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Genomic and microenvironmental landscape of stage I follicular lymphoma, compared to stage III/IV.

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Abstract:
While the genomic and immune microenvironmental landscape of follicular lymphoma (FL) has been extensively investigated, little is known regarding potential biological differences between stage I and stage III/IV disease. Using next generation sequencing (NGS) and immunohistochemistry, 82 FL nodal stage I cases were analysed and compared to 139 FL stage III/IV nodal cases. Many similarities in mutations, chromosomal copy number aberrations (CNAs) and microenvironmental cell populations were detected. However, there were also significant differences in microenvironmental and genomic features. CD8+ T-cells (p=0.02) and STAT6 mutations (FDR<0.001), were more frequent in stage I FL. In contrast, PD1+ T-cells, CD68+/CD163+ macrophages (p<0.001), BCL2 translocation (BCL2trl+) (p<0.0001), KMT2D (FDR=0.003) and CREBBP (FDR=0.04) mutations were found more frequently in stage III/IV FL. By clustering we identified three clusters within stage I, and two within stage III/IV. The BLC2trl+ stage I cluster was comparable to the BCL2trl+ cluster in stage III/IV. The two BCL2trl- stage I clusters were unique for stage I. One was enriched for CREBBP (95%) and STAT6 (64%) mutations, without BLC6 translocation (BCL6trl), whereas the BCL2trl- stage III/IV cluster contained BCL6trl (64%) with less CREBBP (45%) and STAT6 (9%) mutations. The other BCL2trl- stage I cluster was relatively heterogeneous with more CNAs and linker histone mutations. This exploratory study shows that FL stage I is genetically heterogenous with different underlying oncogenic pathways. Stage I FL BCL2trl- is likely STAT6 driven while BCL2trl- stage III/IV appears to be more BCL6trl driven.

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Key points

1. Stage I FL is a heterogeneous disease that has clear genomic and microenvironmental similarities with stage III/IV disease
2. Stage I FL can be classified in three clusters, of which two display different underlying oncogenic pathways compared to Stage III/IV FL

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Abstract

While the genomic and immune microenvironmental landscape of follicular lymphoma (FL) has been extensively investigated, little is known regarding potential biological differences between stage I and stage III/IV disease.

Using next generation sequencing (NGS) and immunohistochemistry, 82 FL nodal stage I cases were analysed and compared to 139 FL stage III/IV nodal cases. Many similarities in mutations, chromosomal copy number aberrations (CNAs) and microenvironmental cell populations were detected. However, there were also significant differences in microenvironmental and genomic features. CD8+ T-cells ($p=0.02$) and *STAT6* mutations (FDR<0.001), were more frequent in stage I FL. In contrast, PD1+ T-cells, CD68+/CD163+ macrophages ($p<0.001$), *BCL2* translocation (BCL2trl+) ($p<0.0001$), *KMT2D* (FDR=0.003) and *CREBBP* (FDR=0.04) mutations were found more frequently in stage III/IV FL.

By clustering we identified three clusters within stage I, and two within stage III/IV. The BLC2trl+ stage I cluster was comparable to the BCL2trl+ cluster in stage III/IV. The two BCL2trl- stage I clusters were unique for stage I. One was enriched for *CREBBP* (95%) and *STAT6* (64%) mutations, without *BLC6* translocation (BCL6trl), whereas the BCL2trl- stage III/IV cluster contained BCL6trl (64%) with less *CREBBP* (45%) and *STAT6* (9%) mutations. The other BCL2trl- stage I cluster was relatively heterogeneous with more CNAs and linker histone mutations.

This exploratory study shows that FL stage I is genetically heterogenous with different underlying oncogenic pathways. Stage I FL BCL2trl- is likely STAT6 driven while BCL2trl- stage III/IV appears to be more BCL6trl driven.

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Introduction

Follicular lymphoma (FL) is the most common indolent non-Hodgkin lymphoma (NHL) in adults, with an incidence of 2.2-5 per 100,000 in the western world.[1-3] The large majority of patients presents with advanced stage disease (stage III/IV) at diagnosis while only 10-15% exhibit limited stage disease at presentation.[4,5]

Patients with limited stage FL, defined by stage I and limited, contiguous stage II disease, may be cured in 45-65% of cases with local radiotherapy (RT) (24Gy involved field RT) without further systemic treatment.[5-11] Adding rituximab (R) or R-chemotherapy has been shown to improve progression-free survival (PFS) but at the cost of mild toxicity and with conflicting results pertaining to improving overall survival (OS).[11-13] Despite the responsiveness of advanced stage FL to current chemo-immunotherapy modalities, the disease course is characterized by multiple relapses and is considered incurable.

The oncogenesis of FL suggests a primary systemic disease with *BCL2* translocation (BCL2trl+) as an early transforming event, most likely occurring in the bone marrow and not in eventual presenting nodal sites. It is intriguing that a lymphoma characterized by a relapsing, protracted, but eventually fatal course, may be cured by local therapy only when presenting in the rare context of limited stage disease. A key question therefore is whether limited-stage FL follows a different oncogenesis and is driven by specific genomic and/or microenvironmental features that may explain this distinctive clinical course.

The most characteristic genomic feature of FL is BCL2trl+, observed in 85-95% of advanced stage FL, but in only in 42-50% of limited stage FL.[14,15] In cases where this translocation has been identified, it results from an aberrant immunoglobulin (Ig) locus rearrangement that occurs most frequently at the pre-B cell stage in the bone marrow and serves as one of the initiating events in FL oncogenesis. Whether other genomic differences occur in limited stage FL compared to advanced stage FL is currently unknown.

