Integration of gene mutations in risk prognostication for patients receiving first-line immunochemotherapy for follicular lymphoma: a retrospective analysis of a prospective clinical trial and validation in a population-based registry

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Summary

Background Follicular lymphoma is a clinically and genetically heterogeneous disease, but the prognostic value of somatic mutations has not been systematically assessed. We aimed to improve risk stratification of patients receiving first-line immunochemotherapy by integrating gene mutations into a prognostic model.

Methods We did DNA deep sequencing to retrospectively analyse the mutation status of 74 genes in 151 follicular lymphoma biopsy specimens that were obtained from patients within 1 year before beginning immunochemotherapy consisting of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP). These patients were recruited between May 4, 2000, and Oct 20, 2010, as part of a phase 3 trial (GLSG2000). Eligible patients had symptomatic, advanced stage follicular lymphoma and were previously untreated. The primary endpoints were failure-free survival (defined as less than a partial remission at the end of induction, relapse, progression, or death) and overall survival calculated from date of treatment initiation. Median follow-up was 7·7 years (IQR 5·5–9·3). Mutations and clinical factors were incorporated into a risk model for failure-free survival using multivariable L1-penalised Cox regression. We validated the risk model in an independent population-based cohort of 107 patients with symptomatic follicular lymphoma considered ineligible for curative irradiation. Pretreatment biopsies were taken between Feb 24, 2004, and Nov 24, 2009, within 1 year before beginning first-line immunochemotherapy consisting of rituximab, cyclophosphamide, vincristine, and prednisone (R-CVP). Median follow-up was 6·7 years (IQR 5·7–7·6).

Findings We established a clinicogenetic risk model (termed m7-FLIPI) that included the mutation status of seven genes (*EZH2, ARID1A, MEF2B, EP300, FOXO1, CREBBP*, and *CARD11*), the Follicular Lymphoma International Prognostic Index (FLIPI), and Eastern Cooperative Oncology Group (ECOG) performance status. In the training cohort, m7-FLIPI defined a high-risk group (28%, 43/151) with 5-year failure-free survival of 38·29% (95% CI 25·31–57·95) versus 77·21% (95% CI 69·21–86·14) for the low-risk group (hazard ratio [HR] 4·14, 95% CI 2·47–6·93; p<0·0001; bootstrap-corrected HR 2·02), and outperformed a prognostic model of only gene mutations (HR 3·76, 95% CI 2·10–6·74; p<0·0001; bootstrap-corrected HR 1·57). The positive predictive value and negative predictive value for 5-year failure-free survival were 64% and 78%, respectively, with a *C*-index of 0·80 (95% CI 0·71–0·89). In the validation cohort, m7-FLIPI again defined a high-risk group (22%, 24/107) with 5-year failure-free survival of 25·00% (95% CI 12·50–49·99) versus 68·24% (58·84–79·15) in the low-risk group (HR 3·58, 95% CI 2·00–6·42; p<0.0001). The positive predictive value for 5-year failure-free survival was 72% and 68% for negative predictive value, with a *C*-index of 0·79 (95% CI 0·69–0·89). In the validation cohort, risk stratification by m7-FLIPI outperformed FLIPI alone (HR 2·18, 95% CI 1·21–3·92), and FLIPI combined with ECOG performance status (HR 2·03, 95% CI 1·12–3·67).

Interpretation Integration of the mutational status of seven genes with clinical risk factors improves prognostication for patients with follicular lymphoma receiving first-line immunochemotherapy and is a promising approach to identify the subset at highest risk of treatment failure.

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Introduction

Follicular lymphoma is the second most common nodal lymphoma worldwide and presents with advanced stage disease in most patients.[1] Several randomised trials have shown that the addition of the monoclonal anti-CD20 antibody rituximab to various first-line chemotherapy regimens improves progression-free survival and overall survival.[2] Additionally, many patients now receive rituximab maintenance after first-line treatment,[2] based on a randomised trial that showed improved progression-free survival.[3]

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Research in context

Evidence before this study

On April 9, 2015, we searched the Cochrane Library for all publications without language or date restrictions using the terms "follicular lymphoma" in the title, abstract, keywords, and "prognosis OR prognostic OR prediction OR predictive" anywhere in the text. This search identified a total of 79 publications. Additionally, we searched PubMed for all original publications (ie, review articles were excluded) using the keywords "follicular lymphoma" in the title, and "gene mutation" and "prognostic OR prognosis OR predictive OR prediction" anywhere in the text. This search identified a total of 49 publications.

The relevant papers addressing the effect of molecular alterations on treatment outcome in follicular lymphoma included conflicting reports on the significance of karyotypic abnormalities, *BCL2* translocation breakpoints and immunohistochemistry, positive reports on immunohistochemistry for MUM-1, copy number alterations, uniparenteral loss of heterozygosity, and gene expression signatures, and a negative report on germ-line polymorphisms in single genes (*TP53*, *MDM2*). Additionally, we identified publications on the clinical significance of somatic alterations in single genes (*TP53*, *TNFRSF14*, *CDKN2A*, *BCL2*). In a heterogeneously treated patient population, *TP53* mutations, although rare at initial diagnosis (6%) were predictive for shorter progression-free survival and overall survival when adjusted for International Prognostic Index (IPI). In one study, mutations and chromosomal deletions affecting *TNFRSF14* at 1p36 were associated with high-risk clinical features (ECOG performance status >1, number of extranodal sites >1, high IPI) and shorter overall survival in non-uniformly treated patients, some of whom also received rituximab. In another study, *TNFRSF14* alterations were not associated with poor prognosis. Inactivation of the *CDKN2A* tumour-suppressor gene by deletion or methylation was associated with poor clinical outcome in one study. Coding sequence mutation in *BCL2*, including silent and non-silent variants have recently been associated with increased risk of transformation and shortened overall survival, assumed to represent a surrogate marker for activation-induced cytidine deaminase-mediated genetic instability. Almost all patients in this study were treated in the pre-rituximab era.

Added value of this study

By contrast with previous studies that focused on single gene alterations, we did a multivariable analysis that included a comprehensive compilation of recurrent gene mutations and clinical risk factors in patients with symptomatic follicular lymphoma receiving first-line immunochemotherapy. To the best of our knowledge, this is the largest study to date of relevant mutations in follicular lymphoma, and the first multivariable assessment of their prognostic relevance. The study uses mature clinical data from two independent cohorts of patients: a uniformly treated clinical trial population as a training cohort and a population-based registry as a validation cohort. The resulting model (m7-FLIPI) is, to the best of our knowledge, the first prognostic model in follicular lymphoma that accounts for both clinical factors and genetic alterations. Our study also contrasts with previous studies with regards to the stringent inclusion criteria: all patients had follicular lymphoma grade 1, 2, or 3A confirmed by reference pathology, advanced stage or bulky disease considered ineligible for curative irradiation, symptomatic disease requiring systemic treatment, and a diagnostic biopsy specimen obtained 12 months or less before therapy initiation. Additionally, all patients received a combination of rituximab and chemotherapy (either CVP or CHOP) as first-line treatment. This stringent approach led to the unexpected finding that *EZH2* mutations (found in about one-quarter of patients and associated with a unique transcriptional signature) are linked to favourable outcome in patients with high-risk FLIPI.

