A network meta-analysis and whole-exome analysis of chronic lymphocytic leukemia patients treated with ibrutinib and chlorambucil

A network meta-analysis and whole-exome analysis of chronic lymphocytic leukemia patients treated with ibrutinib and chlorambucil

By

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I have reviewed this thesis. It represents work done by the author under my guidance/supervision.

Primary Mentor: Dr. Gad Getz, Ph.D.

Signature:

TABLE OF CONTENTS

Page

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Overview

Chronic lymphocytic leukemia (CLL) is a type of blood cancer with progressive accumulation of phenotypically mature malignant B lymphocytes. The CLL patients with Rai stage of 0 have an overall survival of >10 years, and patients with Binet stage of A have an overall survival of 12 years. Under the standard CLL treatment therapies, more than 80% of patients are alive at 3 years, and 5-year survival has significantly increased from 60% to 66% from 2001 to 2014 due to the advance in new therapies for CLL. The first line regimens in the most updated National Comprehensive Cancer Network (NCCN) guideline in 2020 for CLL include preferred regimens (ibrutinib, acalabrutinib, acalabrutinib plus obinutuzumab, venetoclax plus obinutuzumab), and other regimens (such as bendamustine, chlorambucil plus obinutuzumab, ibrutinib plus obinutuzumab, obinutuzumab, fludarabine plus cytoxan plus rituxan, fludarabine plus rituximab, ibrutinib plus rituximab). Although ibrutinib was also approved as an important first-line treatment for CLL and there were a few systematic reviews comparing ibrutinib with fludarabine, bendamustine, or rituximab, comprehensive evidence comparing different CLL therapies, including ibrutinib or chlorambucil, is still lacking. To address this research gap, in Paper 1 we used the data of the published studies to conduct a network meta-analysis to estimate the effect of various therapies (ibrutinib and chlorambucil, alone or combined with other therapies) on both efficacy and safety outcomes in patients with CLL.

CLL does not require treatment once it is diagnosed. Once CLL patients need initial treatment, some biomarkers will be tested, including 17p deletion, *TP53* mutation, *IGHV* mutation, and complex karyotype. Based on the test results of these biomarkers, as well as patients' age, overall health, and medications that the patients are currently taking, the first-line treatment can be provided.

Although ibrutinib has been approved for CLL initial treatment by Food and Drug Administration (FDA) and listed as first-line therapy, the results from a randomized clinical trial showed that the response rate was 86% within a median follow-up period of 18.4 months, but only 4 out of 86 patients achieved complete response. However, there is no research studying the clonal and subclonal evolution of CLL patients under the treatment of ibrutinib and whether this is related to patients' response or associated with whether the patient will develop secondary resistance during the treatment. To explore this research question, in Paper 2 we investigated the clonal and subclonal evolution under the treatment of ibrutinib and the difference between ibrutinib and chlorambucil.

In summary, the significance of our work is summarizing the current evidence of ibrutinib and chlorambucil in the treatment of CLL patients and figuring out the clonal and subclonal evolution under the treatment of ibrutinib.

MANUSCRIPT 1:

Comparative Efficacy and Safety of Ibrutinib and Chlorambucil for Chronic Lymphocytic Leukemia Patients: A Systematic Review and Network Meta-analysis

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Running Title: ibrutinib and chlorambucil for Chronic Lymphocytic Leukemia – network meta-analysis

Abstract

Background

Both ibrutinib and chlorambucil, used alone or in combination, have been used for first-line treatment for CLL. However, a comprehensive comparison among different CLL therapies, including ibrutinib or chlorambucil, is still lacking. In this study, we used network meta-analysis to estimate the effects of these therapies on clinical outcomes.

Methods

We did a network meta-analysis based on a systematic review comparing ibrutinib or chlorambucil against ibrutinib or chlorambucil combined with other medicines, other CLL treatments, placebo, or no treatment. Five databases were searched from inception up to Feb. 12, 2022. Only randomized controlled trials were included for analysis. The primary efficacy outcome was progression-free survival, and the primary safety outcome was based on adverse events. This study was registered with PROSPERO.

Results

A total of 6 eligible studies involving 1618 patients treated with six different treatment therapies were assessed. Compared with chlorambucil alone as reference therapy, both ibrutinib and ibrutinib combined therapies (ibrutinib plus ublituximab) significantly prolonged progression-free survival (HR 0.16, $CI_{95\%}$ [0.04, 0.74]; HR 0.08, $CI_{95\%}$ [0.01, 0.86], respectively). No significant difference in progression-free survival was found between chlorambucil and rituximab, or between ibrutinib and ibrunitib combined therapies. No significant difference in the safety outcome based on adverse events was found between chlorambucil, ibrutinib, ibrutinib plus rituximab, ibrutinib plus ublituximab, and rituximab alone.

Conclusion

Significant differences exist among ibrutinib, chlorambucil, and their combined therapies in terms of progression-free survival. Both ibrutinib and ibrutinib plus ublituximab might be considered over chlorambucil. Further randomized clinical trials directly comparing the interventions such as ibrutinib plus ublituximab versus chlorambucil alone should be designed to validate the results in our study.

Introduction

Chronic lymphocytic leukemia (CLL) is a kind of blood cancer with progressive accumulation of phenotypically mature malignant B lymphocytes. CLL is the most common type of leukemia in developed countries, with an age-adjusted incidence of 4-5 per 100,000 population [1]. The median age at diagnosis is 72 years, with more men than women (2:1) [1]. One of the features of CLL is that it does not require treatment until indications to start therapy develop. The historical treatments of CLL include chemotherapy such as alkylating agents like chlorambucil (by slowing or stopping the growth of cancer cells in the body), cyclophosphamide, and bendamustine, chemoimmunotherapy such as fludarabine plus cytoxan plus rituxan (FCR) or bendamustine plus rituximab (BR), and targeted therapies such as Bruton Tyrosine Kinase (BTK) inhibitor ibrutinib, Splenic Tyrosine Kinase (SYK) inhibitors fostamatinib, Phosphoinositide 3-kinase (PI3K) inhibitors idelalisib, B-cell Lymphoma 2 (BCL2) antagonist venetoclax, as well as Chimeric Antigen Receptor T cell (CAR-T) therapy [2-3]. The first line regimens in the most updated National Comprehensive Cancer Network (NCCN) guideline in 2020 for CLL include preferred regimens (ibrutinib, acalabrutinib, acalabrutinib plus obinutuzumab, venetoclax plus obinutuzumab), and other regimens (such as bendamustine, chlorambucil plus obinutuzumab, ibrutinib plus obinutuzumab, obinutuzumab, FCR, fludarabine plus rituximab, ibrutinib plus rituximab). Although there are many new therapies in the current guideline, we choose to focus on one typical chemotherapy chlorambucil and one typical targeted therapy ibrutinib.

In 2014, a study published in the New England Journal of Medicine (NEJM) showed that ibrutinib was superior to ofatumumab in terms of improving progression-free survival, overall survival, and response rate in patients with previously treated CLL or small lymphocytic lymphoma [4]. In 2015,

another study also published in NEJM showed that ibrutinib significantly improved progression-free survival as compared to chlorambucil as an initial treatment for CLL or small lymphocytic lymphoma patients [5]. In 2018, multi-center randomized controlled trial (RCT) results showed that ibrutinib was superior to bendamustine plus rituximab for older patients with CLL in terms of improving progression-free survival [6]. In 2019, an RCT with 529 participants concluded that ibrutinib-rituximab therapy was superior to FCR in terms of progression-free survival and overall survival in patients 70 years or younger with untreated CLL [7]. In 2018, a systematic review [8] reported that the hazard ratio of progression-free survival comparing ibrutinib with chlorambucil was 0.16 ($CI_{95\%}$ [0.08, 0.31]). However, this study only used the data from the Resonate-2 study with a median follow-up time of 18.4 months published in 2015. Actually, the Resonate-2 study published the results of 5 years follow-up in 2020. Therefore, the systematic review comparing ibrutinib and chlorambucil should be updated. Besides, the indirect comparison between ibrutinib and chlorambucil through a third drug such as rituximab should also be incorporated using network meta-analysis. Moreover, the updated first-line therapies recommended in the 2020 NCCN guideline should also be included in the systematic review, especially the combined therapies such as ibrutinib plus rituximab, ibrutinib plus ublituximab, and chlorambucil plus obinutuzumab. In this way, more than two interventions need to be evaluated. The network meta-analysis can combine both direct and indirect estimates across a network of interventions in a single analysis and then can inform comparative effectiveness of multiple interventions, which should be used in the systematic reviews comparing multiple interventions.

Despite the value of ibrutinib as an important first-line initial treatment for CLL and a few systematic reviews comparing ibrutinib with fludarabine, bendamustine, or rituximab, a

comprehensive comparison between ibrutinib or chlorambucil using both direct and indirect comparisons, is still lacking. We wanted to test (i) whether meta-analysis using only indirect comparison clinical trials could yield results consistent with the direct comparison clinical trial; and (ii) whether meta-analysis using both the direct and indirect clinical trials will result in tighter confidence intervals relative to the direct clinical trial alone. Therefore, in order to investigate the efficacy and safety of ibrutinib and chlorambucil using both direct and indirect comparisons and to obtain the rankings of both ibrutinib or chlorambucil used alone and updated first-line combined therapies, this study used the available data to conduct a network meta-analysis to estimate the effect of various therapies (ibrutinib and chlorambucil, alone or combined with other therapies) on both efficacy and safety in patients with CLL.

Methods

Search strategy and selection criteria

This systematic review and network meta-analysis were registered at PROSPERO (https://library.cumc.columbia.edu/insight/prospero-registry-systematic-review-protocols) and is reported according to the standard of Preferred reporting items for systematic reviews and meta-analysis (PRISMA) extension statement for network meta-analysis [9].

The databases of Medline, Embase, Cochrane Library, Web of Science, and ClinicalTrials.gov were searched from the inception of each database to Feb. 12, 2022. We selected only randomized controlled trials that compared ibrutinib alone or chlorambucil alone as initial therapy for CLL adult patients, against ibrutinib or chlorambucil, combined with other therapies, other CLL treatments, placebo, or no treatment. The primary efficacy outcome of this study was progression-

free survival, and the primary safety outcome of this study was any adverse event. Studies with at least one primary outcome were included. The studies with the intervention and comparison of a single agent that cannot form the loop between chlorambucil and ibrutinib for network-meta analysis were excluded (for example, if there is a trial comparing the single-agent bendamustine with ibrutinib, but there is no trial comparing bendamustine with chlorambucil, then we are not able to form the loop between chlorambucil, ibrutinib, and bendamustine. In such a case, a trial comparing bendamustine with ibrutinib was excluded). The Mesh word "Leukemia, Lymphocytic, Chronic, B-Cell" was used for searching strategies. Details of searching strategies for each of the databases are described in the appendix (Appendix 1).

Data extraction and quality assessment

Two authors independently screened the titles and abstracts of retrieved studies and then evaluated the full text of the articles at the platform of Covidence (https://www.covidence.org/). The study's basic information and data were extracted by the same two reviewers using a standardized extraction form. The abstracted data included study characteristics, patient baseline characteristics, interventions, comparisons, and outcomes. The Cochrane collaboration’s risk of bias assessment 2 (RoB-2) was used to evaluate the risk of bias in each comparison for each outcome of each study [10]. All extracted data were double-checked by the third author, and any discrepancies were resolved by discussion between these three authors.

Type of interventions

The initial therapy for CLL patients in this study included the targeted therapy ibrutinib--a kind of Bruton's tyrosine kinase (BTK) inhibitor, chlorambucil--one of the chemotherapies, given alone

or in combination with other treatments, including any other chemotherapies, targeted therapies, and immunotherapies.

Outcomes

The primary efficacy outcome of this systematic review was progression-free survival, and the secondary efficacy outcome was overall survival. The primary safety outcome was the total number of adverse events of grade ≥ 3.

Quality of evidence

The quality of evidence for network meta-analysis was assessed using GRADEpro (GRADE working group, McMaster University, Canada) based on the method of Salanti [11], including five domains: risk of bias, inconsistency, indirectness, imprecision, and publication bias. Four levels of quality of evidence were used: high, moderate, low, and very low.

Data synthesis and statistical analysis

The relative intervention effects (i.e., risk ratio [RR], hazard ratio [HR]) were estimated for individual studies. A direct meta-analysis with a random-effect model was used to pool RRs or HRs. Heterogeneity was assessed using the I^2 statistic and Cochran Q test. Comparing all treatments using both direct and indirect data was completed by network meta-analysis with a Bayesian consistency model [12]. The inconsistency between direct and indirect estimates was assessed by the global inconsistency test [13]. The ranking plot with probabilities, the surface under the cumulative ranking (SUCRA), and the cluster ranking plot were used to rank the

hierarchy of interventions analyzed in the network meta-analysis [14]. The comparison-adjusted funnel plot was used to detect the publication bias.

Stata version 16.0 [15] using "metan," "mvmeta," "networkplot," and R package "gemtc" [16] were used to do all the analyses. A two-sided p-value of less than 0.05 was regarded as statistically significant for all the tests in the network meta-analysis. (The detailed methods and codes are listed in the Appendix 2)

Results

Study selection

We identified 3040 records by searching Medline, Embase, Cochrane, Web of Science, and ClinicalTrials.gov, in which 773 potentially eligible studies were retrieved. Of these studies, 614 were excluded due to not being relevant to the topic, then 153 studies were excluded due to wrong study design (n=45), wrong intervention (n=17), wrong outcomes (n=29), wrong patient population (n=4), review papers (n=26) or duplicate studies (n=32). Finally, six studies were included in this systematic review, and all of them were used for quantitative analysis. The PRISMA flow diagram (Figure 1) summarized the process of electronic screening and article selection on Covidence.

Study characteristics

The six studies involving 1618 participants were included and assessed in the network meta-analysis. These studies were done from 2014 to 2021; most of them were done in North America

and Europe, and one of them was done in China. The mean age of the patients included in this meta-analysis was 66.3 years. In total, 62.0% of the 1618 participants were male, 47.8% were in Rai stage III or IV, 22.9% obtained Chromosome 11q22.3 deletion, and 60.9% were unmutated IGHV. The median follow-up time of these included studies ranged from 17.8 to 88.8 months. Other characteristics and study designs of the included articles were summarized in Table 1.