The interaction between tumor- and immune microenvironmental cells in FL results in distinctive features and is likely to influence the clinical course and outcome in this disease.[16] The role of specific immune microenvironment populations, such as T-cells and macrophages has not been fully elucidated. Despite extensive studies in advanced stage FL, conflicting conclusions remain regarding the impact on survival.[17-22] Again there is a dearth of knowledge regarding the immune microenvironment of limited stage FL. Only one study has reported microenvironment characteristics in different stages of FL. Stages I to IIIA were combined and considered early disease in that study, which was characterized by a significantly higher number of PD1+ T-cells and a lower number of FOXP3+ T-cells compared to advanced stage (stage IIIB-IV) disease.[23]

In a concerted effort, the Lunenburg Lymphoma Biomarker Consortium (LLBC) has collected a relatively large series of rigorously defined and clinically well-annotated cases of stage I nodal FL from clinical trial cohorts and population-based registry. The genomic and immune microenvironmental characteristics of stage I FL were mapped and subgroups were determined. Subsequently, this information was interpreted in the context of a large cohort of advanced stage FL patients collated in parallel by the LLBC members and analysed with the same techniques.

Methods

Patient selection

Within the LLBC collaboration, samples were collected from 8 different cohorts. Stage I cases were collected from: European Organization for Research and Treatment of Cancer (EORTC) study 20971[24,25], the German Low-Grade Lymphoma Study Group (GLSG) early stage FL study[26,27], and the Haematological Malignancy Research network (HMRN) population-based registry.[28] (supplemental Table 1A for detailed inclusion criteria and treatment protocols)

Stage III/IV cases were collected from: Lymphoma Study Association (LYSA) FL2000 study[29,30], GLSG2000 study[31], together with the population based registries from HMRN and Sweden, and the institutional registries from St Bartholomew's Hospital, London and Stanford University Medical Center, Stanford (supplemental Table 1B for detailed inclusion criteria and treatment protocols).

Patients for the stage I cohort, selected from the 2 studies and the population-based registry, needed to fulfil the following criteria: 1) stage I as determined by standard staging procedures at time of inclusion in study or database, 2) nodal, non-bulky disease (<7 cm) and 3) histologically confirmed FL grade 1-3A. Inclusion criteria for the patients in the stage III/IV cohort were: 1) stage III/IV disease as determined by standard staging procedures, 2) ≥5 nodal areas, with or without bulky disease and 3) histologically confirmed FL grade 1-3A.

All stage I and III/IV cases were staged with computed tomography (CT) and bone marrow biopsy. For both cohorts, availability of complete and detailed clinical information on demographic parameters, staging procedures, and treatment was required as well as a representative diagnostic formalin-fixed paraffin embedded (FFPE) biopsy sample.

Immunohistochemistry analysis of the microenvironment and tumour cells

Tissue microarrays (TMAs) were constructed centrally according to LLBC validated protocols using duplicate cores of 1 mm diameter.[32] Sections of 3 µm were mounted on slides and stained for CD3, CD4, CD8, CD68, CD163, FOXP3, and PD1 according to standard procedures at the Barts Cancer Institute – Centre for Haemato-Oncology Research Laboratory, London, UK (supplemental Table 2).

These microenvironment markers were scored on the whole core by a computerized system with automated scanning microscopy and computerized image analysis (Ariol SL-8, Leica Microsystems, Wetzlar, Germany) as validated in Sander et al.[32] and applied previously in Stevens et al.[18] For more detailed information, see supplemental Methods.

Additionally, tumor cell features were further assessed with immunohistochemistry (IHC) for expression of BCL2, with antibodies to different epitopes (DAKO124, SP66), germinal centre markers (BCL6, CD10, LMO2 and HGAL), a post-germinal centre marker (MUM1), and a putative nodal marginal zone lymphoma (NMZL) marker (MNDA) (supplemental Table 2). MNDA was added as an extra control that these cases were true FL and not MZL. These immunohistochemical stains were performed according to standard procedures at the Department of Pathology, Amsterdam UMC location VUMC, Amsterdam, The Netherlands. All markers were independently scored on duplicate cores in a dichotomized manner as negative or positive, defined as >30% positive tumour cells by two pathologists (DdJ, BS, and AR). All cores with <50% of scorable core surface area were excluded. In case of discordance between the two pathologists, a deciding score by the third pathologist was performed.

Gene mutation and copy number analysis using next generation sequencing (NGS)

NGS library preparation and analysis was performed as previously described[33], in brief: genomic DNA was extracted with the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), and fragmented using a Covaris ME220 (Covaris Inc, Woburn, MA, USA). Subsequently, NGS libraries were made with 100ng sheared DNA and unique indexes (IDT, Coralville, IA) using the KAPA or KAPA Hyper Library Preparation kit (KAPA Biosystems, Wilmington, MA).