Implications of all the available evidence

If validated in subsequent studies, the m7-FLIPI could be highly significant for the medical community, as high-risk patients are clearly underserved by current standard treatment and should be prioritised for innovative treatment options.

Follicular lymphoma is a clinically heterogeneous disease.[4] Identifying the subset of patients at highest risk of early treatment failure is essential, as they are clearly underserved with current standard immuno-chemotherapy. Prognostication for patients with follicular lymphoma relies primarily on clinical and basic laboratory findings.[4] The Follicular Lymphoma International Prognostic Index (FLIPI) is the most widely used risk model and includes five adverse prognostic factors: age older than 60 years, stage III–IV, haemoglobin less than 120 g/L, four or more involved nodal areas, and elevated serum lactate dehydrogenase.[5] For patients who receive rituximab-containing regimens, the FLIPI can distinguish patients into two risk groups—low or intermediate-risk, and high-risk,

for failure-free survival.[6] Whereas about half of patients requiring systemic treatment are classified as high-risk FLIPI, only a third of these will experience treatment failure within 2 years after treatment initiation.[6] Although useful, the FLIPI needs to be further improved to identify those patients at highest risk of early treatment failure. Currently, the FLIPI is not routinely used to guide risk-adapted treatment strategies.

About 90% of follicular lymphomas harbour the t(14;18) translocation, which results in overexpression of BCL2.[7] Recent sequencing studies have catalogued additional recurrent genetic alterations in follicular lymphomas,[7] but their effects on clinical outcome remain unclear. We postulated that integrating gene mutations into prognostic models will improve risk stratification for patients

with follicular lymphoma receiving first-line immuno-chemotherapy. To address this hypothesis, we analysed the full coding sequence of 74 genes that are recurrently mutated in lymphomas across two independent cohorts of patients who received first-line immunochemotherapy.

Methods

Study design and participants

We did a retrospective analysis of gene mutations and clinical risk factors in two cohorts of patients with symptomatic, advanced stage, or bulky follicular lymphoma grade 1, 2, or 3A, who had a biopsy specimen obtained 12 months or less before initiation of a rituximab-containing first-line immunochemotherapy regimen.

The training cohort (figure 1) was derived from the randomised GLSG2000 trial[8] of the German Low-Grade Lymphoma Study Group (GLSG). Between May 4, 2000, and Oct 20, 2010, this trial recruited patients with a diagnosis of stage III/IV follicular lymphoma. All patients needed treatment, as defined by the presence of B symptoms, bulky disease (mediastinal lymphomas >7·5 cm or other lymphomas >5 cm), impairment of normal haemopoiesis, or rapidly progressive disease.[8] Patients eligible for potentially curative radiotherapy were excluded. Other exclusion criteria were age younger than 18 years, pregnancy, Eastern Cooperative Oncology Group (ECOG) performance status 3 or more, or severe organ dysfunction unless due to lymphoma. Patients with other systemic malignancies, previous radiotherapy, chemotherapy, or immunotherapy were ineligible.[8]

Patients were excluded from the training cohort if the diagnosis of follicular lymphoma was not confirmed by central pathology review, or if they were randomised to not receive rituximab, or randomised to receive post-remission high-dose radiochemotherapy followed by autologous stem-cell transplantation (figure 1) because these regimens do not represent current standard practice.[2] The remaining patients received rituximab 375 mg/m², plus cyclophosphamide 750 mg/m², doxorubicin 50 mg/m², and vincristine 1·4 mg/m² (maximum, 2·0 mg) on day 1, and prednisone 100 mg/m² on days 1–5 (R-CHOP), every 3 weeks for a total of eight cycles.[8] Patients achieving at least a partial response after six cycles received two additional cycles of R-CHOP followed by interferon α maintenance.[8] This trial was approved by the institutional review board and patients signed informed consent that included molecular and genetic analyses.

The validation cohort (figure 1) consisted of patients with symptomatic, advanced stage, or bulky follicular lymphoma considered ineligible for curative irradiation, receiving first-line immunochemotherapy with six to eight cycles of R-CVP (rituximab 375 mg/m² plus cyclophosphamide 1000 mg/m², and vincristine 1·4 mg/m² on day 1, and prednisone 100 mg/day, days 1–5) every 3 weeks at the British Columbia Cancer Agency (BCCA). Diagnostic tumour biopsies were collected between Feb 24, 2004, and Nov 24, 2009, as part of a research project approved by the University of British Columbia–BCCA Research Ethics Board that included molecular and genetic analyses. From 2006 onwards, patients achieving at least a partial response were scheduled for rituximab maintenance (375 mg/m² given every 3 months for a total of eight doses; table 1).

Procedures

We analysed the full coding sequence of 74 genes selected based on the presence of recurrent mutations (appendix pp 7, 8). Genomic DNA from formalin-fixed paraffin-embedded pretreatment tumour biopsies was used for customised hybrid-capture target enrichment (SureSelect, Agilent, Santa Clara, CA, USA) and Illumina sequencing, as previously described.[9] Non-tumour DNA from peripheral blood or bone marrow samples was sequenced to exclude germ-line polymorphisms and platform-specific artifacts (appendix p 2). Matched non-tumour samples were obtained from 24 GLSG2000 patients who tested negative or had less than 1% minimal residual disease after being treated, as previously described,[10] and for ten BCCA patients free of lymphoma cell infiltration by routine clinical assessment, allowing for the filtering of somatic mutations. A panel-of-normals filter was generated from all matched (34) and other (25) non-tumour controls (appendix p 2) available for this platform, as previously described.[11] Tumour samples were filtered using the panel-of-normals and variants were rejected as germ-line events or sequencing artifacts if present in two or more normal samples. Known germ-line polymorphisms from the Exome Sequencing Project and the dbSNP (build 142) databases were also excluded. For all genes except for *BCL2*, we analysed only non-silent mutations (missense mutations, nonsense mutations, in-frame or frame-shift insertions or deletions [InDel], translational start site mutations, splice site mutations) with variant allele frequencies of 10% or more. For *BCL2*, any variant with variant allele frequencies of 10% or more was reported as hyper-mutation. 100% (46/46, appendix p 9) of a randomly selected set of mutations were validated by Sanger sequencing. We applied the MutSigCV algorithm[12] to identify genes that were mutated more often than expected by chance given background mutation processes. We used the evolutionary conservation of the affected aminoacid in protein homologues[13] to predict the effect of non-silent mutations.