Risk of bias within studies

Most studies (4 out of 6) contained a low risk of bias; one study with some concerns and one study with high risk were found to deviate from the intended intervention, as is shown in Figure 2.

Network geometry

The network geometry including all the eligible comparisons for the primary efficacy outcome is presented in figure 3A. The six treatment therapies included in this network geometry are ibrutinib, chlorambucil, rituximab, ibrutinib plus rituximab, ibrutinib plus ublituximab, and chlorambucil plus obinutuzumab. There was only one loop between ibrutinib, chlorambucil, and rituximab with both direct and indirect comparisons. The proportions of direct and indirect comparisons in the network meta-analysis were shown in the contribution plot (Figure 3B).

Results of network meta-analysis

The six studies involving 1618 participants assessed both the progression-free survival and safety outcome (adverse events), and five studies assessed overall survival. As is shown in Figure 4, our results showed that both ibrutinib and ibrutinib plus ublituximab were associated with a significantly decreased risk of progression-free survival (HR 0.16, $CI_{95\%}$ [0.04, 0.74]; HR 0.08,

$CI_{95\%}$ [0.01, 0.86], respectively) for chlorambucil alone. As for the overall survival, our results showed that compared with chlorambucil alone, both ibrutinib and chlorambucil plus obinutuzumab were associated with a significantly decreased risk of overall survival (HR 0.44, $CI_{95\%}$ [0.21, 0.90]; HR 0.41, $CI_{95\%}$ [0.23, 0.74], respectively). No significant difference in progression-free survival was found between chlorambucil and rituximab. No significant difference in safety outcome of adverse events with the severity of grade ≥ 3 was found between chlorambucil, ibrutinib, ibrutinib plus rituximab, ibrutinib plus ublituximab, and rituximab alone.

Heterogeneity and inconsistency

The loop-specific inconsistency test was performed and suggested no evidence of inconsistency of treatment effects for progression-free survival and adverse events (p = 0.79), as is shown in Figure 5. The results from direct comparison alone (HR 0.15, $CI_{95\%}$ [0.09, 0.21]) was not the same with the results from indirect comparison alone (HR 0.20, $CI_{95\%}$ [0.01, 6.30]), and the network results based on both direct and indirect comparisons was more precise (HR 0.16, $CI_{95\%}$ [0.04, 0.74]) than the indirect comparison. The I^2 was 0 for the comparisons between two interventions in terms of PFS. The loop heterogeneity tau2 was less than 0.001 for the loop of ibrutinib-chlorambucil-rituximab (p=0.461). The global inconsistency test was also performed, and no evidence of inconsistency was found.

Synthesis of Results

The full findings of this network meta-analysis of the primary outcome (progression-free survival) and safety outcome (adverse events) were shown in Figure 7. Our analysis showed that ibrutinib had a significantly reduced hazard for disease progression as compared with chlorambucil (HR

0.16, $CI_{95\%}$ [0.04, 0.74]). For safety outcome adverse events with the severity of grade ≥ 3, no significant difference was found between ibrutinib and chlorambucil (RR 0.31, $CI_{95\%}$ [0.04, 1.77]). The GRADE quality of evidence was also marked in Figure 6. The quality of evidence for both efficacy and safety was generally rated as low to moderate in most comparisons. The detailed evaluation of the evidence quality was summarized in the Appendix (Appendix 3).

Ranking of the interventions

The probabilities for each intervention of being ranked as the first, second, third, fourth, fifth, and the last were shown in Figure 7 in terms of efficacy (progression-free survival) and safety (adverse events). As for the progression-free survival efficacy outcome, ibrutinib plus ublituximab has the highest probability of ranking the first, ibrutinib plus rituximab has the highest probability of ranking second, ibrutinib alone has the highest probability of ranking third, chlorambucil plus obinutuzumab has the highest probability of ranking the fourth, chlorambucil alone has the highest probability of ranking the fifth, and rituximab alone has the highest probability of ranking the sixth. As for the adverse events safety outcome, rituximab has the highest probability of ranking first, while ibrutinib plus ublituximab has the highest probability of ranking last. As is shown in Figure 8, the cluster rank indicates that ibrutinib plus ubituximab is the intervention that is associated with better efficacy of progression-free survival as well as lower adverse events, based on the calculated SUCRA (surface under cumulative ranking).

Publication bias

Because there was only one study for each direct comparison, the effect size at comparison-specific pooled effect was 0, so we did not detect the publication bias based on the funnel plot for primary

efficacy outcome (PFS). But there might be potential publication bias, considering that all the included studies in this systematic review reported positive results, and we did not include studies from conference abstracts.

Discussion

This study provides a framework for the comparison of efficacy and safety among treatments, including chlorambucil or ibrutinib, for patients with CLL. The results suggest that ibrutinib, in combination with ubituximab was one of the most effective therapy and had an acceptable level of adverse events. Ibrutinib was superior to chlorambucil in terms of efficacy outcome of progression-free survival, and no significant difference in terms of safety outcome of adverse events. Looping back with the hypotheses of this study, we found that the meta-analysis with only indirect comparison gave consistent results with the direct comparison but with larger error-bars, and meta-analysis using both direct and indirect comparisons improved the confidence intervals in the safety comparison but not in the PFS analysis.

The result of our study is consistent with previous systematic review [8] concluding that ibrutinib should be considered over chlorambucil in the treatment of CLL based on the hazard ratio of 0.16 ($CI_{95\%}$ [0.08, 0.31]) for the outcome of progression-free survival. However, our study updated the previous systematic review by using the results of Resonate-2 study of 5 years follow-up published in 2020, instead of the results of Resonate-2 study with a median follow-up time of 18.4 months published in 2015. Moreover, our study added the indirect comparison between ibrutinib and chlorambucil by the third drug rituximab, which enabled us to estimate the effect with larger sample size. Furthermore, our study provided the ranking of both the ibrutinib, chlorambucil, and

their combined therapies, which indicates that ibrutinib plus ublituximab might be considered as the first choice among the six interventions we included in this systematic review.

Our study has some limitations. First, sub-group analysis and sensitivity analysis cannot be performed due to limited information from the original studies and a limited number of included studies. Second, publication bias was only detected by a funnel plot, but there might be potential publication bias, considering that all the included studies in this systematic review reported positive results, and we did not include studies from conference abstracts. Third, we only included the total number of adverse events as the primary safety outcome. Actually, these adverse events included bleeding, bruising, cardiovascular complications, infections, cytopenia, diarrhea, and dermatologic complications that have different clinical implications. Future studies reporting specific adverse events should be designed and included in this systematic review. Forth, the baseline characteristics of the patients reported in the original studies we included in this systematic review only contained the factors such as age, gender, Rai stage, *IGHV* status, and deletion of Chromosome 11q, other prognostic factors such as ZAP-70 and cytogenetic abnormalities were not reported, so that we were not able to summarize and assess the distribution of these prognostic factors across different studies in this systematic review. Fifth, although we reported both the heterogeneity for the comparison between two interventions and the loop heterogeneity between three interventions, we only included one study for each comparison, which made the assessment of heterogeneity difficult (although it may exist).

In summary, our analysis suggests that significant differences exist among ibrutinib, chlorambucil, and their combined therapies in terms of progression-free survival. Based on the results of this

network meta-analysis, both ibrutinib alone and ibrutinib plus ublituximab might be considered over chlorambucil; considering both the efficacy and safety, ibrutinib plus rituximab might be considered the first among the ibrutinib and chlorambucil therapies. However, further randomized clinical trials directly comparing the interventions such as ibrutinib plus ublituximab versus chlorambucil alone should be designed to validate the results of our study.

Tables and Figures

Author Last Name & Year	Study design	Sample size (n)	Population characteristics (Intervention Group)					Population characteristics (Control Group)					Interven-tion	Comparison	Outcomes	Median follow-up (month)
			Age (year)	Male sex (%)	Rai stage III or IV (%)	Chr.11q 22.3 deletion (%)	Unmut. IGHV (%)	Age (year)	Male sex (%)	Rai stage III or IV (%)	Chr.11q2 2.3 deletion (%)	Unmut. IGHV (%)				
Burger 2020[17]	RCT	269	73 (65-89)	88 (65)	60 (44)	29(21)	58 (43)	72 (65-90)	81 (61)	62 (47)	25(19)	60 (45)	Ibrutinib	Chlorambucil	PFS, OS, adverse events	60
Burger 2019[18]	RCT	208	65 (4-83)	75 (72)	38 (37)	27(26)	61 (59)	65 (42-81)	71 (68)	42 (40)	15(14)	62 (60)	Ibrutinib	Ibrutinib+Rituximab	PFS, OS, adverse events	36
Goede 2014[19]	RCT	356	74 (39-88)	140 (59)	NR	33(16)	129 (61)	72 (43-87)	75 (64)	NR	14(15)	58 (59)	Chloramb ucil+Obinutuzumab	Chlorambucil	PFS, OS, adverse events	NR
Sharman 2021[20]	RCT	224	66 (62-74)	44 (69)	31(51)	30(47)	53 (83)	67 (62-74)	46 (74)	26 (44)	27(44)	52 (84)	Ibrutinib+ Ublituximab	Ibrutinib	PFS, adverse events	41.6
Huang 2018[21]	RCT	160	65 (39-87)	77 (73)	79(79.8)	22(20.8)	63 (59.4)	67(21-86)	36 (66.7)	37 (72.5)	12(22.2)	35 (64.8)	Ibrutinib	Rituximab	PFS, OS, adverse events	17.8
Zucca 2017[22]	RCT	401	62.5 (26-79)	64 (46)	63(45.6)	Not reported	Not reported	60 (26-80)	69 (52.7)	53 40.6)	Not reported	Not reported	Rituximab	Chlorambucil	PFS, OS, adverse events	88.8

PFS: progression-free survival; OS: overall survival; NR: not reported

Table 1. Basic characteristics of studies included in the network meta-analysis

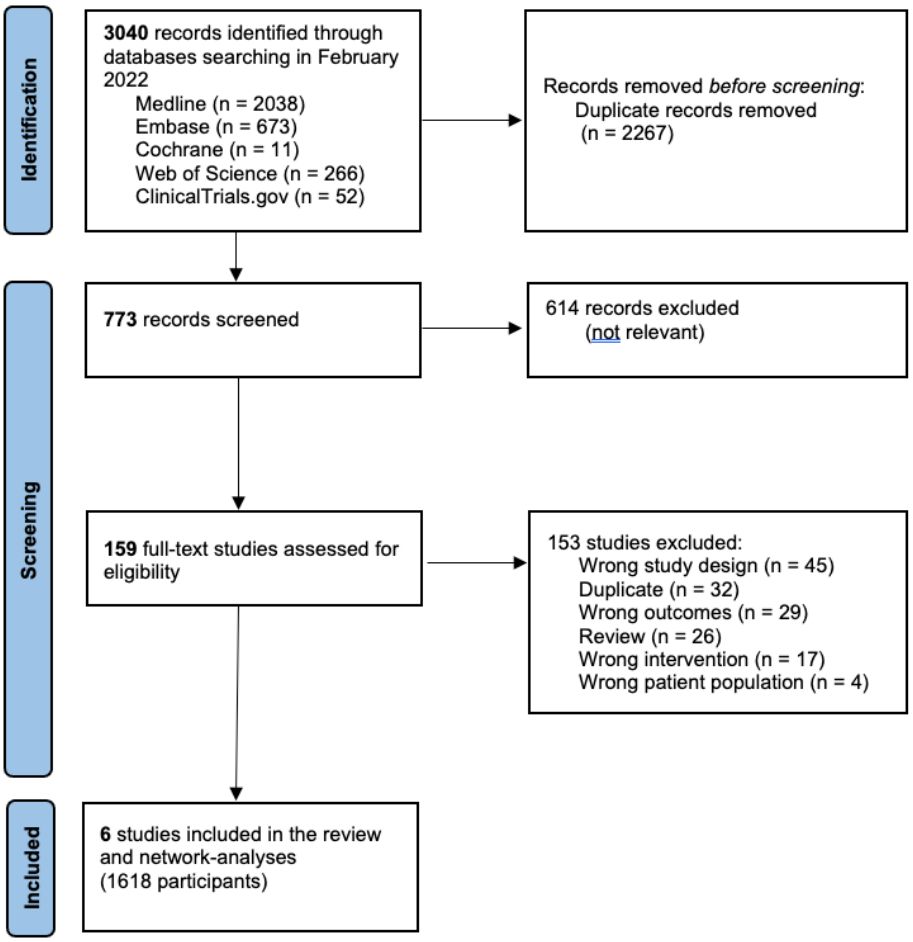

Figure 1. PRISMA flow diagram of screening and selecting the studies searched on databases.

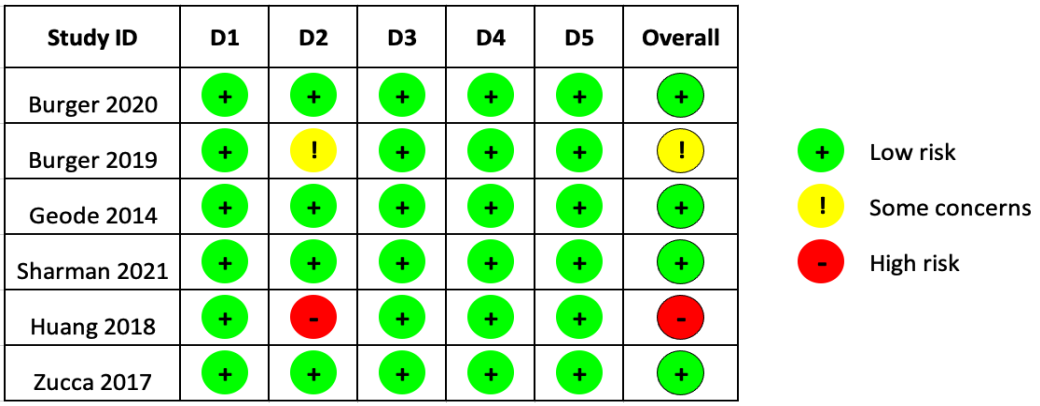

Figure 2. Risk of bias within included studies.
The risk of bias was evaluated as low risk in green, some concerns in yellow, and high risk in red, in terms of five domains (D1: domain 1, randomization; D2: domain 2, deviations; D3: domain 3, missing outcome data; D4: domain 4, measurement of the outcome; D5: domain 5, selection of the reported result).