For copy number aberration (CNA) 50-bp single-read shallow whole-genome sequencing (WGS) was performed on a HiSeq 4000 (Illumina, San Diego, CA). Sequence reads were aligned against the reference genome (GRCh37/hg19) with the Burrows-Wheeler Alignment tool (BWA aln; v0.7.12)[34] and deduplicated with picardtools (v2.15). Copy number analysis was performed with QDNAseq (v1.12.0)[35], NoWaves (v.0.6)[36], DNAcopy (v1.50.1)[37], ACE (v.0)[38], CGHcall (v2.38.0)[39] and CGHregions (v1.34).[40]

For mutation and translocation analysis a 3Mb SeqCapEZ capture panel was designed in collaboration with Roche, containing coding regions of 369 genes and 12 genomic regions (Roche NimbleGen, Madison, WI; order ID 43712) (supplemental Table 3 and 4); eight samples were equimolarly pooled to 1 ug for the capture, and three pools together were sequenced 150-bp paired-end on a HiSeq 4000 (illumina, San Diego, CA).

Sequenced reads were trimmed with SeqPurge (v0.1-104)[41], aligned with BWA mem (v0.7.12)[34], realigned with ABRA (v2.19)[42] and duplicates were removed with picardtools (v2.4.1; using the setting ASSUME SORT ORDER=queryname). Mutations were detected by LoFreq (v2.1.3.1)[43] and Mutect2 (v4.1.7).[44]

Translocation detection was performed with BreaKmer (v0.0.4), GRIDDS (v1.4.2), Wham (v1.7.0) and novoBreak (v,0.0.6)[45-48]. Translocations needed to be detected by at least 2 of the used tools. For more detailed information, see supplemental Methods.

All sequence data have been deposited at the European Genome-Phenome Archive under accession number EGAS00001005755

Ethical Committee statement

The study and protocols to obtain human archival tissues and patient data were approved by the local ethical committee of the VU University Medical Center, Amsterdam (FWA00017598) for all collaborating centres and comply with the Code for Proper Secondary Use of Human Tissue in the Netherlands (http://www.fmwv.nl).

Statistical analysis

Clinical characteristics were summarized with descriptive statistics (median (range) for quantitative and frequency (percent) for qualitative variables) and compared using Chi-Square or Mann-Whitney test. Kaplan-Meier survival curves were constructed. PFS defined from start of treatment to progression/transformation. OS defined from start of treatment to death by any cause.

The average of the IHC biomarker scores from two cores was calculated, and compared between groups of patients with Kruskal-Wallis test corrected for multiple testing with the Benjamini & Hochberg method.

Comparison of frequencies of mutations and translocations was performed with Fisher-exact test and false discovery rates (FDRs) were controlled with the Benjamini & Hochberg method. P-values and FDRs for comparisons between copy number regions were calculated with CGH test.

Complete-linkage hierarchical clustering was performed with the function 'hclust' of the 'stat' package (https://stat.ethz.ch/R-manual/R-devel/library/stats/html/00Index.html). Features included in clustering were somatic mutations and focal and chromosomal arm level aberrations present in more than 5% of the samples, and *BCL2* and *BCL6* translocations. The distance measure used for the clustering was defined as $1\text{-cor}_{spearman}$ for both the genes and the patient samples, implemented by the 'cor' function, also from the 'stat' package. The stability of the clusters was assessed by subsampling as described by Monti et al.[49] All analyses were performed in R (version 3.5.1) and a two-sided p-value of less than 0.05 was considered statistically significant.

Results

In total, 216 patients with stage I disease from two clinical trials and one population-based registry fulfilling the clinical selection criteria were included in this study. Complete targeted NGS data for mutations, translocations and genome-wide copy-number variations could be generated for 82 cases of which 73 also had complete microenvironmental data (Figure 1 and supplement Table 5A-B).

A cohort of 391 stage III/IV patients, who fulfilled the inclusion criteria, were selected from two clinical trials and 4 registries. For the final analysis 139 of 391 cases with complete NGS data were included of which 120 had complete microenvironment data available (Figure 1 and supplemental Table 5A-B).

Clinical characteristics for the 82 stage I and 139 stage III/IV patients with complete NGS data are shown in table 1. The study cohort is representative of the complete cohort of 602 patients with FL that fulfilled the initial clinical inclusion criteria (supplemental Table 6). Clinical variables such as presence of B-symptoms, higher FLIPI score, low hemoglobin and elevated LDH were as expected significantly more frequent in the stage III/IV cohort. The 10 years PFS and OS of the stage I cohort were 56% and 83% respectively (supplemental Figure 1).

The eight IHC markers (BCL2 DAKO124 and SPS66, CD10, BCL6, HGAL, LMO2, MNDA and MUM1), used to confirm true stage I FL, could be evaluated in 75/82 patients. Concordance had to be reached with a third pathologist in 4% of the scored markers. In 91% cases a minimum of 3 of 4 germinal centre markers were scored positive (CD10 91%, BCL6 96%, HGAL 80%, LMO2 95%) (supplemental Table 7). Moreover, MNDA was not expressed in any of the cases,[50] nor was MUM1, underlining the germinal centre features. These findings exclude alternative diagnoses such as NMZL and support classification of FL in all included cases.

The immune microenvironment in stage I and stage III/IV follicular lymphoma

Microenvironment analysis was available for 193 of 221 cases with complete NGS data (stage I n=73, stage III/IV n=120). In stage I disease, a significantly denser infiltrate of CD8+ cytotoxic T-cells was observed (median stage I 13.7% vs stage III/IV 10.9%, p=0.02), while PD1+ follicular T-helper cells (median stage I 1.8%, vs stage III/IV 4.7%, p<0.001), and macrophages (CD68+ median stage I 2.7%, vs stage III/IV 3.6%, p<0.001 and CD163+ median stage I 2.3%, vs stage III/IV 4.1%, p<0.001) were more frequent in stage III/IV disease. Other cell populations as measured by T-cell markers CD3, CD4 and FOXP3 showed no significant differences (Figure 2A and supplemental Table 8). It should be noted however that although statistically significant differences were observed for CD8+ and PD1+ T-cell populations and macrophage contribution, the absolute differences were minor and may only be appreciated using automated image analysis.