Statistical analysis

For the training set, we did univariable and multivariable analyses for all genes mutated in five or more patients for stability reasons of the models, as previously performed.[14] Genes were studied for their correlation with the binary FLIPI (high-risk *vs* low or intermediate-risk) and its individual components, ECOG performance status and sex. We used the binary FLIPI because no significant
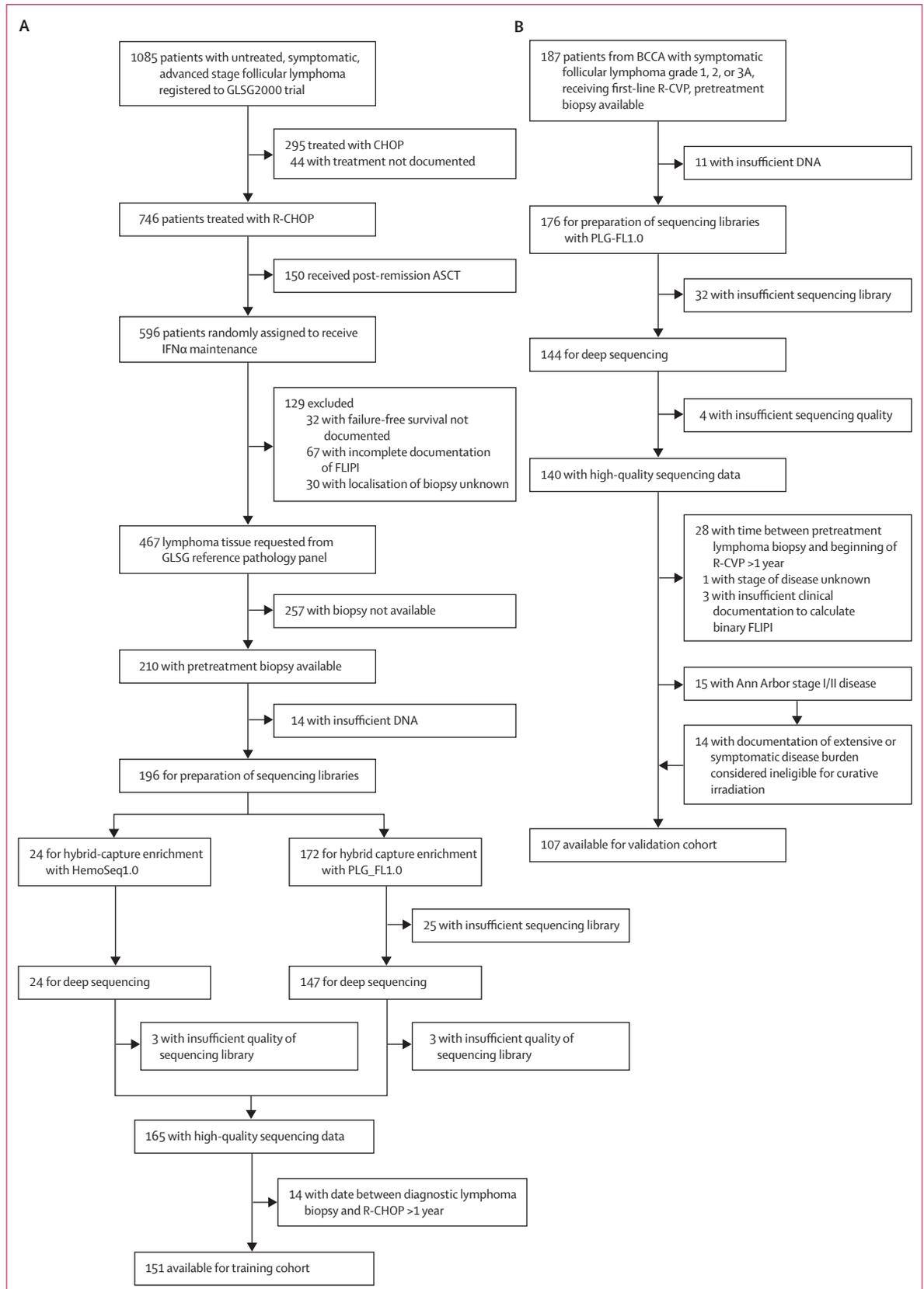
Figure 1: **Patient flow for the GLSG2000 training cohort (A) and BCCA validation cohort (B)**
HemoSeq1.0 and PLG_FL1.0 are the exon capture strategies (appendix pp 7, 8). BCCA=British Columbia Cancer Agency. ASCT=autologous stem-cell transplantation. IFNα=interferon α. FLIPI=Follicular Lymphoma International Prognostic Index. GLSG=German Low-Grade Lymphoma Study Group.

A

1085 patients with untreated, symptomatic, advanced stage follicular lymphoma registered to GLSG2000 trial

→ 295 treated with CHOP
  44 with treatment not documented

746 patients treated with R-CHOP

→ 150 received post-remission ASCT

596 patients randomly assigned to receive IFNα maintenance

→ 129 excluded
  32 with failure-free survival not documented
  67 with incomplete documentation of FLIPI
  30 with localisation of biopsy unknown

467 lymphoma tissue requested from GLSG reference pathology panel

→ 257 with biopsy not available

210 with pretreatment biopsy available

→ 14 with insufficient DNA

196 for preparation of sequencing libraries

24 for hybrid-capture enrichment with HemoSeq1.0 | 172 for hybrid capture enrichment with PLG_FL1.0

→ 25 with insufficient sequencing library

24 for deep sequencing | 147 for deep sequencing

→ 3 with insufficient quality of sequencing library   → 3 with insufficient quality of sequencing library

165 with high-quality sequencing data

→ 14 with date between diagnostic lymphoma biopsy and R-CHOP >1 year

151 available for training cohort

B

187 patients from BCCA with symptomatic follicular lymphoma grade 1, 2, or 3A, receiving first-line R-CVP, pretreatment biopsy available

→ 11 with insufficient DNA

176 for preparation of sequencing libraries with PLG-FL1.0

→ 32 with insufficient sequencing library

144 for deep sequencing

→ 4 with insufficient sequencing quality

140 with high-quality sequencing data

→ 28 with time between pretreatment lymphoma biopsy and beginning of R-CVP >1 year
  1 with stage of disease unknown
  3 with insufficient clinical documentation to calculate binary FLIPI

→ 15 with Ann Arbor stage I/II disease

→ 14 with documentation of extensive or symptomatic disease burden considered ineligible for curative irradiation

107 available for validation cohort

differences for failure-free survival were seen between low-risk and intermediate-risk patients treated with R-CHOP in a previous study,[6] and a recent update of this analysis with a median follow-up of 7·8 years (appendix p 13). Univariable Cox regression was done with and without adjustment for binary FLIPI and ECOG performance status. The Bonferroni-Holm procedure was used to adjust for multiple testing.

We generated risk models for failure-free survival using different sets of predictors. One consisted only of recurrent gene mutations and the second also included the binary clinical variables FLIPI and ECOG performance status. The latter were chosen because they were confirmed to be independent risk factors for failure-free survival in a large cohort of assessable patients from the GLSG2000 trial who received R-CHOP (appendix p 13). Additionally, we calculated prognostic models with selected genes grouped into well-described functional pathways (appendix p 5). L1-penalised Cox regression (Lasso) with 10-fold cross-validation was used to select gene mutation predictors. To generate prognostic risk models for failure-free survival, we used all 31 genes with mutations in five or more patients irrespective of results from univariable testing. We postulated that clinical variables might add prognostic information beyond tumour biology, and calculated risk models with and without inclusion of the previously well-established risk factors, FLIPI[5] and ECOG performance status.[5,15,16] We favoured FLIPI for inclusions in the model over the previously described FLIPI-2[17] because β-2-microglobulin serum concentrations are not routinely measured, particularly in North America, and the original FLIPI is more commonly used.[18] β-2-microglobulin serum concentrations were only available from a subset of patients from the GLSG2000 trial (84/151) and not for BCCA patients. We also calculated the FLIPI-2,[17] which is comprised of elevated β-2-microglobulin, longest diameter of largest node greater than 6 cm, bone marrow involvement, haemoglobin less than 120 g/dL, and age older than 60 years, for 126 assessable patients with available data from the GSLG2000 cohort.