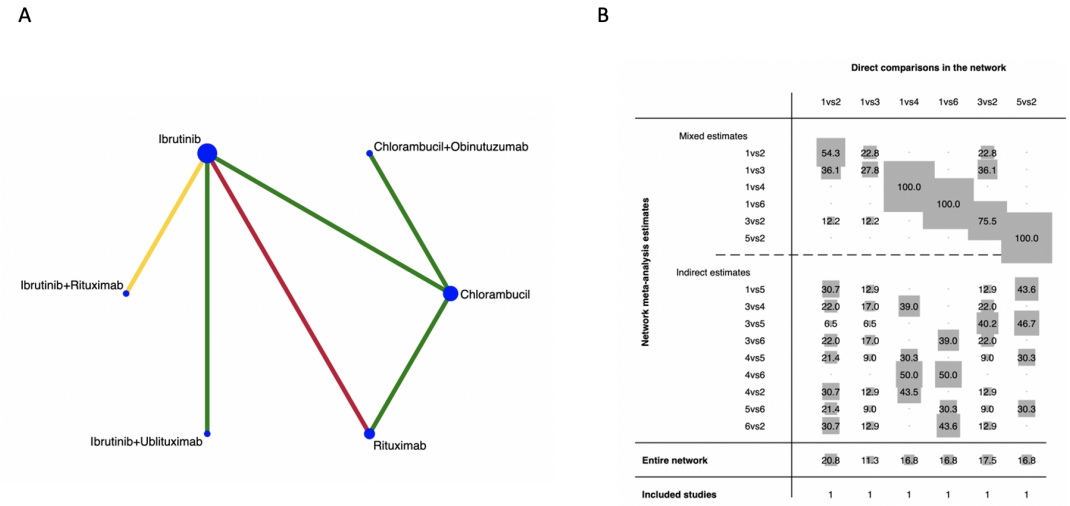

Figure 3. Network of eligible comparisons and contribution plot for primary efficacy outcomes.
(A) network of interventions. Line in green, yellow, and red represents low risk of bias, some concerns, and high risk of bias, respectively. The wider the line between interventions, the larger number of RCTs comparing those two interventions.
(B)contribution plot of direct and indirect comparison. 1: Ibrutinib, 2: Chlorambucil, 3: Rituximab, 4: Ibrutinib_Rituximab, 5: Chlorambucil_Obinutuzumab, 6: Ibrutinib+Ublituximab

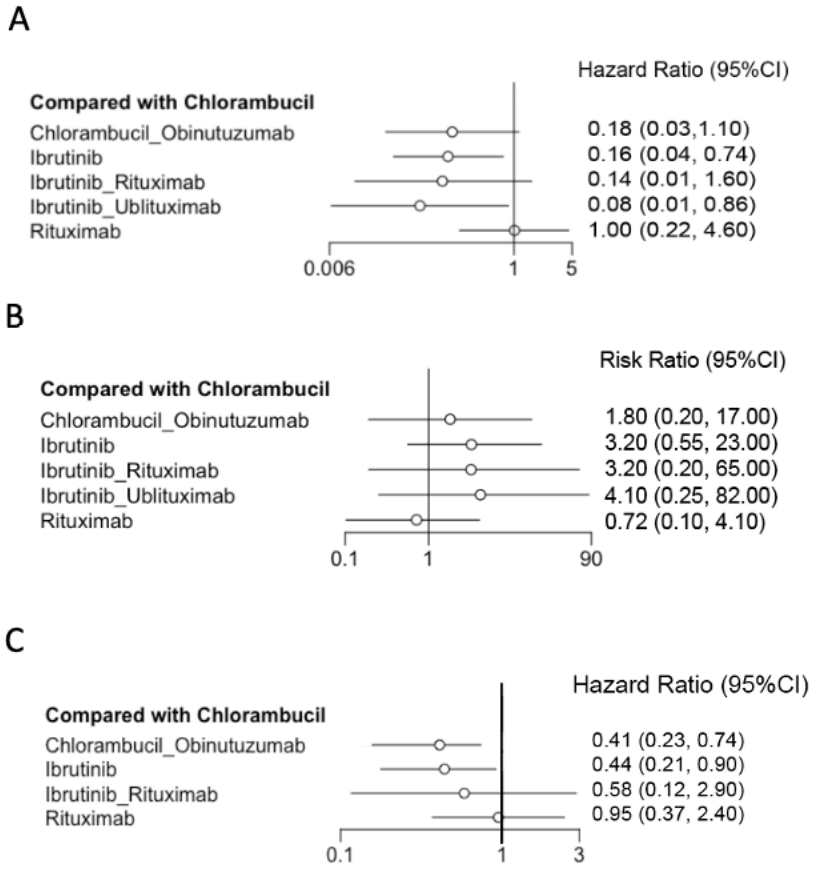

Figure 4. **Forest plots comparing 6 interventions in efficacy and safety.**
(A) Primary efficacy outcome (progression-free survival). (B) Safety (adverse events). (C) Secondary efficacy outcome (overall survival)

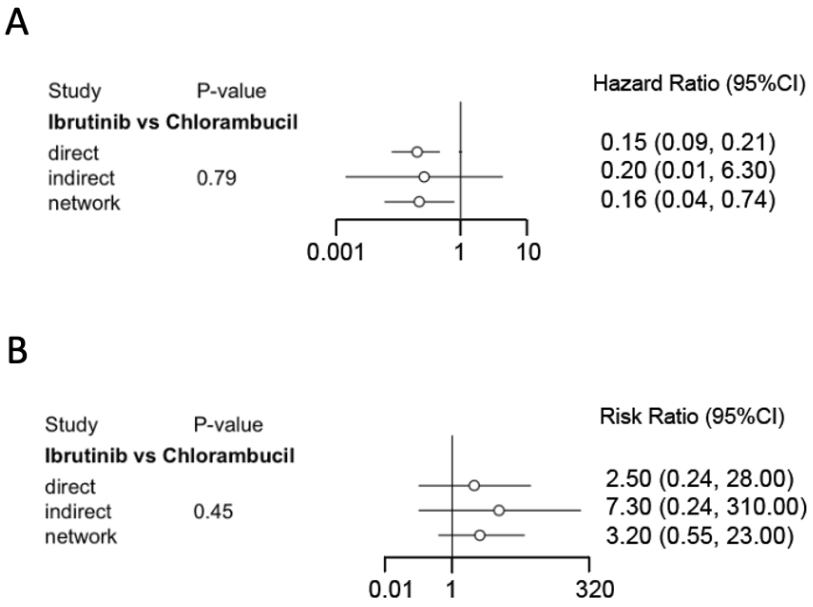

Figure 5. Inconsistency test for efficacy and safety.
(A) Progression-free survival. (B) Safety (adverse events). The P-value is for the test between direct and indirect results.

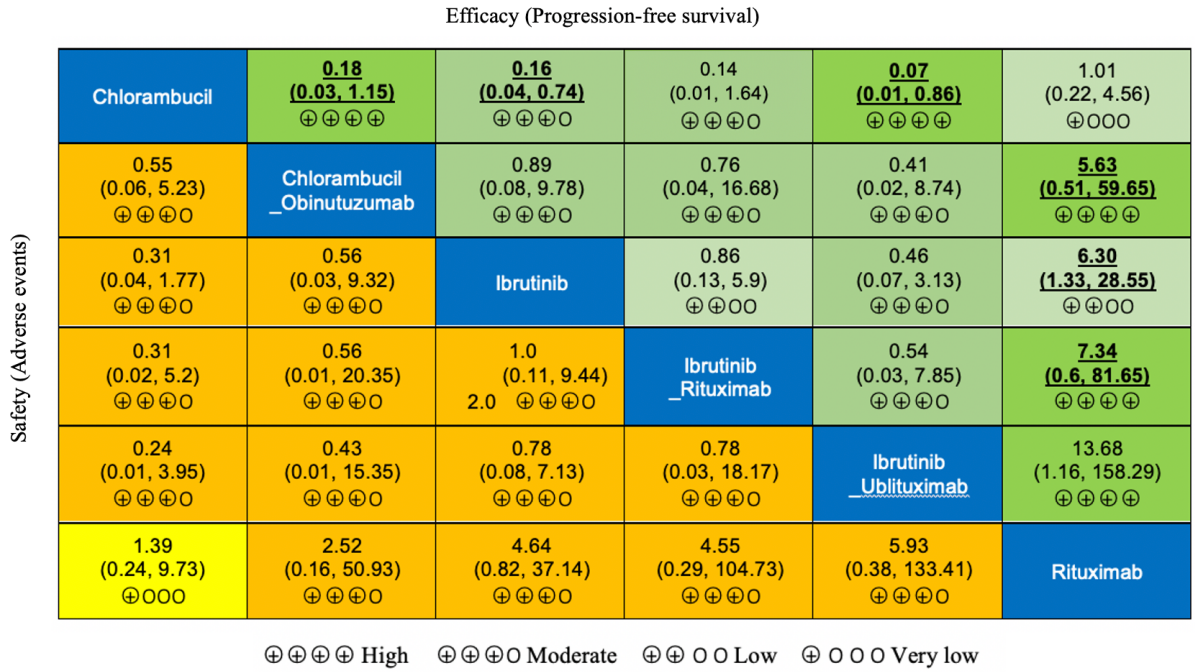

Figure 6. Network meta-analysis of primary efficacy (progress-free survival) and safety (adverse events) outcomes.

The upper right cells in green show the efficacy outcome (progress-free survival) in HRs (95%CI) based on included RCTs, and the lower-left cells in yellow show the safety outcome (adverse events) in RRs (95%CI). The results are the combined estimates from the network meta-analysis comparing the row-defining intervention and column-defining intervention. The comparisons should be read from left to right. The numbers in bold represent statistically significant results. The scale of the evidence quality corresponds to the colors of the cell, with the darker the color the higher the quality

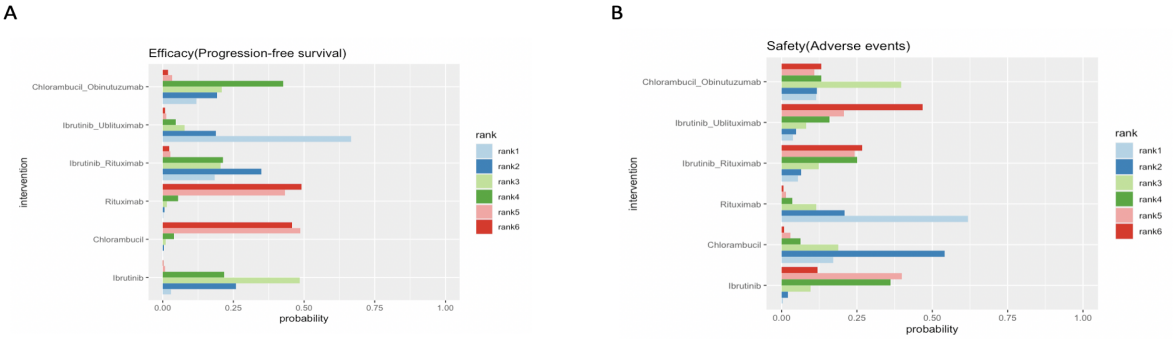

Figure 7. **Ranking plot for efficacy and safety using probabilities.**
This bar plot shows the probability of each intervention that has a certain rank such as ranking the first (rank 1) in terms of efficacy (A) and safety (B).

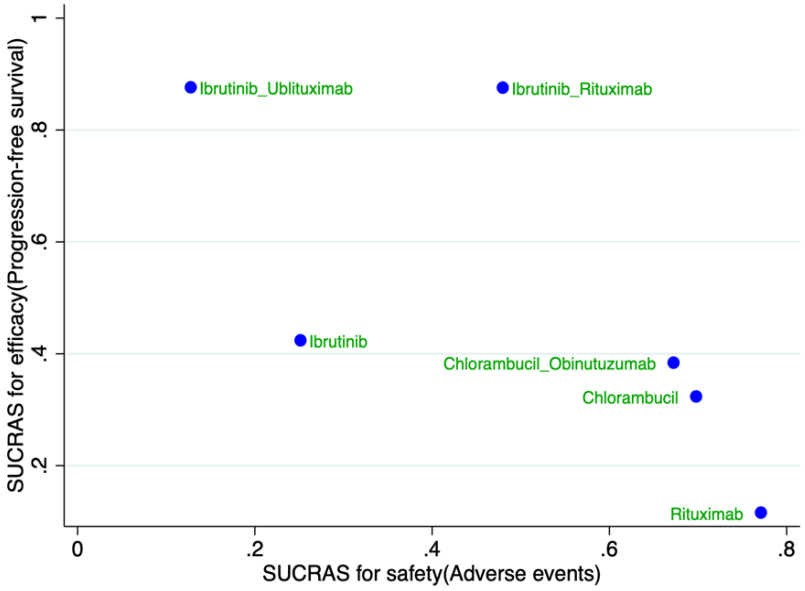

Figure 8. Cluster ranking plot for the intervention network.
The treatments are ranked by the surface under the cumulative ranking curve (SUCRA). The relative ranking for efficacy and safety was presented jointly. The dashed lines represent the different quadrants of the risk estimates. Treatments lying in the upper right corner are considered to perform better for both outcomes.