Genomic and microenvironmental features of stage I FL in comparison with stage III/IV

BCL2trl were detected with significantly lower frequency in stage I cases, 59% as compared to 91% of cases in stage III/IV disease (p<0.001) (Figure 2B). There was no differences of the breakpoint locations between the stages (supplemental Figure 2A-F and supplemental Table 9). In addition to classical *BCL2*/IGH translocations (n=171), rare other translocation partners were also found: IGL (n=2), IGK (n=1) and *HLA-DRA* (n=1), all present in stage III/IV.

BCL6 translocations (BCL6trl) were observed in 6% of stage I cases and 17% of stage III/IV cases (p=0.07) (Figure 2B). Translocation partners for *BCL6* were diverse. (supplemental Figure 2G-H and supplemental Table 9).

Other IGH translocations were observed in 13% of stage I cases and 16% of stage III/IV cases, with diverse translocation partners. Additionally, most recurrent other translocations detected were *MYC* (n=1 stage I, n=3 stage III/IV) and *TBL1XR1* (n=1 stage I, n=3 stage III/IV) translocations. (supplemental Table 9).

High quality genome-wide CNA plots were obtained by shallow WGS for all cases. Overall, stage I and III/IV disease showed comparable frequencies of aberrations and the spectrum of alterations did not differ significantly (Figure 2B and supplemental Table 10). The copy number load per stage is similar (supplemental Figure 3A). The overall landscape of CNA included focal gains of known FL-related genes such as *REL* and *BCL11A* (2p16.1) and focal losses of *TNFRSF14* (1p36.32), *PRDM1* (6q21), *TNFAIP3* (6q23.3), *CDKN2A* (9p21-22) and *PTEN* and *FAS* (10q23.31). The focal loss of 9p21-22 containing *CDKN2A*, and a small region on 6q12 without a specific gene, were significantly more common in stage III/IV (Figure 2C).

The median number of non-synonymous and splice-site mutations was comparable between stage I (median 11 mutations/case, range 0-29) and stage III/IV (median 12 mutations/case, range 0-116) (supplemental Figure 3B and supplemental Table 11). Regarding *BCL2* somatic hypermutations (SHM) (defined for the purpose of this study as: $\leq$2 mutations in known SHM target genes), there was a significant difference between the number of cases with SHM in stage I 36% and III/IV 71% (p=0.017), which correlated with more BCL2trl in stage III/IV. Comparing the number of SHM-related mutations only in the BCL2trl+ cases there was no difference (stage I

63% n=26/41 vs stage III/IV 77% n=97/127, p=1). SHM in *BCL6* (stage I n=1) and *PIM1* (stage I n=4, stage III/IV n=3) was seen in only a few cases, and no SHM was found in *MYC* (supplemental Table 11).

Of the genes included in the LLBC-NGS targeted panel, the following were most frequently affected by non-synonymous mutations in stage I FL: *KMT2D* (52%), *CREBBP* (50%), *BCL2* (35%), *EZH2* (35%), *TNFRSF14* (35%), *STAT6* (30%) and *MEF2B* (18%). The most frequently affected genes in stage III/IV FL were: *KMT2D* (76%), *CREBBP* (69%), *BCL2* (54%), *TNFRFS14* (31%), *EZH2* (20%), and *ARID1A* (17%). A comparison of mutation frequencies showed that *STAT6* was mutated at a significantly higher frequency in stage I compared to stage III/IV (FDR<0.001) while *KMT2D* and *CREBBP* were mutated at a significantly higher frequency in stage III/IV malignancies (FDR=0.003 and FDR=0.04) (Figure 2D).

Overall, the mutational landscape between stage I and stage III/IV FL was highly similar with a dominant involvement of epigenetic and chromatin modifying genes (*KMT2D, CREBBP, EP300, EZH2, MEF2B*), but at different frequencies. In 94% of stage I and in 99% of stage III/IV cases, at least 1 of these 5 genes was mutated, indicating a critical role of epigenetic deregulation in the development of FL.

Integrated analysis of translocation, CNA and mutation data

Next, we performed an integrated analysis of all molecular modalities by an unsupervised hierarchical clustering strategy to explore the potential heterogeneity and integrated profiles within stage I disease. (Figure 3) For this analysis, 81 stage I cases were included, excluding one case with a very low level of shared mutations. The Dunn index, estimates 4 clusters for stage I as optimal (supplemental Figure 4). Cluster 1 (CL1) (n=44) was characterized by presence of BCL2trl in all cases in concert with frequent mutations in *BCL2.* CL1 was further characterized by classic FL mutations (*KMT2D, EZH2*, *CREBBP, TNFRSF*14, *MEF2B)*. (supplemental Figure 5 Table 12). Cluster 2 (CL2) (n=15) was characterized by a relatively high level of CNAs (median 19%, range 0-58%) (supplemental Figure 6) and mutations in 40% of the cases in one or both linker histone genes (*HIST1H1E* 27% and *HIST1H1C* 20%). In CL2, BCL2trl and BCL6trl and *STAT6* mutations occurred at intermediate frequencies, while epigenetic modifying genes (KMT2D, CREBBP, EZH2) were mutated at relatively low levels (supplemental Table 12). The last two clusters were defined by absence of BCL2trl and presence of STAT6 and CREBBP mutation. They differ in presence of TNFRSF14 and KMT2D mutations, which are both tumor suppressor genes. TNFRSF14 is controlled by KMT2D[51] , the biological pathways of these two clusters are likely identical, so we combine these two cluster into cluster 3 (CL3) (n=22). CL3 has the “Classical” FL-related genes with the exception of *MEF2B*. (Figure 3, supplemental Figure 7 and Table 12). The mean consensus index of two samples from the same cluster was 87%, indicating that the clustering was stable. (supplemental Figure 8).