For the model that included FLIPI and ECOG performance status, these categorical variables were not penalised and thus forced into the model. A prognostic score was calculated as the sum of predictor values weighted by Lasso coefficients. The cutoff value maximising the log-rank statistics dichotomised patients into high-risk and low-risk groups. We used bootstrap on the training cohort and selected the model with the largest bootstrap-corrected hazard ratio (HR) for failure-free survival of high-risk versus low-risk groups. Outcome data for the validation cohort remained masked until final validation. Sensitivity and specificity of the final model were estimated with the inverse probability of censoring weighting (IPCW) approach, as previously described.[19] Additionally, we calculated the C-index (Harrell) for prognostic discrimination.

	GLSG2000 training cohort	BCCA validation cohort	p value
Patients			
Number of assessable patients*	151	107	
Male	78 (52%)	59 (55%)	0·67
Female	73 (48%)	48 (45%)	
Clinical risk factors			
>60 years	57 (38%)	59 (55%)	0·0083
>4 nodal sites	106 (70%)	78 (73%)	0·74
Lactate dehydrogenase elevated	49 (32%)	22 (21%)	0·074
Haemoglobin <120 g/L	32 (21%)	12 (11%)	0·062
ECOG performance status >1	8 (5%)	16 (15%)	0·016
FLIPI high-risk	77 (51%)	53 (50%)	0·92
Treatment			
First-line treatment	R-CHOP†	R-CVP‡	
Maintenance treatment	IFNα	Rituximab	
Number of patients intended for maintenance treatment	151	93	
Outcome			
5-year failure-free survival (95% CI; number of events)	66·22% (58·63–74·79; 63)	58·43% (49·73–68·66; 48)	
5-year overall survival (95% CI; number of deaths)	83·25% (77·20–89·78; 33)	74·40% (66·50–83·23; 32)	
Median (IQR) follow-up for overall survival, years	7·7 (5·5–9·3)	6·7 (5·7–7·6)	

ECOG=Eastern Cooperative Oncology Group. FLIPI=Follicular Lymphoma International Prognostic Index. *Assessable patients fulfilled all of the following inclusion criteria: confirmed diagnosis of follicular lymphoma by reference pathology, symptomatic disease requiring treatment, advanced stage disease or bulky disease considered ineligible for curative irradiation, initial treatment regimen contained rituximab, no dose-intensified consolidative treatment, diagnostic lymphoma tissue or DNA available, time between pretreatment biopsy and treatment initiation less than 1 year, and high-quality sequencing data available. †R-CHOP: eight cycles of rituximab 375 mg/m², cyclophosphamide 750 mg/m², doxorubicin 50 mg/m², vincristine 1·4 mg/m² (day 1), and prednisone 100 mg (days 1–5), every 3 weeks, no consolidative transplant; patients achieving at least a partial response received interferon α (IFNα) maintenance (3–5 MioU per week). ‡R-CVP: six to eight cycles of rituximab 375 mg/m², cyclophosphamide 1000 mg/m², vincristine 1·4 mg/m² (day 1), and prednisone 100 mg/day (days 1–5), every 3 weeks; from 2006 onwards, rituximab maintenance (375 mg/m² given every 3 months for a total of eight doses) was given to patients achieving at least a partial response.

Table 1: Patient and disease characteristics

All statistical analyses were done using the statistical software R (version 3.1.0) and the R-packages ggplot2 (version 1.0.1), corrplot (version 0.73), MEMo (version 1.0), survival (version 2.38-1), timeROC (version 0.3), penalised (version 0.9-42), pec (version 2.4.4), and SAS 9.2.

Additional methods are described in the appendix (pp 2–4).

Role of the funding source

The funding source had no involvement in study design, collection, analysis, and interpretation of the data, and in writing of the report or decision to submit this paper for publication. AP, RK, MM, PVH, MDu, HPS, RDG, DMW, and OW had access to the raw sequencing data. VJ, EH, MU, and OW had access to raw clinical data of GLSG2000 patients. VJ, RK, EH, AAM, MU, RDG, and OW had access to raw clinical data of BCCA patients. The corresponding author had full access to all the data in the study and the final responsibility for the decision to submit for publication.

Results

The training cohort consisted of 151 GLSG2000 patients (table 1, figure 1). The median age was 57 years (IQR 50–64), 78 (52%) were male, and 77 (51%) had a high-risk FLIPI. After a median follow-up of 7·7 years (IQR 5·5–9·3), 5-year failure-free survival was 66·22% (95% CI 58·63–74·79; 63 events overall) and 5-year overall survival was 83·25% (95% CI 77·20–89·78; 33 deaths overall; table 1).

The median number of targeted genes with non-silent mutations per patient was four (IQR 3–5·5; appendix p 14). Nine genes had non-silent mutations in 10% or more of patients (KMT2D 79% [n=119], CREBBP 70% [105], TNFRSF14 32% [48], EZH2 22% [33], ARID1A 15% [22], EP300 14% [21], CARD11 12% [18], STAT6 11% [16], and MEF2B 10% [15]). BCL2 was hypermutated in 76% (115) of patients. Mutation details on all significantly mutated genes[12] and on eight additional genes with non-silent mutations in more than 5% of patients are shown in the appendix (p 15).

Overall, 146 (97%) follicular lymphomas harboured non-silent mutations in epigenetic modifiers, with predominantly disruptive mutations found in KMT2D, CREBBP, EP300, ARID1A, and BCL7A. Additional disruptive mutations affected TNFRSF14, the B-cell receptor components CD79A and CD79B, the NFκB pathway inhibitor TNFAIP3, and the transcription factors IRF8 and ETS1. Overall, 70 follicular lymphomas (46%) harboured one or more non-silent mutation in lymphoid transcription factors (appendix p 15).

A subset of mutations clustered at known hotspots. Mutations in EZH2, the catalytic subunit of the polycomb repressor complex 2 (PRC2), were clustered at Tyr641 (29 [88%] of 33 patients) and Ala677 (three [9%] of 33 patients).[20,21] Other hotspot and clustered mutations affected CARD11 (16 [80%] of 20 in the coiled-coil domain),[22] CXCR4 (WHIM-like mutations in five [100%] of five),[23] exon 1 of FOXO1 (nine [90%] of 10),[24] STAT6 at Asp419 (seven [44%] of 16),[25] and MEF2B mutations (15 [100%] of 15 within the N-terminal domain;[26] appendix p 16). By contrast, PIM1 and BCL2, which are known targets of aberrant somatic hypermutation,[27] predominantly harboured transition mutations (appendix p 17) with low predicted functional effect. Additional analyses for co-occurring mutations are included in the appendix (pp 5, 18).