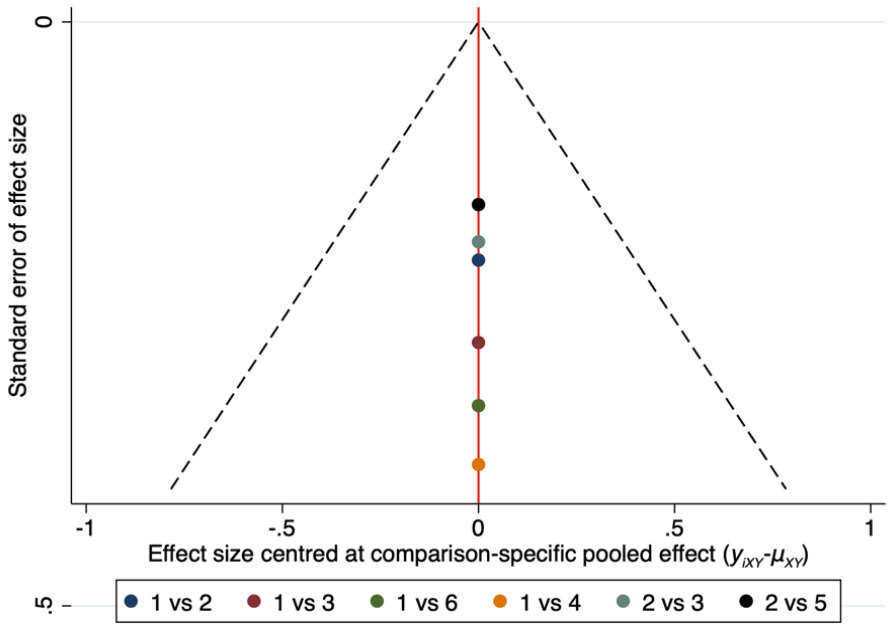

Figure 9. Funnel plot for primary efficacy outcome.

1: Ibrutinib, 2: Chlorambucil, 3: Rituximab, 4: Ibrutinib+Rituximab, 5: Chlorambucil_Obinutuzumab, 6: Ibrutinib+Ublituximab

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Supplement

Appendix 1. Search strategies for each of the databases.
The searching strategies for the databases of Medline, Embase, Cochrane library, Web of Science, and Clinicaltrial.gov were as follows.

Medline
#1 "Leukemia, Lymphocytic, Chronic, B-Cell"[Mesh] OR CLL[tiab] OR Chronic Lymphocytic Leukemia[tiab] OR Chronic Lymphocytic Leukemia[tiab] OR Lymphocytic Leukemia[tiab] OR Leukemia[tiab] OR B-Cell Lymphoma[tiab] OR Lymphoma[tiab] OR lymphocytic leukemias[tiab]
#2 "ibrutinib" [Supplementary Concept] OR Bruton's Tyrosine Kinase Inhibitor*[tiab] OR “BTK inhibitor*”[tiab]
#3 "Chlorambucil"[Mesh] OR LEUKERAN[tiab]
#4 #1 AND (#2 OR #3)

Embase

#1 ('chronic lymphatic leukemia'/exp OR 'cll':ab,ti,kw OR 'lymphocytic leukemia':ab,kw,ti OR 'leukemia':ab,kw,ti OR 'b-cell lymphoma':ab,kw,ti OR 'lymphoma':ab,kw,ti OR 'lymphocytic leukemias':ab,kw,ti) AND [embase]/lim

#2 'ibrutinib'/exp OR 'bruton tyrosine kinase inhibitor*':ab,kw,ti OR 'btk inhibitor*':ab,kw,ti

#3 'chlorambucil'/exp OR leukeran:ab,kw,ti

#4 #1 AND (#2 OR #3)

Cochrane library (12/02/2022)

#1 MeSH descriptor: [Leukemia, Lymphocytic, Chronic, B-cell] explode all trees
#2 MeSH descriptor: [Chlorambucil] explode all trees
#3 (ibrutinib):ti,ab,kw
#4 (#2 OR #3) AND #1

Web of Science
((TS=(ibrutinib)) AND TS=(Chlorambucil)) AND TS=(Chronic Lymphocytic Leukemia)

Clinicaltrial.gov 33+20-1=52
Intervention/treatment: Ibrutinib or Chlorambucil
Condition or disease: Chronic Lymphocytic Leukemia
Status: Completed
Study type: Interventional Studies (Clinical Trials)

Appendix 2. Detailed network meta-analysis methods and codes

In this study, we included six interventions. Therefore, in order to combine and integrate the results of varied studies comparing multiple treatments, a Bayesian framework model was used to conduct the network meta-analysis. Basically, direct comparisons were analyzed using data comparing the interventions directly in the original studies; indirect comparisons allowed us to estimate the relative effects of the two interventions that have not been compared directly in the original studies. For instance, in our study we had direct comparison between ibrutinib and chlorambucil by studies that directly compared these two in the original studies, we also got the indirect comparison between these two based on the studies comparing ibrutinib and rituximab, and the studies comparing rituximab and chlorambucil. If the estimates from direct comparison and indirect comparison are consistent, we combined the estimates to get the mixed estimate from both direct and indirect comparisons using inverse-variance weighted average. In order to confirm this assumption, we tested the consistency as measured using the I2 statistic. All of these analyses were done using the R package “gemtc”. The contribution plot, funnel plot, and ranking cluster plot were done using Stata package “metan”, “mvmeta”, and “networkplot”.

The codes for R were as follows.

```{r network analysis for survival data}
#library the package
library("gemtc")

#import the log scale of hr and se
network_sample <- read.table(textConnection('
study diff std.err treatment
"Burger 2015" NA NA Chlorambucil
"Burger 2015" -1.832 0.289 Ibrutinib
"Burger 2020" NA NA Chlorambucil
"Burger 2020" -1.924 0.203 Ibrutinib
"Burger 2019" 0.15 0.378 Ibrutinib
"Burger 2019" NA NA Ibrutinib_Rituximab
"Goede 2014" -1.715 0.156 Chlorambucil_Obinutuzumab
"Goede 2014" NA NA Chlorambucil
```

```
"Sharman 2021" NA NA Ibrutinib
"Sharman 2021" -0.777 0.328 Ibrutinib_Ublituximab
"Huang 2018" NA NA Rituximab
"Huang 2018" -1.715 0.2745 Ibrutinib
"Zucca 2017" 0.0953 0.188 Rituximab
"Zucca 2017" NA NA Chlorambucil'), header = TRUE)

#run the network model
network_sample$diff <- as.numeric(network_sample$diff)
network_sample_data <- mtc.network(data.re = network_sample, description = "Network",
treatments = NULL)

plot(network_sample_data)

sample_model <-mtc.model(network_sample_data, type="consistency",
n.chain=4,likelihood="binom",link="cloglog",linearModel="random")
sample_results <- mtc.run(sample_model, n.adapt = 20000, n.iter = 50000, thin = 1)

summary(sample_results)
summary(relative.effect(sample_results, "Chlorambucil"))

a <- round(exp(relative.effect.table(sample_results)),2)
write.csv(a, "network_meta_pfs.csv")

#make forest plot
forest(relative.effect(sample_results, "Ibrutinib_Ublituximab"))
forest(relative.effect(sample_results, "Ibrutinib"))
forest(relative.effect(sample_results, "Chlorambucil"))
forest(relative.effect(sample_results, "Rituximab",col.square="green",col.diamond="blue"))
forest(relative.effect(sample_results, "Rituximab"), xlim=c(0.01,70))

#calculae the ranking and making the ranking plot
gelman.plot(sample_results)
ranks <- rank.probability(sample_results)
print(ranks)
plot(ranks)
write.csv(ranks, "ranks_pfs_original.csv")

ggplot(data=ranks_pfs,aes(x=intervention, y=probability, fill=rank))+geom_bar(stat="identity",
position=position_dodge())+scale_fill_brewer(palette="Paired") +
scale_x_discrete(limits=c("Ibrutinib", "Chlorambucil",
"Rituximab","Ibrutinib_Rituximab","Ibrutinib_Ublituximab","Chlorambucil_Obinutuzumab")) +
coord_flip()+labs(title="Efficacy(Progression-free survival)") + scale_y_continuous(limits = c(0,
1, by = 0.1))

#calculate the sucra for stata to plot the rank cluster plot
```

```
sucra_pfs <- sucra(ranks)
sucra_pfs <- sucra(ranks,lower.is.better=TRUE)
sucra_pfs
write.csv(sucra_pfs, "sucra_pfs.csv")

#inconsistancy
resultnodesplit <-
mtc.nodesplit(network_sample_data,likelihood="binom",link="cloglog",linearModel="random")
print(summary(resultnodesplit))
plot(summary(resultnodesplit))

#heterogeneity
resultanohe <-
mtc.anohe(network_sample_data,likelihood="binom",link="cloglog",linearModel="random")
print(summary(resultanohe))
plot(summary(resultanohe))
```

The codes for Stata were as follows.

```
#import the analyzed results from R in the format of ‘treatment’, ‘safety’, and ‘efficacy.
#make the rank cluster plot
clusterank safety efficacy treatment
label variable efficacy "SUCRAS for efficacy(Progression-free survival)"
label variable safety "SUCRAS for safety(Adverse events)"


#import the data from R in the format of ‘study’, ‘treatment’, and ‘rr’ or ‘hr’.
network setup r n, studyvar(study) trtvar(treatment) format(augment) rr
#make the contribution plot
netweight _y _stderr _t1 _t2

#make the funnel plot
netfunnel _y _stderr _t1 _t2 , random bycomp add(lfit _stderr _ES_CEN)noalpha ylabel(0 0.1
0.2 0.3)
```

Appendix 3. Quality of evidence assessment of GRADE in 5 domains.

Intervention	Comparison	Outcome	Study limitations	Indirectness	Inconsistency	Imprecision	Publication bias	Quality of evidence
Ibrutinib	Chlorambucil	pfs	Not serious	Serious	Not serious	Not serious	Not serious	Moderate
Ibrutinib	Chlorambucil	safety	Not serious	Serious	Not serious	Not serious	Not serious	Moderate
Ibrutinib	Ibrutinib+ Rituximab	pfs	Serious	Not serious	Not serious	Serious	Not serious	Low
Ibrutinib	Ibrutinib+ Rituximab	safety	Serious	Not serious	Not serious	Serious	Not serious	Moderate
Chlorambucil + Obinutuzumab	Chlorambucil	pfs	Not serious	Not serious	Not serious	Not serious	Not serious	High
Chlorambucil + Obinutuzumab	Chlorambucil	safety	Not serious	Not serious	Not serious	Serious	Not serious	Moderate
Ibrutinib+ Ublituximab	Ibrutinib	pfs	Not serious	Not serious	Not serious	Serious	Not serious	Moderate
Ibrutinib+ Ublituximab	Ibrutinib	safety	Not serious	Not serious	Not serious	Serious	Not serious	Moderate
Ibrutinib	Rituximab	pfs	Very serious	Serious	Not serious	Serious	Not serious	Low
Ibrutinib	Rituximab	safety	Very serious	Serious	Not serious	Serious	Not serious	Moderate
Rituximab	Chlorambucil	pfs	Not serious	Very serious	Not serious	Serious	Not serious	Very low
Rituximab	Chlorambucil	safety	Not serious	Very serious	Not serious	Serious	Not serious	Very low

MANUSCRIPT 2:

Clonal evolution in response to Ibrutinib, Chlorambucil, or Watch-and-Wait regimens in Chronic Lymphocytic Leukemia

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Running Title: ibrutinib and chlorambucil in Chronic Lymphocytic Leukemia – clonal evolution

Abstract

Background

Although ibrutinib has been approved for CLL initial treatment by the Food and Drug Administration and listed as the first-line therapy by the National Comprehensive Cancer Network (NCCN) guideline, clonal evolution of CLL under the treatment of ibrutinib is still understudied.

Method

We used the samples from ibrutinib and chlorambucil group in an open-label randomized clinical trial (NCT01724346) from 108 centers in 16 countries and recruited the watch-and-wait observational cohort at the study center of University Of California San Diego. The primary outcome of this study was response rate. Whole-exome sequencing was used for both the tumor and normal samples at baseline, 1 year, and 2 years after treatment initiation. Drivers were identified using MutSig2CV, and PhylogicNDT was used to analyze clonal evolution and dynamics.

Results

A total of 216 patients with a mean age of 69.7 years (SD 6.9) were included in this study. The overall response rate was 28.6% in the chlorambucil group, as compared with 76.7% in the ibrutinib group ($p < 0.001$). There was a significant difference in the number of changing subclones across watch-and-wait group (6 changing subclones out of 84 subclones), chlorambucil group (13 changing subclones out of 64 subclones), and ibrutinib group (30 changing subclones out of 66 subclones) ($p < 0.001$). In the ibrutinib cohort, the significantly mutated genes included *NOTCH1*, *SF3B1*, *BCOR*, *NRAS*, and *BIRC3*. *BIRC3* mutation in the increasing subclone was significantly associated with the clinical outcome of stable disease ($p = 0.013$).

Conclusion

The subclonal diversity in CLL patients treated with ibrutinib changes more than with chlorambucil or watch-and-wait. *NOTCH1*, *SF3B1*, *BCOR*, *NRAS*, and *BIRC3* are drivers in expanding subclones under ibrutinib therapy. *BIRC3* mutations are significantly associated with clinical response in the CLL patients treated with ibrutinib.

Introduction

Chronic lymphocytic leukemia (CLL) is a type of leukemia that affects white blood cells. White blood cells are derived from both the myeloid and lymphoid cell lineages. Lymphocytes include 3 types of cells: B cells, T cells, and natural killer cells. CLL affects lymphocytes and is characterized by abnormally many B cells that are often found in blood, bone marrow, and lymphoid tissues.

CLL does not require treatment once it is diagnosed; instead, oncologists assess the cancer regularly and decide to start therapy when some specific indications appear and meet the criteria for treatment initiation. This method is called watch-and-wait. Once CLL patients need initial treatment, some biomarkers will be tested, including 17p deletion, *TP53* mutation, *IGHV* mutation status, and complex karyotype. Patients with 17p deletion, where *TP53* resides, or who have a *TP53* mutated indicate more abnormal and aggressive cancer B cells that can more frequently resist chemotherapy; CLLs with *IGHV* mutations that are acquired in the germinal center, can form more mature leukemia cells, that develop more slowly and often responds to chemotherapy. Based on these biomarkers, as well as the patients' age, overall health, and other medications, the first-line treatment is chosen. The first-line preferred regimens for CLL patients with or without 17p deletion and *TP53* mutation include ibrutinib alone, acalabrutinib alone, acalabrutinib plus obinutuzumab, venetoclax plus obinutuzumab [1].