Integrated analysis of the stage III/IV FL cases showed BCL*2*trl as the most frequent genetic alteration. Resulting in a relatively homogeneous cluster of BCL2trl+ FL (n=128) and a separate cluster lacking the BCL2trl and concomitant *BCL2* mutations (n=11) (supplemental Figure 7,9-10). In this BCL2trl negative (BLC2trl-) group, *BCL6*trl were present at high frequency (64%). Mostly “classical” FL mutations were seen, albeit at different frequencies for BCL2trl positive vs BCL2trl negative cohort (*KMT2D* (79% vs 55%), *EZH2* (22% vs 0%) *HIST1H1E* (15% and 0%) and *HIST1H1C* (6% vs 27%). (supplemental Table 12)

The stage III/IV BCL2trl-negative cluster lacked the distinct characteristics of the stage I BCL2trl- CL3 and was not enriched for *CREBBP* and *STAT6* mutations while also characteristic high level CNA of CL2 were less prominent. Altogether the results

showed that within stage I there are 2 distinct molecularly driven clusters in addition to a “canonical” FL cluster.

Copy number aberrations, mutations and translocations of the subset with complete microenvironment information was representative of the dataset with complete NGS, nor would the clustering have been affected if this subset was used to perform the analysis (supplemental Figure 11-13).

After the identification of the three clusters in stage I disease, we explored whether these clusters might underlie a distinct immune microenvironment signature. For 183 of 220 cases included in the hierarchical cluster analysis, complete microenvironment information was available for an integrated analysis (stage I n=69: CL1 n=37, CL2 n=11, CL3 n=21 and stage III/IV n=114: BCL2trl+ n=107, BCL2trl- n=7) (supplemental Figure 14 and supplemental Table 13). While there seemed to be a lower level of PD1 positive cells in CL2, due to the few cases per cluster and the minimal differences observed in the scoring results, no statistical analysis could be performed, which precluded biological interpretation of the data.

Discussion

Tumor and microenvironmental analyses of the largest series of nodal stage I FL thus far allow us to conclude that stage I FL has mostly genomic and microenvironmental abnormalities similar to stage III/IV disease, but some significant differences were found.

In both groups, the same mutations and CNAs are observed with *KMT2D, CREBBP, BCL2, TNFRSF14, EZH2* as the most frequently mutated genes. The most frequent copy number gains of chromosomes 1q, 2, 7, 12 and 18 in the present series are in agreement with other published reports.[52-58] The immunophenotype of the tumor cells is also consistent with germinal center cell derivation and the overall composition of the immune microenvironment shows no clinical significant differences between stage I and stage III/IV disease. The higher frequency of CD8+ T-cells, lower frequency of PD1+ T-cells, and CD68/CD163+ macrophages noted in stage I FL, are suggestive of a biological role for these cell populations, yet the small absolute differences cannot be appreciated without automated image analysis and are therefore unlikely to be useful in clinical practice. Thus, our results are consistent with the currently accepted view that stage I FL in general is not a distinct biological entity.

The major difference observed between stage I and stage III/IV is the frequency of BCL2trl, as previously reported.[15] Studies focusing on BCL2trl- FL have reported a higher frequency of BCL6trl, [59] as well as more frequent mutations in *STAT6, CREBBP,* and *TNFRSF14.*[60-63] Our data allows a broader perspective as we now identify signatures in their specific clinical contexts showing essentially different signatures for BCL2trl- stage I and stage III/IV disease. A unique BCL2trl- cluster, CL3, is recognized as highly specific for stage I FL. CL3 is characterized by enrichment for *CREBBP* (95%), *STAT6* (64%), *EZH2* (50%) and *TNFRSF14* (50%) mutations and absence of BCL6trl, while stage III/IV BCL2trl- FL is enriched for BCL6trl (64%) with low frequency of the most frequently mutated genes in CL3. These differences suggest that different sets of specific molecular events may drive the pathogenesis of FL.

We identify the same three most frequent hotspots in STAT6 previously reported by Yildiz et al. E372K (stage I n=5, stage III/IV n=1), E377K (stage I n=3, stage III/IV n=3) and D419G (stage I n=5, stage III/IV n=2), (supplemental Figure 15A-B), which are activating mutations in the IL4/JAK/STAT pathway.[64] This pathway may indeed be capable of overriding the important role of the absent BCL2trl in the pathogenesis

of stage I FL. For example, follicular T-helper cells are an important source of IL4 which via STAT6 can directly regulate BCL2 expression.[65] Due to the low number of cases in each of the 3 clusters and minimal differences in frequency of PD1+ follicular T-helper cells in the microenvironment, we are however not yet able to draw firm conclusions about the interaction between *STAT6* mutations and the number of PD1+ follicular T-helper cells. Strikingly, there is only 1 BCL2trl- sample with a STAT6 mutation in the stage III/IV group.