The FLIPI and many individual FLIPI components were associated with specific gene mutations in univariable analyses (appendix p 10). However, none of these associations were statistically significant after correction for multiple testing. In univariable analyses, mutations in EP300 (HR 1·99, 95% CI 1·08–3·68; p=0·028) and FOXO1 (HR 2·74, 1·23–6·09; p=0·013) were associated with shorter failure-free survival, whereas mutations in EZH2 (HR 0·46, 0·22–0·93; p=0·030) were associated with longer failure-free survival. Adjustment for FLIPI and ECOG performance

status also revealed that ARID1A mutations correlated with longer failure-free survival (HR 0·40, 95% CI 0·16–0·996; p=0·049) and TP53 mutations with shorter failure-free survival (HR 2·85, 1·12–7·27; p=0·029; appendix p 19). After correction for multiple testing by Bonferroni-Holm, no single mutation (including BCL2 hypermutation) was significantly associated with failure-free survival (appendix pp 19, 20).

TP53 mutations were associated with inferior overall survival as a single factor (HR 4·70, 95% CI 1·80–12·23; p=0·0015) and with adjustment for FLIPI and ECOG performance status (HR 6·32, 2·35–17·01; p=0·00026). CARD11 mutations were prognostic for inferior overall survival in univariable analysis (HR 2·43, 95% CI 1·09–5·39; p=0·029) and when adjusted for FLIPI and ECOG performance status (HR 3·71, 1·59–8·67; p=0·0024). After correction for multiple testing, only TP53 mutations remained significantly associated with overall survival (adjusted p=0·0081; appendix p 19).

We generated multivariable risk models for failure-free survival using different sets of predictors: one consisted only of recurrent gene mutations, and the second also included the binary clinical variables FLIPI and ECOG performance status. Additional risk models are described in the appendix (pp 5, 12). Internal validation by bootstrap analysis showed superiority of the model that integrated gene mutations and clinical factors compared to the model of only gene mutations and the other models (appendix p 12). This clinicogenetic model, which we termed m7-FLIPI, was calculated as the sum of predictor values weighted by Lasso coefficients, and included high-risk FLIPI (β_{Lasso}=+0·79), poor ECOG performance status (>1, β_{Lasso}=+0·38), and non-silent mutations in seven genes: EZH2 (β_{Lasso} –0·53), ARID1A (–0·4), EP300 (+0·33), FOXO1 (+0·26), MEF2B (–0·07), CREBBP (+0·05), and CARD11 (+0·04; figure 2). To divide the risk score into high-risk and low-risk cohorts, a cutoff of 0·8 was calculated to be optimum (appendix p 3). The m7-FLIPI identified a high-risk group (43 [28%] of 151 patients) with 5-year failure-free survival of 38·29% (95% CI 25·31–57·95) and a low-risk group (108 [72%] of 151 patients) with 5-year failure-free survival of 77·21% (69·21–86·14 [HR 4·14, 2·47–6·93; p<0·0001; bootstrap-corrected HR 2·02]), and outperformed a prognostic model of only gene mutations (HR 3·76, 2·10–6·74; p<0·0001; bootstrap-corrected HR 1·57). In the patients with available FLIPI-2 scores (n=126), the m7-FLIPI (p<0·0001) outperformed the FLIPI-2 (p=0·00088; appendix p 21).

The validation cohort consisted of 107 BCCA patients (figure 1). Median age was 62 years (IQR 54–69) and 59 (55%) were male. 93 (87%) of 107 patients received rituximab maintenance. Compared with the GLSG2000 cohort, the BCCA cohort included more patients older than 60 years and more with ECOG performance status greater than 1, whereas elevated lactate dehydrogenase and haemoglobin less than 120 g/L were less frequent

(table 1). The fraction of high-risk FLIPI patients was similar between the two cohorts (53 [50%] of 107 vs 77 [51%] of 151; p=0·92). After a median follow-up of 6·7 years (IQR 5·7–7·6) in the BCCA cohort the 5-year failure-free survival was 58·43% (95% CI 49·73–68·66) and the overall survival was 74·40% (66·50–83·23).

The median number of mutations in the validation cohort was five (IQR 3–6; appendix p 14). The targeted mutational landscape of the validation cohort is summarised in the appendix (p 22). Compared with the training cohort, there were no significant differences in the mutation frequencies of any of the 74 genes after correction for multiple testing. m7-FLIPI defined high-risk (24 [22%] of 107 patients) and low-risk (83 [78%] of 107 patients) groups with 5-year failure-free survival of 25·00% (95% CI 12·50–49·99) and 68·24% (58·84–79·15), repectively (HR 3·58, 2·00–6·42; p<0·0001; figure 2, table 2, appendix p 11). The m7-FLIPI validated and outperformed FLIPI alone (HR 2·18, 95% CI 1·21–3·92), and FLIPI combined with ECOG performance status (HR 2·03, 95% CI 1·12–3·67; figure 2, appendix p 20). Performance metrics for m7-FLIPI and FLIPI based on 5-year failure-free survival in both cohorts are outlined in table 2.
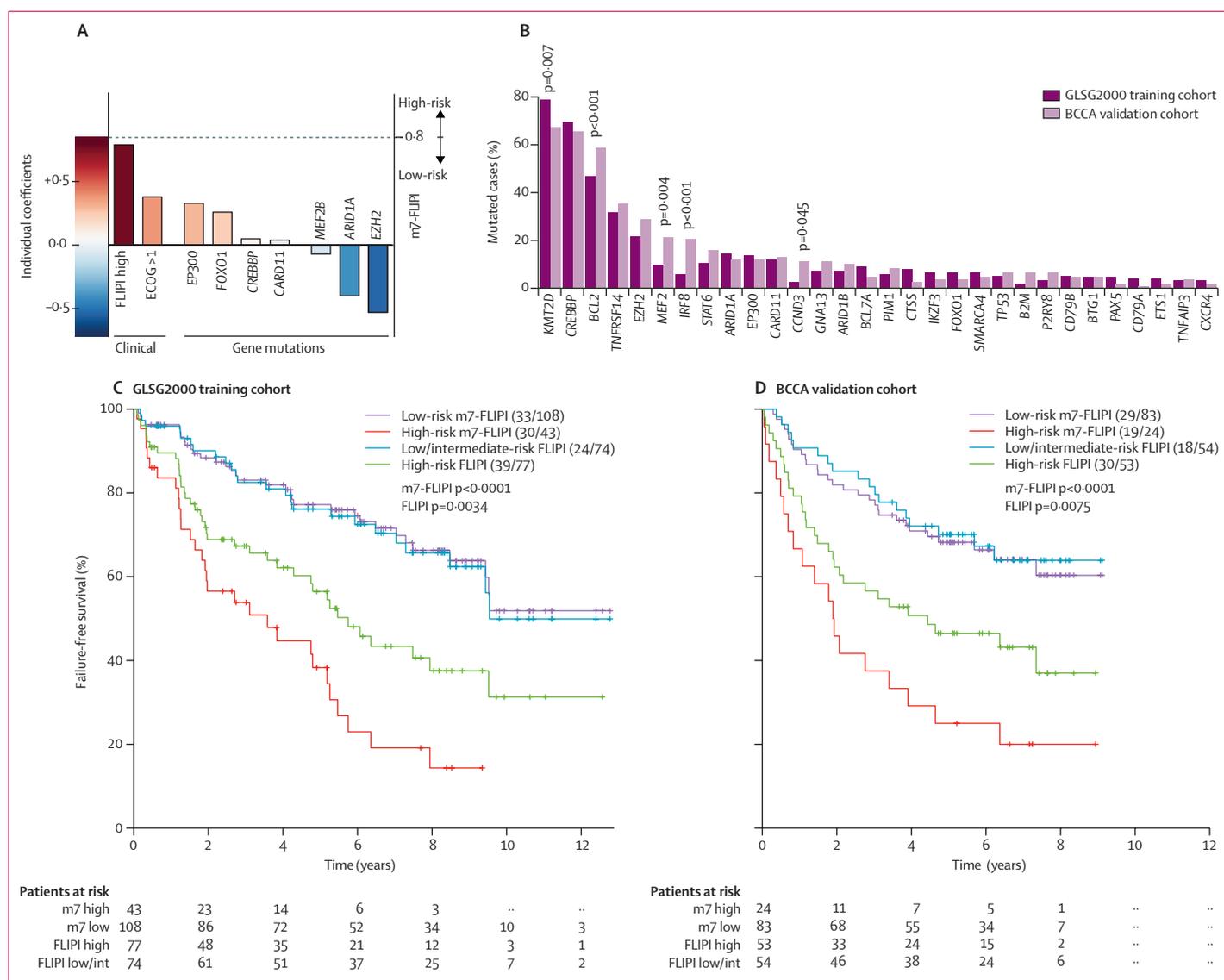
Figure 2: The clinicogenetic risk model m7-FLIPI
(A) The m7-FLIPI (m7) is calculated as the sum of individual clinical and gene mutation predictor values weighted by their individual coefficients. (B) Mutation frequencies of the GLSG2000 training and the BCCA validation cohorts. p values by Fisher's exact test, without correction for multiple testing. Depicted are all significantly mutated genes[12] and genes with non-silent mutations in more than 5% of cases from the GLSG2000 training cohort. Detailed mutation plots for both cohorts are shown in the appendix (pp 15, 22). (C) Kaplan-Meier curves for failure-free survival for the GLSG2000 training cohort by FLIPI and by m7-FLIPI. (D) Kaplan-Meier curves for failure-free survival for the BCCA validation cohort by FLIPI and by m7-FLIPI. Numbers in parentheses show number of patients with event/number of patients per cohort. FLIPI low/int=low or intermediate-risk FLIPI.