Although ibrutinib has been approved for CLL initial treatment by the Food and Drug Administration (FDA) and listed as the first-line therapy, the results from a randomized clinical trial [2] showed that the response rate was 86% within a median follow-up period of 18.4 months,

but only 4 out of 86 patients achieved complete response. Moreover, subgroup analysis indicated that the progress-free survival (PFS) is consistent across subgroups in the Rai stage, or *IGHV* status, which means traditional biomarkers cannot explain the heterogeneity of patients' response to ibrutinib. Previous studies illustrated that receiving chemotherapy in CLL patients can be associated with expansion of subclones with high-risk genetic abnormalities like *TP53* and *NOTCH1* mutation, thus increasing the risk of recurrence [3]. However, there is no research systematically studying clonal evolution and dynamics of CLL patients under the treatment of ibrutinib and whether this is related to patients' response or associated with whether they will develop resistance during therapy. Our hypothesis is that ibrutinib treatment can affect the clonal evolution of CLL, and it is different from chlorambucil and watch-and-wait, and the clonal evolution and driver mutations are related to the patients' response. Therefore, our study aimed to investigate clonal evolution under the treatment of ibrutinib and the difference between ibrutinib and chlorambucil.

Methods

Study design and population

Firstly, Group 1 (ibrutinib group) and group 2 (chlorambucil group) enrolled patients who received the treatment of ibrutinib, or chlorambucil, respectively. We selected these patients whose blood samples of both baseline and 1 year and 2 years after the treatment were collected from the open-label randomized clinical trial comparing ibrutinib and chlorambucil in CLL patients (NCT01724346) [4], the patients in this study were enrolled from 108 study centers in 16 countries including Australia, Belgium, Canada, China, Czechia, Ireland, Israel, Italy, New Zealand, Poland, Russian, Federation, Spain, Turkey, Ukraine, United Kingdom, and United States. Then Group 3

(watch-and-wait group) recruited patients who did not meet the criteria for treatment initiation so received no treatment and matched to groups 1 and 2 patients with respect to age and time from diagnosis to baseline, and these patients were recruited at the study center of University Of California San Diego.

The diagnosis of CLL was based on the following criteria [5-6]: flow cytometry shows absolute lymphocytosis defined as more than 5×10^9/L monoclonal B lymphocytes in peripheral blood, and the leukemia cells should be small lymphocytes with narrow cytoplasm border, discernible nucleoli lacking a nucleus, and partially aggregated chromatin (larger, atypical lymphocytes or prolymphocytes must not be over 55%).

Watch-and-wait is the situation when CLL does not need to be treated once the diagnosis of CLL is established. In this case, the disease is assessed regularly, and the treatment starts when patients develop treatment indications, including the following criteria [7]: (1) progressive bone marrow failure as evidenced by anemia, thrombocytopenia, or both; (2) massive (>6 cm below the left costal margin), symptomatic, or progressive splenomegaly; (3) progressive lymphocytosis: >50% increase in 2 months or lymphocyte doubling time of fewer than 6 months; (4) autoimmune anemia or thrombocytopenia not responsive to standard therapies; (5) constitutional symptoms: unintentional weight loss greater than 10% over the preceding 6 months, unexplained night sweats for more than 1 month, unexplained fevers (>38.1℃) for 2 weeks.

Exposures and outcomes

The exposures of this study include the intervention of ibrutinib or chlorambucil. The patients in group 1 were treated with oral ibrutinib at a dose of 420 mg once daily until the disease progressed or an unacceptable level of toxicity developed. The patients in group 2 were treated with 12 cycles of chlorambucil at a dose of 0.5 mg per kilogram of body weight on days 1 and 15 of each 28-day cycle, the dose can be increased, if well tolerated, in increments of 0.1 mg/kg on Day 1 of each cycle to a maximum of 0.8 mg/kg. Chlorambucil was stopped if there was disease progression, lack of efficacy, or development of unacceptable toxicity. The patients in group 3 did not receive any CLL therapy.

The primary efficacy outcome of this study was the response rate at the follow-up of 2 years treatment. The definition of response after the treatment was established by the criteria based on the guidelines for CLL from the International Workshop on Chronic Lymphocytic Leukemia [8], including complete remission (CR), partial remission (PR), nodular partial response (nPR), partial response with lymphocytosis (PR-L), progressive disease (PD), and stable disease (SD). The efficacy outcome assessment included a physical examination and evaluation of the blood and bone marrow, and two groups of parameters assessing the lymphoid tumor load and constitutional symptoms and hematopoietic system were documented.

Sample collection and sequencing

A total of 763 samples from 216 patients were collected in this CLL cohort with 3 time points (before the treatment, 1 year after the treatment, and 2 years after the treatment), including both tumor and germline samples. Peripheral blood mononuclear cells were isolated, and DNA was

extracted and sequenced by whole-exome sequencing (WES). All the sequencing data were processed on the platform Terra (https://terra.bio/).

Driver mutation and copy number variant identification

The WES data were analyzed on the Terra platform using the Getz lab Cancer Genome Analysis WES pipeline. After quality control, a tool named DeTiN [9] was used to estimate the tumor in normal contamination and another tool named ContEst [10] was used to estimate contamination from other individuals, then somatic mutations were called using MuTect, MuTect2 (a method that applies a Bayesian classifier to detect somatic mutations with very low allele fractions), and Strelka2 (a method for somatic SNV and small indel detection from sequencing data of matched tumor-normal samples) [11-12]. Somatic copy number variants (CNV) were called using the Genome Analysis Toolkit (GATK4) CNV pipeline [13]. Driver discovery was done using MutSig2CV (MutSig, Mutation Significance, analyzes lists of mutations to identify genes that were mutated more often than expected by chance given background mutation) [14], and the tool of Genomic Identification of Significant Targets in Cancer (GISTIC2.0) [15] was used to discover drivers affected by copy number alterations.

Clonal and subclonal evolution

For participants with paired samples of sufficient quality, purity and ploidy were called using ABSOLUTE [16]. PhylogicNDT [17] was used to infer the clonal structure, phylogenic tree, and evolutionary trajectory for each patient under the treatment of ibrutinib or chlorambucil or receiving no treatment (Watch-and-wait). To quantify the subclone changes, we defined the changing subclones based on the following two conditions: (i) the CCF change from baseline is

larger than 0.15; (ii) there is a significant difference between CCF change of the parent subclone and the sum of the CCF changes of all the child subclones.

Statistical analysis

For the baseline characteristics analysis, one way ANOVA was used for continuous variables with normal distribution, Kruskal-Wallis (KW) test was used for not normally distributed continuous variables, and Chi-squared test or Fisher's exact test was used for count data. The primary outcome response rate was analyzed by the Cochran-Mantel-Haenszel chi-square. The propensity score was calculated by a logistic regression model with group assignment as an outcome and confounding factors as predictors. The confounding factors in the development of the propensity score included the variables of sex, age at diagnosis, *TP53, NOTCH1, SF3B1, BIRC3, IGHV* status, and WBC counts. We generated the matched groups of ibrutinib and chlorambucil based on the propensity score matching (caliper with 0.1, matching ratio of 1). In order to quantify the change of subclones across groups, we define the changing subclones as increasing or decreasing if mean CCF change from baseline is larger than 0.15 and there is a significant difference between CCF change of the parent subclone and the sum of the CCF changes of all the child subclones, then Chi-squared test or Fisher exact test was used to analyze the difference in number of changing subclones between groups in the propensity score-match cohort. The analysis was conducted using the R4.1.1, the propensity score matching was conducted using the R package of "MatchIt", plots were generated using Python 3.9.4 packages of "matplotlib.pyplot", "plotly.express", and "comut".

Results

Patient population

From the 269 patients in the open label randomized clinical trial, we included a total of 164 patients who had the blood samples for the ibrutinib and chlorambucil group. For the watch-and-wait, we recruited 52 CLL patients who did not meet the criteria of treatment initiation. In total, we included 216 patients with a mean age of 69.7 years (SD 6.9) in the final analytic set. Table 1 summarizes the baseline characteristics of the included patients. Among these patients, 40% had mutated *IGHV*, 39% were female, *TP53* mutations were found in 3% of patients, *NOTCH1* mutations in 10%, *SF3B1* mutations in 10%, *BIRC3* mutations in 3%, deletion of 17p in 3%, the deletion of 11q in 1%, and 14% had an amplification of chromosome 12.

Clinical response

During a follow-up period of 2 years, we lost the clinical response data of one patient in the ibrutinib group. In general, 7.1% (4 in 56) patients developed progressive disease (PD) after a 2-year treatment with chlorambucil, compared with no patients with PD in the ibrutinib group. 64.3% of patients in the chlorambucil group kept stable disease in the 2-year follow-up, and 22.4% of the patients receiving ibrutinib maintained stable disease after a 2-year treatment. The overall response rate was 28.6% in the chlorambucil group, as compared with 76.7% in the ibrutinib group ($p < 0.001$), as is shown in Figure 1. As for Richter's syndrome transformation, only 4 out of 108 patients in ibrutinib group, 2 out of 56 patients in chlorambucil group, and none of the patients in watch-and-wait group obtained the Richter's transformation, and there was no significant difference across these three groups ($p = 0.375$).

Coverage and purity

In order to control the quality of the WES analysis, we calculated the coverage of the sequencing in this study, as was shown in Figure 2A. Almost all (97%) of the samples have a median coverage over 200, and less than 1% of the samples failed due to low median coverage (i.e., less than 20), possibly due to the total amount of DNA in the sample being too low for sequencing. The purity based on matched tumor-normal samples for each pair in this study is shown in Figure 2B. Less than 10% of the pairs had an estimated purity of less than 50%, and about 50% of the pairs had an estimated purity between 90% and 100%. Based on the quality of the sequencing and the estimated purity, we only included a total of 80 participants in downstream analyses (34 participants from the Watch-and-wait cohort, 22 participants from the chlorambucil cohort, and 24 participants from the ibrutinib cohort).

Clonal evolution

Using PhylogicNDT we analyzed the 34 watch-and-wait patients, 24 ibrutinib patients, and 22 chlorambucil patients. As is shown in Figure 3 for watch-and-wait cohort, Figure 4 for chlorambucil cohort, and Figure 5 for ibrutinib cohort, there was little change in the subclonal composition of the Watch-and-wait group from baseline to the 2-year follow-up, but more changes were observed across the time points after treatment with chlorambucil or ibrutinib. We also observed different evolutionary patterns in the chlorambucil and ibrutinib cohorts. First, although they were under chlorambucil or ibrutinib treatment, some patients' clonal composition stayed relatively stable, such as in participants CH10 and IB115; Second, some patients underwent a large change in subclonal structure within the first year of treatment with chlorambucil or ibrutinib, such as participant CH3 and IB55; Third, some patients were stable for the first year of the treatment but gained the changes after 1 year of treatment, such participant IB8.

To quantify these changes, we calculated the number of changing subclones with the CCF change from baseline larger than 0.15. There was a significant difference in the number of changing subclones across watch-and-wait group (6 changing subclones out of 84 subclones), chlorambucil group (13 changing subclones out of 64 subclones), and ibrutinib group (30 changing subclones out of 66 subclones) ($p < 0.001$). In the matched cohort of ibrutinib group and chlorambucil group based on propensity score, there was a significant difference in the number of changing subclones between ibrutinib group (18 changing subclones out of 32 subclones) and chlorambucil group (10 changing subclones out of 37 subclones) ($p = 0.016$).

Driver mutations

MutSig2CV analysis of both clonal and subclonal mutations at baseline for the three groups (ibrutinib, chlorambucil, and watch-and-wait) showed that the significantly recurrently mutated genes, among the list of known CLL drivers from the CLL-map project [18] included *XPO1, SF3B1, NOTCH1, NFKBIE, MGA, KRAS, KLHL6, IKZF3, BIRC3*, and *ATM*, as is shown in Figure 6. The number of patients who did not obtain any of the mutations on the list of known CLL drivers at baseline were significantly different across three cohorts (chlorambucil group: 5 out of 22 patients; ibrutinib group: 0 out of 24 patients; watch-and-wait group: 9 out of 34 patients, $p = 0.011$). The MutSig2CV analysis only on subclonal clusters with increasing CCFs across from year 1 to year 2 after treatment with ibrutinib ($n = 24$) found as candidate drivers (among known drivers) included *NOTCH1, SF3B1, BCOR, NRAS*, and *BIRC3*. Among these driver mutations in the ibrutinib cohort, the Fisher exact test also showed that *BIRC3* mutation in the increasing subclone was significantly associated with the clinical outcome of stable disease ($p = 0.013$), as is

shown in the CoMut plot (Figure 7). Considering both figure 5 and figure7, I found that patients who obtained an increasing growth rate of child subclone with the *BIRC3* driver mutation, did not respond to the treatment of ibrutinib and maintained stable disease, such as patients IB2 and IB3. Patients who obtained a decreasing growth rate of child subclones with *BIRC3* mutations, responded to ibrutinib therapy and achieved partial response, such as the patient IB26.

The patient with a relatively shrinking subclone with both *NOTCH1* and *BIRC3* achieved a partial response. One of the assumptions that might explain the partial response of patient IB26 is as follows. The prognosis of CLL was evaluated by Rai and Binet staging systems, patients with Rai stage of 0 have an overall survival of >10 years and patients with Binet stage of A have an overall survival of 12 years [19]. Under the standard CLL treatment therapies, more than 80% of patients are alive at 3 years, and 5-year survival has significantly increased from 60% to 66% from 2001 to 2014 [20]. However, CLL has the possibility of transformation into an aggressive lymphoma, which is known as Richter syndrome, then the median survival ranges from 8 to 16 months after the Richter syndrome transformation [21]. *NOTCH1* mutation is recurrent in about 30% Richter transformation, thus it can be a biomarker to identify CLL patients with a risk of Richter transformation to diffuse large B-cell lymphoma [22]. The deletion of a CT dinucleotide in *NOTCH1* (p.P2515Rfs*4) can result in the removal of the C-terminal of the proline-glutamic acid-serine-threonine domain and then reduce the switch off the activated *NOTCH1* signaling [22]. Human diffuse large B-cell lymphoma cell lines with active B cell receptor signaling were inhibited by ibrutinib selectively [23]. The assumption is that if the subclones with *NOTCH1* decrease, the patients are more likely to have a better prognosis.