The CL2 cluster appears to represent a distinct group defined by a higher number of CNAs and higher frequency of HIST1H1E and HIST1H1C mutations (supplemental Figure 15C-E). Loss of function of these linker histone genes has been shown to drive lymphomagenesis due to higher fitness of germinal center B-cells, and enhanced self-renewal potential.[66] The small number of cases in this cluster and the relatively heterogeneous features, however, preclude definite interpretation.

It should be noted that though supported by mathematical and biological evidence, clustering should not be regarded as a definitive classification, but rather a means to obtain biological insight and a step toward finding the driver genes and pathways of FL.

A limitation in our study is that the majority of patients were diagnosed prior to including FDG-PET as a staging procedure, and therefore, this cohort may contain some patients who would be classified as a higher stage with current staging techniques. As indicated in the literature, up to 30% of patients may be upgraded to a higher stage with PET-CT scans.[67] However, the unique and specific mutational landscape characteristics of the two distinct clusters described above are not recognized in advanced stage, suggesting that the majority of these cases were true stage I FL.

The identification of three different clusters raises the question if each subtype follows a distinct clinical course. Due to the diverse origin of the samples, treatment modalities, follow-up strategies and the limited number of samples per cluster, our study is not able to address answers with regard to clinical outcome per cluster.

In conclusion, with this relatively large cohort we demonstrate that stage I FL is a genetically heterogeneous group divided over three distinct and unique clusters, for which the two BCL2trl- clusters suggest different underlying oncogenetic pathways in comparison with stage III/IV FL. Our results suggest that BCL2trl- stage I disease follows a different pathogenesis than BCL2trl- stage III/IV.

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Contribution:

The Lunenburg Lymphoma Biomarker Consortium, M.J.K, ,B.Y., and D.d.J. designed the study;

G.T.L.-d.V., W.B.C.S., E.v.D., C.L.J., A.J.C., P.S., M.M., N.J.H. performed experiments;

G.T.L.-d.V., W.B.C.S., E.v.D., C.L.J, M.G.M.R, M.M., B.S., A.R., D.M.B., B.Y., and D.d.J. analyzed and interpreted the data;

G.T.L.-d.V., W.B.C.S, E.v.D., D.M.B., C.L.J., B.Y., and D.d.J. wrote the manuscript; and all authors critically revised the manuscript and were involved in its editing and gave final approval of the submitted and published versions.

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Table 1: demographic and clinical characteristics of stage I and stage III/IV patients included for analysis in the study

		Stage I n = 82	Stage III/IV n = 139	P value
Age at diagnosis				**0.96[1]**
	Median (range)	58 (28-85)	57(27-95)	
Gender				
	Female	32 (39%)	62 (45%)	**0.42[2]**
	Male	50 (61%)	77 (55%)	
B-symptoms				**<0.001[2]**
	Present	5 (6%)	41 (30%)	
	Absent	77 (94%)	97 (69%)	
	Missing		1 (1%)	
FLIPI risk categories				**<0.001[2]**
	Low	68 (83%)		
	Intermediate	6 (7%)	53 (38%)	
	High		77 (55%)	
	Missing	8 (10%)	9 (7%)	
Haemoglobin				**0.002[2]**
	<12 g/l	2 (2%)	26 (19%)	
	≥12 g/l	78 (95%)	111 (80%)	
	Missing	2 (2%)	2 (1%)	
LDH elevated				**<0.001[2]**
	Yes	6 (7%)	44 (32%)	
	No	71 (87%)	94 (67%)	
	Missing	5 (6%)	1 (1%)	
Stage				
	Stage I	82 (100%)		
	Stage III		38 (27%)	
	Stage IV		101 (73%)	
Bulky disease				
	<7 cm	82 (100%)	96 (69%)	
	≥7cm		38 (27%)	
	Missing		5 (4%)	
ECOG Performance status				
	<2	81 (99%)	128 (93%)	
	≥2		10 (7%)	
	Missing	1 (1%)	1 (1%)	

Bone marrow involvement		
Yes		83 (60%)
No	82 (100%)	49 35%)
Missing		7 (5%)
Number of involved nodal sites		
Median (range)	1 (1-1)	8 (5-14)
First line therapy		
R-chemotherapy	2 (2%)	117 (84%)
chemotherapy		5 (4%)
IF-RT	38 (46%)	
IF-RT + TBI	14 (17%)	
IF-RT + R	23 (28%)	
Watch & Wait		3 (2%)
other	1 (1%) *	
Unknown	4 (5%)**	14 (10%)

Abbreviations: ECOG, Eastern Cooperative Oncology Group; PS, performance score; FLIPI, follicular lymphoma international prognostic index; IF-RT, involved field radiotherapy; TBI, total body irridation; R, rituximab

[1] Mann Whitney p-value; [2] Chi-Square p-value;

* surgical removal; ** IF-RT +/-TBI TBI

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Figure 1: Outline of follicular lymphoma (FL) cases included in the study.