	High-risk patients, % (n/N)	High-risk vs non-high-risk 5-year failure-free survival (95% CI)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	C index (95% CI)
GLSG2000 cohort m7-FLIPI	28% (43/151)	38·29% (25·31–57·95) vs 77·21% (69·21–86·14)	52%	85%	64%	78%	0·80 (0·71–0·89)
BCCA cohort m7-FLIPI	22% (24/107)	25·00% (12·50–49·99) vs 68·24% (58·84–79·15)	41%	89%	72%	68%	0·79 (0·69–0·89)
GLSG2000 cohort FLIPI	51% (77/151)	56·47% (45·61–69·92) vs 76·14% (66·54–87·12)	65%	60%	45%	77%	0·70 (0·58–0·82)
BCCA cohort FLIPI	50% (53/107)	46·49% (34·69–62·30) vs 70·08% (58·81–83·52)	64%	62%	55%	71%	0·70 (0·58–0·83)

Sensitivity, specificity, positive predictive value, and negative predictive value for patients remaining without failure at 5 years. Harrell's C index or concordance C is a generalisation of the area under the receiver operating characteristic curve for survival data and quantifies prognostic discrimination. FLIPI=Follicular Lymphoma International Prognostic Index.

Table 2: Performance metrics for the m7-FLIPI and FLIPI based on 5-year failure-free survival

Although m7-FLIPI was developed to predict failure-free survival, we tested its prognostic utility for overall survival. High-risk m7-FLIPI was associated with an inferior 5-year overall survival of 65·25% (95% CI 51·40–82·84) versus 89·98% (84·26–96·08; p=0·00031) in the training cohort, and 41·67% (25·95–66·89) versus 84·01% (76·39–92·39; p<0·0001) in the validation cohort (appendix p 21). In each cohort, the m7-FLIPI again outperformed the FLIPI alone (appendix p 23).

Distinct molecular features exist between t(14;18)-positive and t(14;18)-negative follicular lymphomas.[28] Fluorescence-in-situ hybridisation (FISH) data using a BCL2 break-apart probe (appendix p 4) was available for 97 GLSG2000 patients and 104 BCCA patients. t(14;18) was present in 88 (91%) assessable GLSG2000 patients and 92 (88%) assessable BCCA patients. Similar to the overall study population, m7-FLIPI outperformed the FLIPI and was significantly associated with failure-free survival in the subset of t(14;18)-positive cases from both cohorts (appendix p 24). Because of the small number of t(14;18)-negative cases in each cohort, it was not feasible to test the prognostic utility of m7-FLIPI specifically within this population.

In both cohorts, the improved performance with m7-FLIPI resulted from reclassification of a subset of patients with high-risk FLIPI into the low-risk m7-FLIPI category (figure 3). 34 (44%) of 77 patients in the training cohort and 29 (55%) of 53 patients in the validation cohort that were classified as high-risk by FLIPI were re-classified into the low-risk m7-FLIPI group (figure 3). Tumours from these patients were enriched for mutations in *EZH2* (50% [17/34] vs 0% [0/43] and 55% [16/29] vs 0% [0/24]), *MEF2B* (29% [10/34] vs 0% [0/43] and 28% [8/29] vs 13% [3/24]), and *ARID1A* (32% [11/34] vs 2% [1/43] and 31% [9/29] vs 4% [1/24]). By contrast, samples from patients classified as high-risk by m7-FLIPI were enriched for mutations in *EP300* and *CREBBP* in both the training and validation cohorts (figure 3, appendix p 25).

The finding that all 66 patients across both cohorts with *EZH2* mutations were classified as low-risk m7-FLIPI led us to ask whether *EZH2* expression defines a unique biology in follicular lymphoma. To address this, we profiled gene expression in the 107 BCCA patients from the validation cohort and 33 additional BCCA patients that were excluded from the validation cohort for stringency reasons (28 patients, time between lymphoma biopsy and initiation of R-CVP >1 year; five patients, insufficient clinical documentation; figure 1). Whole-genome gene expression profiling using the Illumina cDNA-mediated annealing, selection, extension, and ligation assay (DASL; appendix p 4) was done for 140 BCCA tumour samples with available high-quality sequencing data (figure 1) and successful for 138 cases, including 106 patients from the validation cohort.

All assessable tumour samples were divided into mutated and non-mutated cases for the 15 most common gene mutations and compared for differentially expressed genes (p<0·05; appendix p 26). A false discovery rate approach to adjust individual p values revealed that *EZH2* was associated with the highest number (129) of differentially expressed genes at q values less than 0·05 (appendix p 26). These genes were used to define a distinct gene expression signature for *EZH2* mutation status by unsupervised clustering of the 106 patients from the validation cohort (appendix p 27). This signature significantly correlated with both failure-free survival and overall survival (appendix p 28). Gene set enrichment analysis of our *EZH2* signature showed significant enrichment of a previously reported *EZH2* signature[29] (figure 4).

