34a in regulating different cellular pathways.

Further research is needed to elucidate the mechanisms underlying the context-dependent effects of miR-34a on NOTCH1 and to identify additional factors that may modulate this relationship in CLL. A better understanding of these processes may guide the development of targeted therapeutic strategies for high-risk CLL patients with *TP53* alterations, ultimately improving disease management and patient outcomes. In particular, the potential synergistic effects of combining p53-reactivating agents, such as MDM2 inhibitors, with NOTCH1 or BCL-2 inhibitors should be explored to overcome treatment resistance and enhance the efficacy of targeted therapies in CLL.

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Data availability statement

For this study no datasets have been deposited. Additional information on original experimental data can be requested from the corresponding author.

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