233 stage I cases were initially submitted from 2 clinical trials and 1 population-based cohort, 216 fulfill all clinical inclusion criteria (European Organization for Research and Treatment of Cancer (EORTC) study 20971 (n=143), German low-grade Lymphoma Study Group (GLSG), Rituximab and involved Field Radiotherapy in early stage FL (MIR) study (n=39) and Haematological Malignancy Research network (HMRN) population-based registry (n=34)). Of 82/216 cases was with targeted next generation sequencing (NGS) a complete data set on translocation, copy number aberration (CNA) and mutations were successfully obtained, meeting all quality measures, meaning sufficient amount of DNA (>100ng) from FFPE material, and sequencing results with a minimum mean target coverage off >30 reads for paired-end sequencing and 300000 reads for shallow sequencing. For 73/82 cases, complete immunohistochemistry (IHC) data of 7 markers (CD3, CD4, CD8, PD1, FOXp3, CD68 and CD163) of the microenvironment was available, meeting all quality measures, meaning sufficient amount of FFPE material to obtain two 1 mm cores, and the cores should contain >50% tumor tissue to score.

As a reference cohort 667 stage III/IV cases were selected from 2 clinical trials and 4 population based cohorts of which 391 fulfilled all clinical inclusion criteria (Lymphoma Study Association (LYSA) FL2000 study (n=163), GLSG2000 study (n=98), HMRN population-based registry (n=100), Sweden population based registry (n=19), St Bartholomew's Hospital, London (n=6) and Stanford university hospital, Stanford (n=5)). In 139/391 a complete NGS data meeting all quality measures was obtained. For 120/139 cases also complete microenvironment information meeting all quality measures was available. Depicted in the yellow box are the cases which are incorporated in the analysis.

Abbreviations: FFPE: formalin-fixed paraffin-embedded, IHC: immunohistochemistry, NGS: next generation sequencing

Figure 2: Microenvironment, mutations, translocations and copy number landscape of stage I versus stage III/IV follicular lymphoma.

A: percentage of positive nucleated cells for CD4, CD8, CD3, FOXP3 and PD1 depicted as boxplots. For CD163 and CD68 the percentage of positive area of the total cell area computer assisted scored are plotted in the boxplots, stage I in green (n=73) and stage in blue III/IV (n=120). Significant differences are seen for CD8, PD1, CD163 and CD68 with a p value adjusted for multiple testing *p=0.02, **p=0.002 and ***p<0.001

B: comparison plots for CNAs between stage I as filled areas (n=82) and stage III/IV as lines (n=139) are percentages of the number of cases with gains (positive value red) and losses (negative value blue), sorted for chromosome position (x-axis)

C: frequency plots with P-values (orange) calculated with a 2-sided Rank-Sum Test with 10000 permutations and false discovery rate (FDR, striped segments) of the difference in CNAs, the horizontal dotted lines show the significance thresholds p-value < 0.05 in red, and the FDR in blue <0.1. If the difference in CNA level crosses the p-value, and the FDR-level is lower than 0.1, the difference is considered significant which are indicated by * 6q23.3 and 9p21-22 (*p-value < 0.05)

D: frequency of *BLC2* and *BLC6* translocations and top 20 mutated genes according to stage I in green(n=82) and stage III/IV in blue (n=139), significant differences are indicated by * q< 0.05, (Fisher-exact test and false discovery rated using Benjamini & Hochberg method)

Figure 3: Hierarchic cluster analysis of stage I follicular lymphoma.

features of stage I (n=81) included in unsupervised hierarchical clustering are somatic mutations present in more than 5% of the cases, *BCL2* and *BCL6* translocations, and focal and chromosomal arm level aberrations present in more than 5% of the samples with Spearman correlation. Each column represents one patient, cluster 1 (CL1) (green, n=44), cluster 2 (CL2) (yellow, n=15) and cluster 3 (CL3) (orange, n=22). Mutations (green), translocations (turquoise) and copy number aberrations (gains red, losses light blue and multiple losses dark blue) are ordered in the rows.

Figure 1

Figure 1: Outline of follicular lymphoma (FL) cases included in the study.