Ibrutinib, one of the targeted therapies for CLL, binds covalently to the cysteine Cys-481 of Bruton's tyrosine kinase (*BTK*) and inactivates this kinase, thus blocking BCR signaling, which prevents cancer cell proliferation [24]. *BIRC*3 gene encodes the protein which can inhibit apoptosis by binding to tumor necrosis factor receptor-associated factors *TRAF1* and *TRAF2*. The assumption is that patients with increasing size of a subclone with a *BIRC3* mutation did not respond to ibrutinib and kept as stable disease.

Discussion

In this clinical study with 216 CLL patients, we found that the overall response rate was 76.7% in the ibrutinib group compared to 28.6% in the chlorambucil group with a 2-year follow-up. In 80 participants with high sequencing quality (over 200 median coverage) and high sample quality (over 90% purity), clonal analysis showed that the subclonal diversity changes from baseline to the 2-year follow-up time were larger in the ibrutinib cohort compared to the watch-and-wait cohort and chlorambucil cohort. Under the treatment of ibrutinib for 2 years, recurrent mutations in the clonal and subclonal evolution included *NOTCH1*, *SF3B1*, *BCOR*, *NRAS*, and *BIRC3*. In addition, *BIRC3* was also significantly associated with the clinical response in the ibrutinib cohort.

One of the previous studies showed that 31% of the patients receiving ibrutinib during the first year obtained clonal shifts (change > 0.1 in CCF) [25]. In our study, 15 out of 24 patients (63%) of patients harbored changing subclones (change > 0.15 in CCF) after two years of treatment with ibrutinib. Furthermore, previous studies showed that the recurrent driver mutations in CLL patients included *TP53, SF3B1, MYD88, XPO1, MED12, ATM, POT1, RPS15, EGR2, HIST1H1E, DDX3X, NOTCH1, NRAS*, and *IGLL*5 [26]. The drivers *NOTCH1, SF3B1*, and *NRAS* were consistent

between previous studies and our study. The protein encoded by the *BCOR* gene was identified as an interacting corepressor of *BCL6*, which is a POZ/zinc finger transcription repressor that may influence apoptosis, and *BIRC3* encodes the protein which can interfere with the action of caspases and inhibit apoptosis by binding to tumor necrosis factor receptor-associated factors *TRAF1* and *TRAF2*. If there is a mutation of *BIRC3* in the increasing subclones that could cause the increasing inhibition of apoptosis, patients might obtain the worse prognosis. Based on these driver mutations in the clonal and subclonal evolution, we raised the assumption that patients with subclones with *NOTCH1* decreasing growth rate are more likely to have better prognosis, and that patients with a subclone with an increasing CCF with a of *BIRC3* mutation did not respond to ibrutinib. This hypothesis should be tested in a larger cohort of patients receiving ibrutinib and can also be tested in animal and cell models.

One of the strengths of this study was that propensity score matching was used for adjusting the confounding factors. Some biomarkers have been identified to predict the prognosis of CLL during the process of understanding the genetic biology of CLL. For instance, *TP53* mutation predicts an aggressive CLL disease course [27], so as the unmutated *IGHV* status [28]. Because we used the matched watch-and-wait cohort, those factors that are related to the prognosis of CLL disease and have the potential of unbalanced distribution among groups should be controlled. Otherwise, the differences we found can also be related to these confounding factors, instead of the interventions alone. Therefore, we calculated the propensity score based on the confounding factors of age, gender, *IGHV* status, *TP53*, *NOTCH1*, *BIRC3*, and *SF3B1* mutations, as well as Chromosome 12 trisomy and white blood cell counts at baseline, then used the propensity score to match the patients between groups.

Our study also has limitations. First, we only included 80 participants in the PhylogicNDT analysis among a total of 216 participants in this cohort, because the rest of the participants' samples had unacceptable contaminations of tumor cells in the normal samples. The normal blood samples are currently re-processed so that we could perform the full PhylogicNDT analysis in the future once we get the normal samples with better quality. Second, the propensity score matching limited the sample size in the evolution analysis—we only included 24 participants in the subclonal analysis, because we lost some participants that could not be matched to anyone in the other group, even if we set the caliper parameter to 0.1. In this way, we had limited power to do the statistical test. There were more changing subclones in the ibrutinib group than in the chlorambucil group in our dataset (30 changing subclones out of 60 subclones in ibrutinib group and 13 changing subclones out of 64 subclones in chlorambucil group; $p < 0.001$), which should be further tested once we get more samples with high purity and more available patients for the PhylogicNDT analysis. Third, there were unmeasured confounding factors such as ZAP-70, cytogenetic abnormalities, and other prognostic factors. We failed to include these unmeasured confounding variables in the development of propensity score matching. Forth, although we used the propensity score matching to control for confoundings, the association between subclonal evolutions and the interventions can also be mediated by other factors such as progression to Richter's syndrome. For instance, the CLL patient might develop Richter's syndrome under the treatment of ibrutinib, the progression to Richter's syndrome can cause the change in clonal and subclonal evolution. Because of very limited number of patients who developed the Richter's syndrome (4 patients in the ibrutinib group and 2 patients in the chlorambucil group), we failed to figure this potential mediation out in our study. Besides, we were not able to do subgroup analysis or multiple regression model adding

interaction term to investigate the potential effect modifiers of the treatment on the subclonal evolution, such as age, gender, subtype of CLL, comorbid diseases. For example, the subclonal evolution pattern might be different between different sex, or subtype of CLL even though the patients received the same intervention ibrutinib. Fifth, we did not collect the data on the comorbid disease of the included patients at baseline; instead, we collected the data on the white blood cell count at baseline is the most important potential confounding factors that influence both the intervention and the disease progression outcome.

We concluded that the subclonal diversity under the treatment of ibrutinib in CLL patients changes more than for chlorambucil or watch-and-wait. *NOTCH1, SF3B1, BCOR, NRAS*, and *BIRC3* are drivers in expanding subclones under the treatment of ibrutinib in patients with CLL. *BIRC3* mutation in the increasing subclone was significantly associated with the clinical outcome of stable disease.

Tables and Figures

Characteristic	WW (n = 52)	CH (n = 56)	IB (n = 108)	p-value
Age at diagnosis, mean (SD)	68.2 (6.0)	70.8 (6.7)	70.0 (7.3)	0.12
Female (%)	25 (42.4%)	25 (43.9%)	37 (34.3%)	0.39
White Blood Cell (10^9/L)	15.5 (13.5, 16.5)	66.9 (13.6, 138.1)	49.5 (20.1, 115.9)	0.13
Hemoglobin (g/L)	211.1 (15.3)	116.7 (19.2)	114.6 (19.5)	<0.001
Platelets(10^9/L)	202.6 (60.7)	152.5 (68.1)	141.7 (56.5)	<0.001
IGHV mutated (%)	37 (73%)	20 (44%)	32 (39%)	<0.001
TP53 (%)	4 (6.8%)	0 (0.0%)	3 (2.8%)	0.11
NOTCH1 (%)	6 (10.2%)	5 (8.8%)	11 (10.2%)	0.95
SF3B1 (%)	3 (5.1%)	6 (10.5%)	14 (13.0%)	0.28
BIRC3 (%)	2 (3.4%)	0 (0.0%)	4 (3.7%)	0.35
del_17p (%)	2 (3.4%)	0 (0.0%)	4 (3.7%)	0.35
del_11q (%)	0 (0.0%)	1 (1.8%)	1 (0.9%)	0.60
tri_12 (%)	6 (10.2%)	11 (19.3%)	14 (13.0%)	0.34

Table 1. Basic characteristics of the participants.
WW: Watch-and-Wait, CH: Chlorambucil, IB: Ibrutinib.

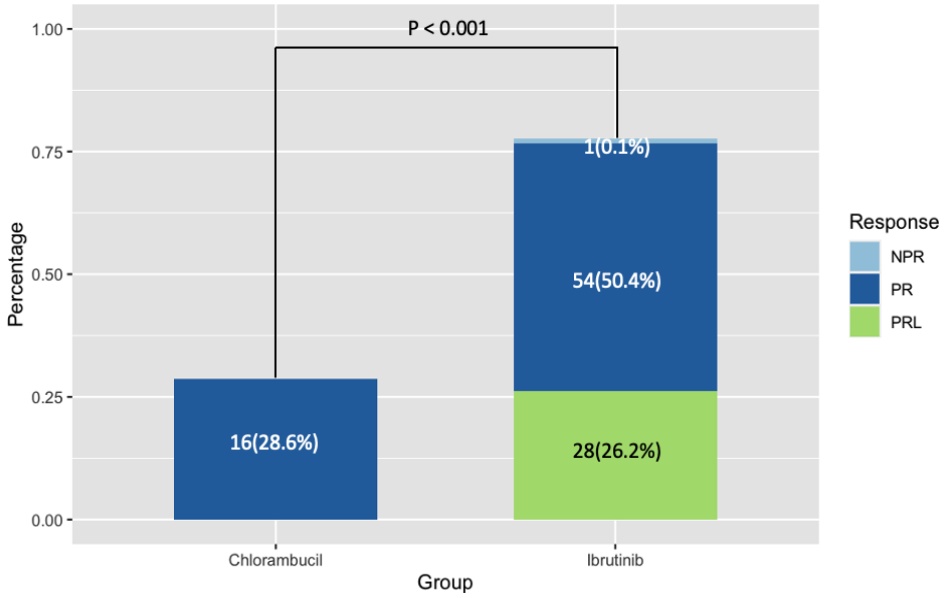

Figure 1. **Response rate with chlorambucil vs. ibrutinib.**
Categories of the clinical response shown in this figure included partial remission (PR), nodular partial response (nPR), partial response with lymphocytosis (PR-L).

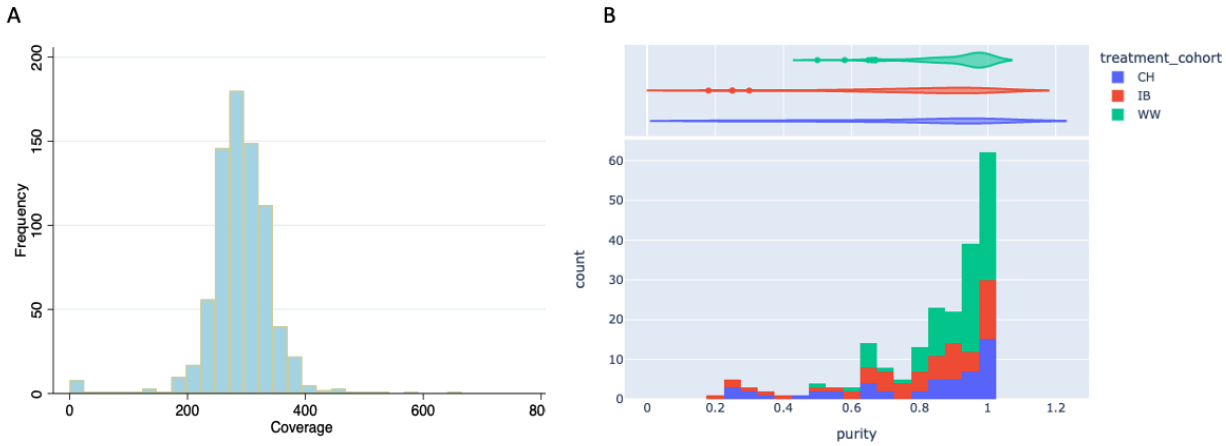

Figure 2. **The sequencing coverage and purity.**
(A) the histogram of the median sequencing coverage of samples in this study. (B) the histogram of purity of tumor-normal matched pairs across the three cohorts in this study. CH: Chlorambucil, IB: Ibrutinib, WW: Watch-and-wait.

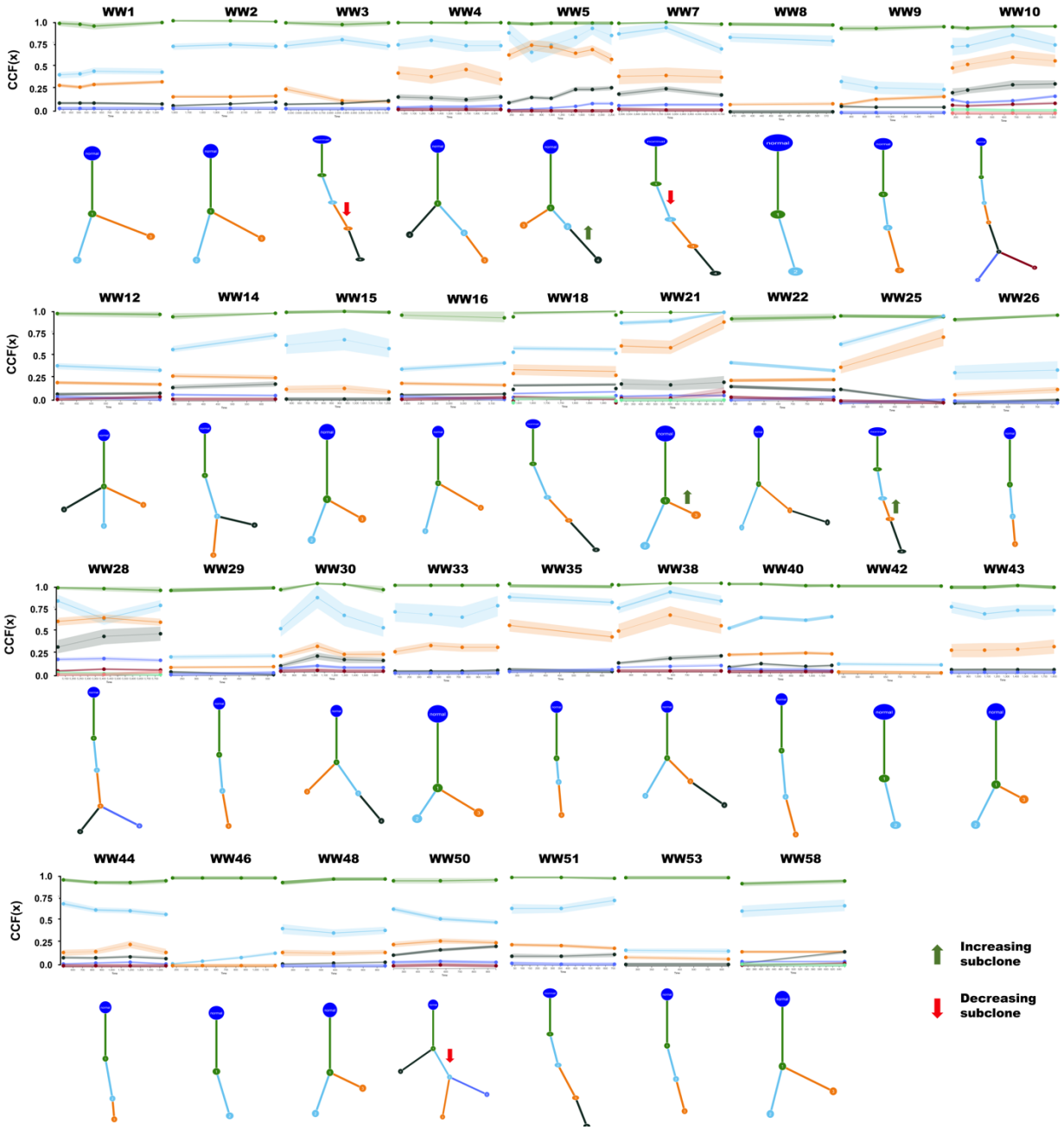

Figure 3. Clonal evolution and phylogenetic tree from PhylogicNDT analysis based on WES sequencing data in watch-and-wait cohort (n = 34).