To measure how well this gene expression signature correctly identified the presence or absence of *EZH2* mutation, we calculated the accuracy (ie, the proportion of true positives and true negatives in the population). Overall, the *EZH2* mutation status was correctly allocated by this gene expression signature in 93 (88%) of 106 tumour samples, indicating that the mutation is truly associated with a distinct transcriptional profile. The presence of *EZH2* mutations was associated with a significant improvement in both failure-free survival
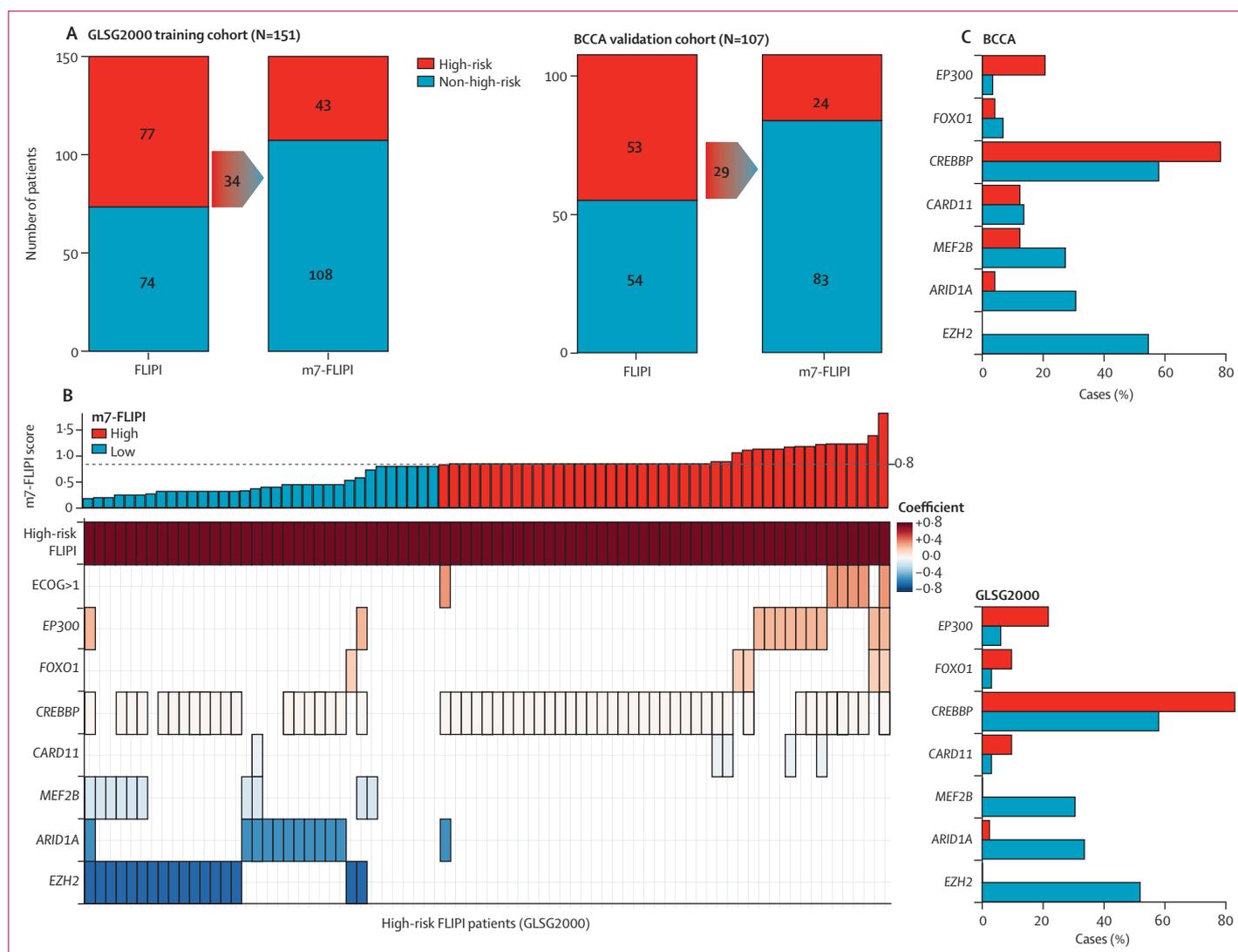
Figure 3: Reclassification of risk category by m7-FLIPI

(A) Migration plot showing reclassification of patients by m7-FLIPI in both cohorts. (B) m7-FLIPI score for all high-risk FLIPI patients from the GLSG2000 cohort, along with the Eastern Cooperative Oncology Group performance status (ECOG) and molecular predictors. Boxes indicate high-risk FLIPI, an ECOG performance status of more than 1, or a mutation in the indicated gene, and the colour code indicates the coefficient of the individual m7-FLIPI predictor. The corresponding Kaplan-Meier curves for failure-free survival and overall survival for patients classified as high-risk by FLIPI and reclassified as high-risk versus low-risk by m7-FLIPI are shown in the appendix (pp 30, 31). Results from the BCCA cohort are shown in the appendix (p 25). On the right, relative frequencies of molecular predictors by m7-FLIPI category in high-risk FLIPI patients from the GLSG2000 cohort are shown. (C) Relative frequencies of molecular predictors by m7-FLIPI category in high-risk FLIPI patients from the BCCA cohort.

(figure 4) and overall survival (appendix p 29) in both cohorts, but only among patients with high-risk FLIPI.

Discussion

To our knowledge, we report the largest study of recurrent and significant mutations in patients with symptomatic follicular lymphoma who received first-line immunochemotherapy and the first to assess prognostic relevance of mature data from two independent cohorts. By adding the mutational status for seven genes to established clinical risk factors, we developed an improved prognostic algorithm to help clarify which

patients are likely to have poor outcome after standard immunochemotherapy. Our cohorts span two continents and include both a clinical trial population (GLSG2000) and a population-based registry (BCCA). We believe the fact that the validation cohort received a different immunochemotherapy and maintenance regimen and gave virtually the same results supports the broad applicability of the m7-FLIPI.