Clonal cluster was shown in green. Mutations with similar cancer cell fraction (CCF) are clustered as evolution in the same subclones, as were marked in different colors other than green. Each mutation was assigned to an independent cluster via a Markov chain Monte-Carlo (MCMC) iteration process. The CCF was shown in mean estimate represented as points and middle lines in the plot as well as the 95% confidence interval as upper and lower lines around the mean. The x axis represents the time of date from first diagnosis of CLL disease.

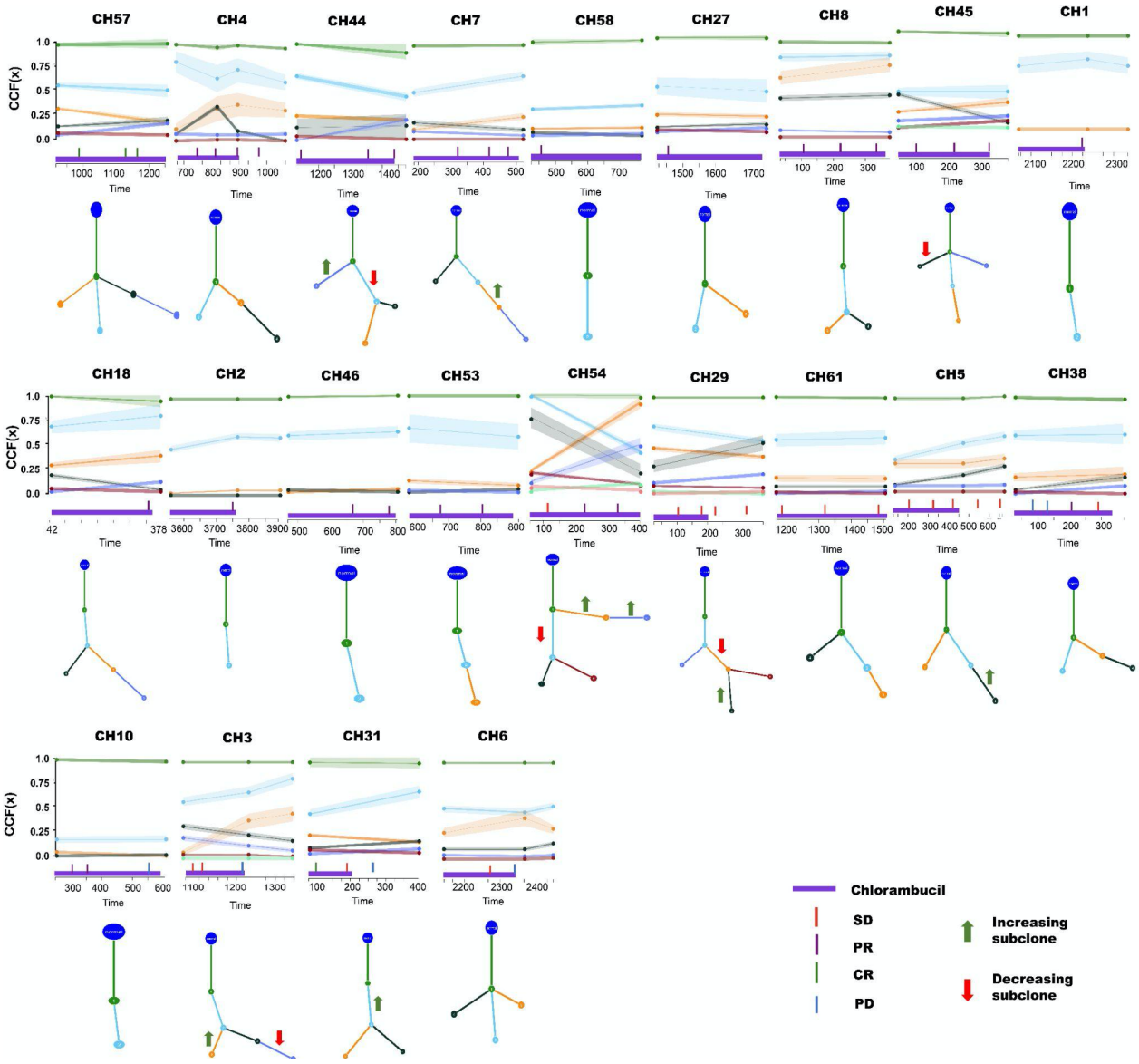

Figure 4. Clonal evolution and phylogenetic tree from PhylogicNDT analysis based on WES sequencing data in chlorambucil cohort (N = 22).

The patients are sorted in decreasing order of the Shannon index change between baseline to year 1. The clonal mutations are shown in green. Mutations with similar cancer cell fraction (CCF) are clustered as evolution in the same subclone, as were marked in different colors other than green. Each mutation was assigned to an independent cluster via a Markov chain Monte-Carlo (MCMC) iteration process. The CCF was shown in mean estimate represented as points and middle lines in the plot as well as the 95% confidence interval as upper and lower lines around the mean. The x axis represents the time of date from first diagnosis of CLL disease. The purple bar above the time points represents the chlorambucil intervention duration for each patient, and the vertical short lines above the treatment bar represents the clinical outcomes, including complete remission (CR), partial remission (PR), stable disease (SD) and progressive disease (PD).

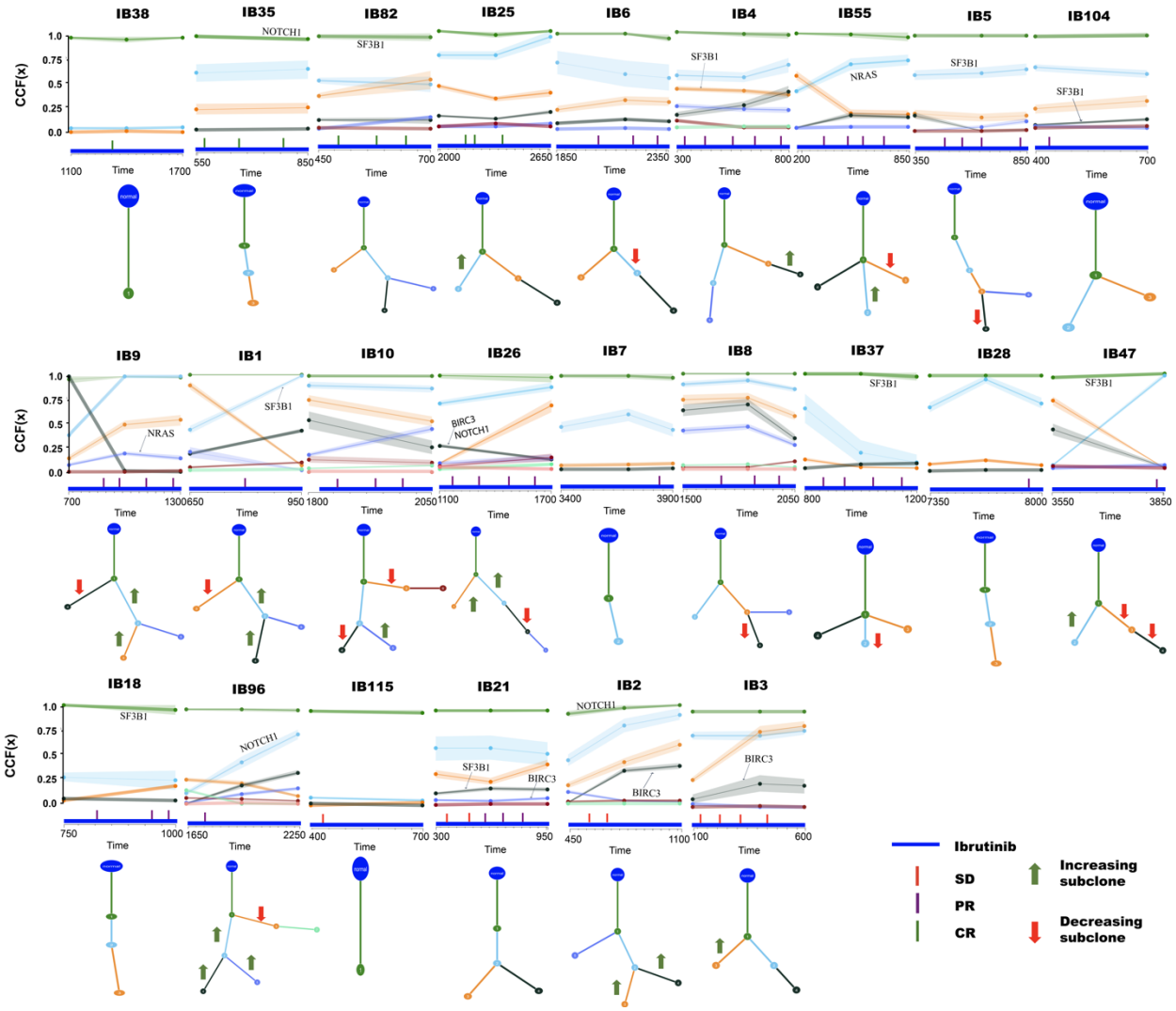

Figure 5. Clonal evolution and phylogenetic tree from PhylogicNDT analysis based on WES sequencing data in ibrutinib cohort (N = 24).

The plots were sorted in decreasing sequence of the Shannon index change from baseline to 1 year. Clonal cluster was shown in green. Mutations with similar cancer cell fraction (CCF) are clustered as evolution in the same subclone, as were marked in different colors other than green. Each mutation was assigned to an independent cluster via a Markov chain Monte-Carlo (MCMC) iteration process. The CCF was shown in mean estimate represented as points and middle lines in the plot as well as the 95% confidence interval as upper and lower lines around the mean. The x axis represents the time of date from first diagnosis of CLL disease. The blue bar above the time points represents the ibrutinib intervention duration for each patient, and the vertical short lines above the treatment bar represents the clinical outcomes, including complete remission (CR), partial remission (PR), nodular partial response (NPR), partial response with lymphocytosis (PRL), and stable disease (SD).

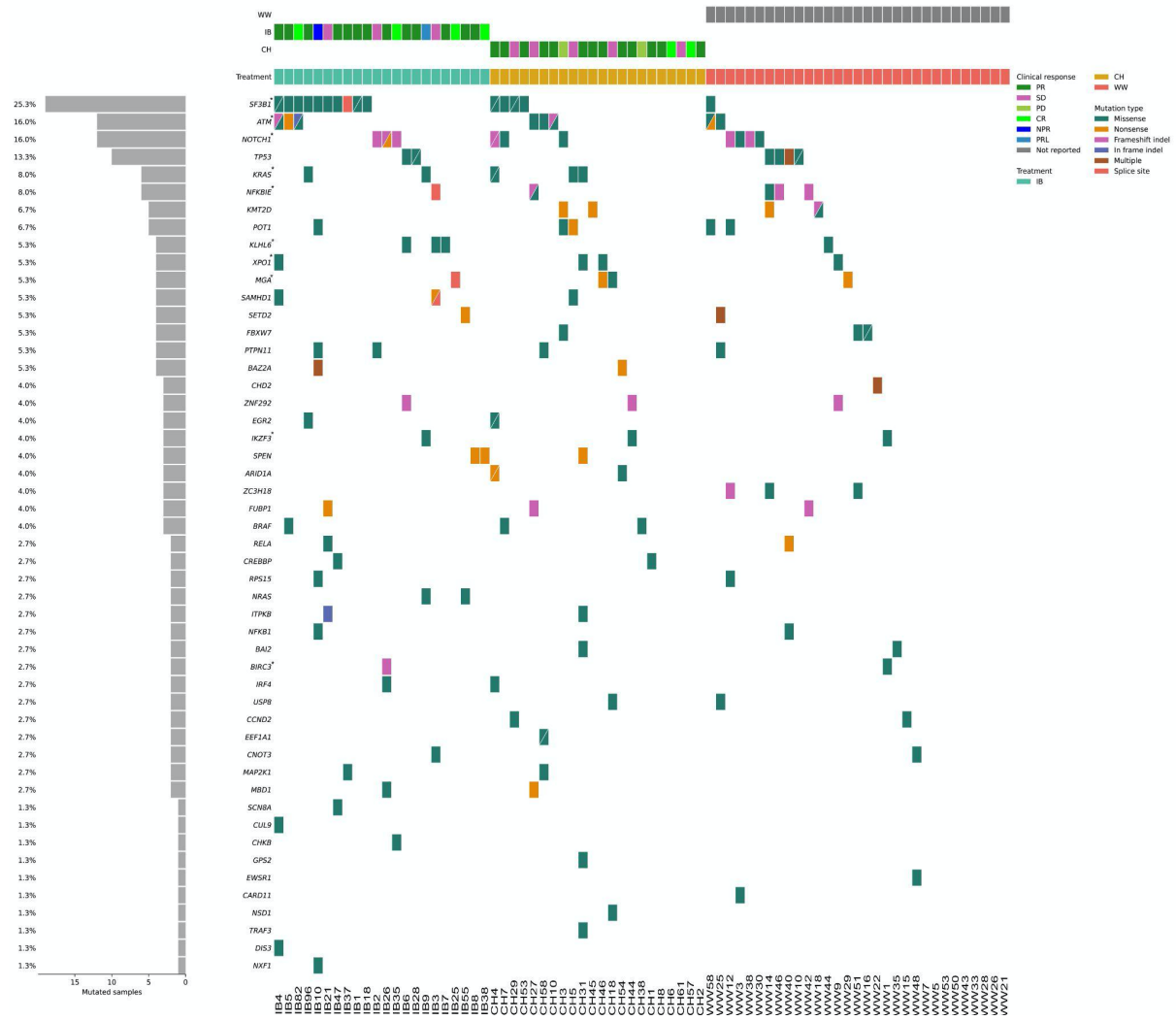

Figure 6. Recurrent mutation events at baseline in both ibrutinib, chlorambucil, and watch-and-wait cohorts.

The driver list was provided by the CLL-map project [17]. *q < 0.1 were significantly recurrent mutation events. Complete remission (CR), partial remission (PR), nodular partial response (NPR), partial response with lymphocytosis (PRL), progressive disease (PD), stable disease (SD).

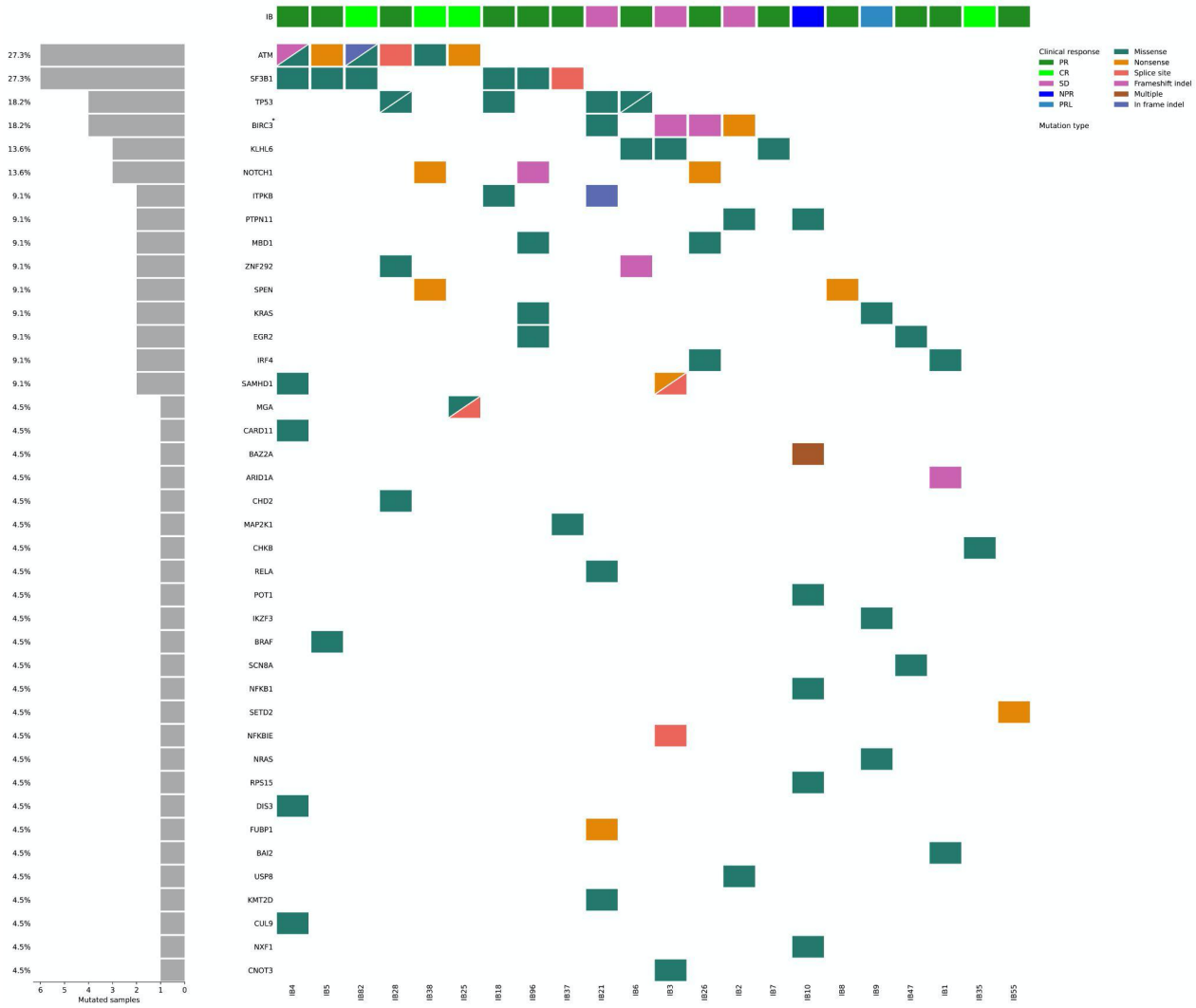

Figure 7. **Recurrent mutation events in both 1 year and 2 years after the treatment in the ibrutinib cohort.**

The driver list was provided by the CLL-map project [17]. *p = 0.013 was the recurrent event that was significantly different between subgroups of stable disease and response patients in ibrutinib cohort. Complete remission (CR), partial remission (PR), nodular partial response (NPR), partial response with lymphocytosis (PRL), progressive disease (PD), stable disease (SD).

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Supplement

participantID	gender	age_at_diagnosis	tp53	notch1	sf3b1	birc3	tri_12	mut IGHV	WBC (10^9/L)	treatment	distance	weights	matching
PCYC_CH27	M	68.66	0	0	0	0	0	0	52.17	CH	0.38	1	1
PCYC_IB10	F	66.98	0	0	0	0	0	1	105.25	IB	0.38	1	1
PCYC_CH61	M	64.81	0	0	0	0	0	0	62.09	CH	0.35	1	2
PCYC_IB115	M	69.60	0	0	0	0	1	0	347.61	IB	0.37	1	2
PCYC_CH58	F	67.51	0	0	0	0	1	0	78.3	CH	0.42	1	3
PCYC_IB25	F	71.80	0	0	0	0	0	1	95.55	IB	0.41	1	3
PCYC_CH38	M	74.68	0	0	0	0	1	0	8.02	CH	0.41	1	4
PCYC_IB3	M	78.62	0	0	0	0	0	0	191.81	IB	0.41	1	4
PCYC_CH2	F	55.63	0	0	0	0	0	1	86.86	CH	0.27	1	5
PCYC_IB35	M	69.51	0	1	0	0	0	0	61.75	IB	0.27	1	5
PCYC_CH3	F	70.18	0	1	0	0	1	0	165.89	CH	0.30	1	6
PCYC_IB37	F	67.94	0	1	0	0	0	0	31.65	IB	0.30	1	6
PCYC_CH57	M	69.42	0	0	0	0	0	1	6.07	CH	0.36	1	7
PCYC_IB38	M	66.96	0	0	0	0	0	0	2.44	IB	0.37	1	7
PCYC_CH7	M	68.33	0	1	1	0	0	0	119.26	CH	0.55	1	8

PCYC_IB47	M	56.20	0	0	1	0	0	1	20.89	IB	0.54	1	8
PCYC_CH54	F	65.93	0	0	0	0	0	0	3.12	CH	0.41	1	9
PCYC_IB55	M	74.36	0	0	0	0	0	0	10.71	IB	0.41	1	9
PCYC_CH44	F	64.22	0	0	0	0	0	0	14.38	CH	0.40	1	10
PCYC_IB7	F	64.63	0	0	0	0	1	0	9.7	IB	0.40	1	10
PCYC_CH46	M	69.68	0	0	0	0	0	0	82.63	CH	0.39	1	11
PCYC_IB9	F	66.58	0	0	0	0	0	1	13.18	IB	0.39	1	11
PCYC_CH29	M	68.39	0	0	1	0	0	0	128.44	CH	0.68	1	12
PCYC_IB96	M	69.39	0	0	1	0	1	0	66.79	IB	0.69	1	12

M: male, F: female, IB: ibrutinib, CH: chlorambucil, WBC: white blood cell

Supplement Table 1. Matching list in ibrutinib cohort based on propensity score.

Summary of conclusions

This thesis project focused on two goals: first, combining and evaluating the current evidence of ibrutinib and chlorambucil for the treatment of CLL patients. The second, explores clonal evolution under the 2-year treatment of ibrutinib or chlorambucil and compares to the watch-and-wait controls.

In the first project, based on a total of 6 eligible studies involving 1618 patients, we found that both ibrutinib and ibrutinib combined therapies (ibrutinib plus ublituximab) significantly prolonged progression-free survival (HR 0.16, $CI_{95\%}$ [0.04, 0.74; HR 0.08, $CI_{95\%}$ [0.01, 0.86], respectively) compared with chlorambucil alone (the quality of evidence was moderate to high). No significant difference in the safety outcome based on adverse events was found between chlorambucil, ibrutinib, ibrutinib plus rituximab, ibrutinib plus ublituximab, and rituximab alone. Ibrutinib plus rituximab has the highest probability of ranking the first in terms of progression-free survival efficacy outcome. Based on these results, ibrutinib used alone and ibrutinib plus ublituximab might be considered over chlorambucil. This finding contributed to the gap on this topic since it was the only network meta-analysis using both the direct and indirect comparison to estimate the efficacy and safety of ibrutinib, chlorambucil, and their combined therapies.

In the second project, we observed that the overall response rate was 28.6% in the chlorambucil group, as compared with 76.7% in the ibrutinib group ($p < 0.001$) in this CLL cohort study, and we also found a significant difference in the number of changing subclones across watch-and-wait group (6 changing subclones out of 84 subclones), chlorambucil group (13 changing subclones out of 64 subclones), and ibrutinib group (30 changing subclones out of 66 subclones) ($p < 0.001$). We

concluded that the subclonal diversity under the treatment of ibrutinib in CLL patients changes more than for chlorambucil. *NOTCH1, SF3B1, BCOR, NRAS*, and *BIRC3* are drivers in expanding subclones under the treatment of ibrutinib in patients with CLL. *BIRC3* mutation in the increasing subclone was significantly associated with the clinical outcome of stable disease ($p = 0.013$). *BIRC*3 gene encodes the protein which can inhibit apoptosis by binding to tumor necrosis factor receptor-associated factors *TRAF1* and *TRAF2*. Ibrutinib, one of the targeted therapies for CLL, binds covalently to the cysteine Cys-481 of Bruton's tyrosine kinase (*BTK*) and inactivates this kinase, thus blocking BCR signaling, which prevents cancer cell proliferation. Therefore, the assumption is that patients with increasing size of a subclone with a *BIRC3* mutation causes the inhibition of apoptosis of cancer cells, then negatively influence the effect of ibrutinib on inactivating the Bruton's tyrosine kinase, thus these patients kept as stable disease, as compared with the patients with a decreasing subclone of *BIRC*3 achieving partial response. This observation started to fill the knowledge gap of how clonal evolution under the treatment of ibrutinib, and these results suggest the potential use of clonal evolution and driver mutations for the prediction of the patients' response to the ibrutinib treatment, which may help guide the clinical decision-making in CLL treatment.

Discussion and perspectives

This study highlights the differences in efficacy between ibrutinib, chlorambucil, and their combined therapies. Both ibrutinib plus ublituximab and ibrutinib might be considered over chlorambucil alone. The result of our study is consistent with previous systematic review [1] concluding that ibrutinib should be considered over chlorambucil in the treatment of CLL based on the hazard ratio of 0.16 ($CI_{95\%}$ [0.08, 0.31]) for the outcome of progression-free survival. However, our study updated the previous systematic review by using the results of Resonate-2 study of 5 years follow-up published in 2020, instead of the results of Resonate-2 study with a median follow-up time of 18.4 months published in 2015. Moreover, our study added the indirect comparison between ibrutinib and chlorambucil by the third drug rituximab, which enabled us to estimate the effect with a larger sample size. Furthermore, our study provided the ranking of both the ibrutinib, chlorambucil, and their combined therapies, which indicates that ibrutinib plus ublituximab should be considered as the first choice among the six interventions we included in this systematic review. However, we were not able to do the subgroup analysis on whether ibrutinib has the advantage in subgroups such as *IGHV* mutated or unmutated subgroup, because of the limited information provided by the original studies. These subgroup analyses could be more informative in terms of helping the clinical decision-making process. Besides, further randomized clinical trials directly comparing the interventions such as ibrutinib plus ublituximab versus chlorambucil alone should be designed to validate the results in our study.

Furthermore, this study showed that the clonal evolution under ibrutinib treatment (a targeted therapy) was different from chlorambucil (a chemotherapy) or Watch-and-wait (natural progression) in subclonal diversity. Previous studies illustrated that receiving chemotherapy in

CLL patients can be associated with expansion of subclones with high-risk genetic abnormalities like *TP53* and *NOTCH1* mutation, thus increasing the risk of recurrence [2]. As for targeted therapy, 31% of the patients receiving ibrutinib can develop clonal shifts (change > 0.1 in CCF) during the first year of follow-up, and these clonal shifts are associated with negative outcomes [3]. Comparing ibrutinib (targeted therapy) with chlorambucil (chemotherapy), we found that subclonal diversity under the treatment of ibrutinib in CLL patients changes more than for chlorambucil, and that *NOTCH1, SF3B1, BCOR, NRAS*, and *BIRC3* are drivers in expanding subclones under the treatment of ibrutinib in patients with CLL. *BIRC3* mutations in the increasing subclones are significantly associated with the clinical response of stable disease in the CLL patients receiving ibrutinib therapy. To identify child subclones with increasing growth rates than parent subclones is more important to uncover the mechanisms underlying the patients' clinical responses. We would benefit from having access to more participants in each cohort and having samples with higher purity. This will enable constructing a prediction model of patients' clinical responses based on the driver mutations in their subclones that can be used for assisting clinical decision-making.

In conclusion, the main body of this work reflects the difference of ibrutinib and chlorambucil in clinical efficacy as well as clonal evolution and dynamics. Further studies should be designed to construct a prediction model of patients' response after the treatment of ibrutinib, which will outline a potential avenue for an assistance tool for clinical decision-making in treating CLL patients.

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