By contrast with previous studies that focused primarily on single gene alterations,[30–33] we took a multivariable approach that included a comprehensive compilation of recurrent gene mutations and clinical
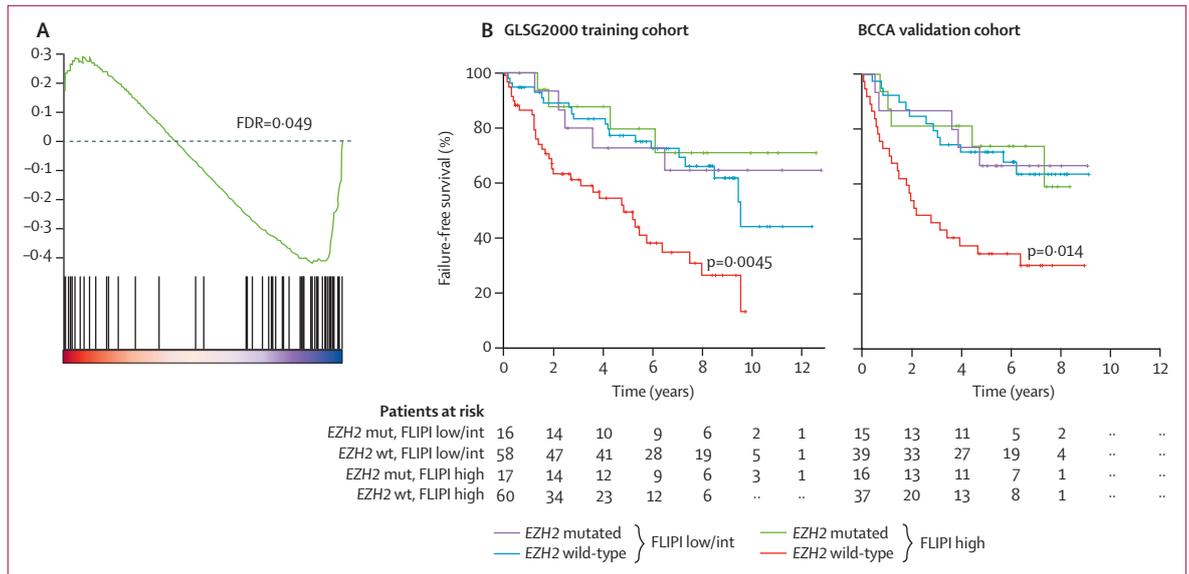
Figure 4: **Biological and prognostic relevance of *EZH2* mutations in follicular lymphoma**
(A) Gene set enrichment analysis of the *EZH2* signature from an independent cohort in our *EZH2* signature.[29] (B) Effect of *EZH2* mutation status combined with binary FLIPI risk score on failure-free survival. p values are shown for the comparison of patients classified as high-risk by FLIPI and do not have *EZH2* mutations versus patients that are classified as high-risk by FLIPI and have *EZH2* mutations. FDR=false discovery rate. low/int=low or intermediate. wt=wild-type. mut=mutant.

risk factors. The advantage of multivariable modelling is that it is unbiased by biological assumptions, and thereby reflects the fact that interactions between distinct gene mutations and clinical factors are complex, interdependent, and largely unknown. This approach does not require that single gene mutations have a significant effect on outcome. In fact, gene mutations significantly associated with outcome by univariable analysis (eg, in *TP53*) can drop out. The superior performance of the m7-FLIPI shows that both clinical factors that reflect the patient's performance status and extent of disease as well as gene mutations affect treatment outcome, and should be combined to provide optimum prognostic information.

In both cohorts, about half the patients classified as high-risk using FLIPI were classified as low-risk using m7-FLIPI and these patients had outcomes indistinguishable from those with low-risk FLIPI. This reclassification of risk category using the m7-FLIPI score primarily results from gene mutations, in particular mutation of *EZH2*, that lower the risk of a failure-free survival event after immunochemotherapy. By contrast, the remaining high-risk patients were enriched for poor outcome, with 5-year failure-free survival in the GLSG2000 cohort of only 38·29% (95% CI 25·31–57·95) and 25·00% (12·50–49·99) in the BCCA cohort.

Although m7-FLIPI was developed for failure-free survival, it was also prognostic for overall survival. However, low-risk m7-FLIPI does not necessarily indicate a more indolent disease course, as all patients studied had required treatment. We did not study asymptomatic patients who did not require therapy;

doing so is particularly challenging because lead time (ie, the time between diagnosis and symptomatic disease requiring treatment) depends on many variables other than disease biology. It is also important to note that all patients in both cohorts had biopsies obtained within 12 months before beginning treatment. The genetics of untreated follicular lymphoma might change within a patient over the course of time, so it remains unclear whether m7-FLIPI is applicable to patients whose sequenced biopsy was obtained many years before receiving first-line immunochemotherapy.

A previous study reported improved prediction of failure-free survival using the FLIPI-2 score.[17] However, we were only able to compare m7-FLIPI with FLIPI-2 in a subset of GLSG2000 patients; thus, it will be important to further validate m7-FLIPI in additional cohorts that have complete documentation of all FLIPI-2 variables. Additionally, the predictive value of m7-FLIPI will need to be assessed in patients who receive treatment regimens containing other chemotherapeutics (eg, bendamustine) or alternative anti-CD20-directed antibodies. Further studies are needed in patients with t(14;18)-negative follicular lymphoma, as the small numbers of t(14;18)-negative cases in our cohorts precluded an adequately powered assessment of the prognostic utility specifically within this rare population. Studies are also needed to determine whether mutations present at less than 10% variant allele frequency can further guide prognostication. Finally, with evolving omics and other technologies, future studies will be needed to iteratively improve m7-FLIPI by adding or substituting genetic, epigenetic, proteomic, or other factors.

Many B-cell lymphomas, both with and without activating *EZH2* mutations, might depend on EZH2 function.[34] As a result, inhibitors of EZH2 activity are in clinical trials for patients with relapsed and refractory lymphomas.[34] Mutations in *EZH2* affected 33 (22%) of 151 GLSG200 patients and 31 (29%) of 107 BCCA patients and clustered at the Tyr641 and Ala677 hotspots that are known to promote hypertrimethylation of lysine 27 on histone H3 (H3K27)[20,21] and B-cell transformation.[35] Unexpectedly, these mutations were strongly associated with low-risk m7-FLIPI, improved failure-free survival and overall survival, and defined a unique transcriptional signature. Thus, patients who harbour *EZH2* mutations are likely to have good outcomes after conventional immunochemotherapy.

In summary, by adding the mutational status for seven genes to established clinical risk factors, we developed an improved prognostic algorithm that can be applied to patients receiving first-line immunochemotherapy. A freely accessible online tool is now available to calculate the m7-FLIPI. If the m7-FLIPI is further validated in subsequent studies, it could serve as a valuable biomarker to select patients for trials of risk-adapted treatment strategies (eg, dose intensification or novel molecular targeted agents).

Contributors
AP and VJ contributed to data analysis, data interpretation, and figures. RK provided patient samples, did experiments, and contributed to data collection, data analysis, data interpretation, and figures. EH contributed to study design, data analysis, data interpretation, and figures. AMS, MS, CP, HH, and DE provided patient samples, and contributed to data collection. NK, AM, and AS did experiments. MM and AAM contributed to data collection. EL did experiments and contributed to data analysis. PVH, MDu, HPS, and CH contributed to data analysis. JMC and LHS collected patient data and contributed to data interpretation. MDr collected patient data. DN contributed to study design, data analysis, and data interpretation. PM, ACF, MLH, HS, AR, GO, and WK provided patient samples. MU contributed to data collection, data analysis, and data interpretation. WH contributed to data collection and data interpretation. RDG provided patient samples, collected patient data, and contributed to study design, data interpretation, and manuscript writing. DMW contributed to study design, data analysis, data interpretation, and manuscript writing. OW did experiments, collected patient data, and contributed to study design, data analysis, data interpretation, figures, and manuscript writing.

Declaration of interests
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