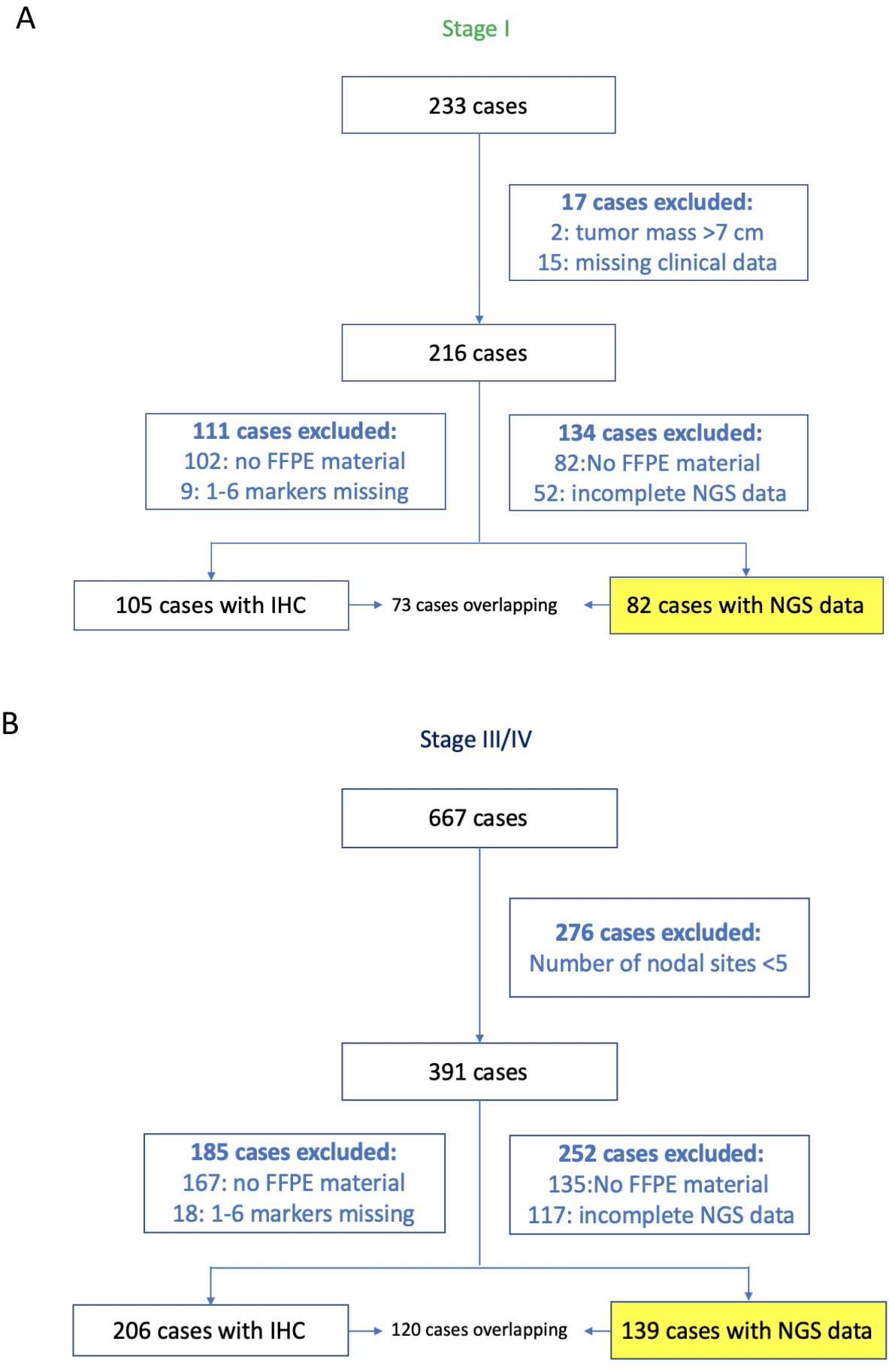
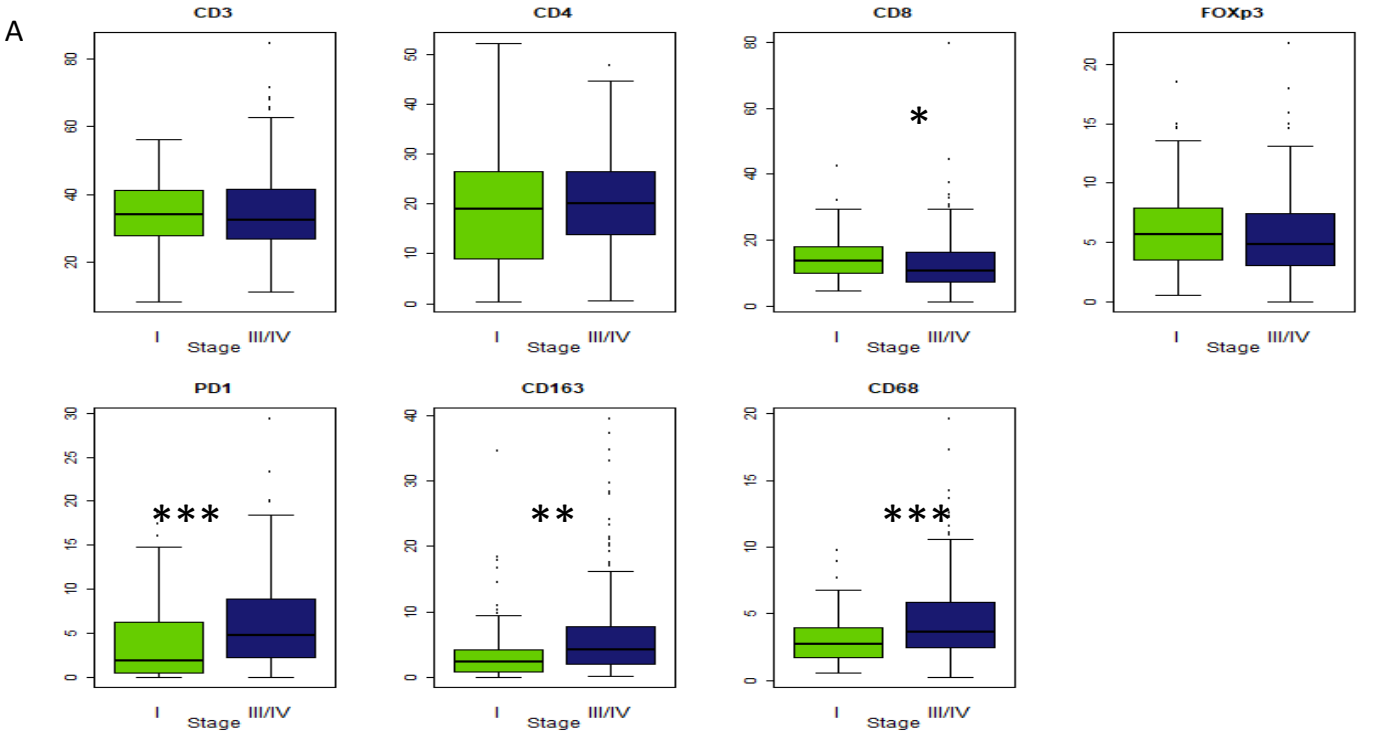
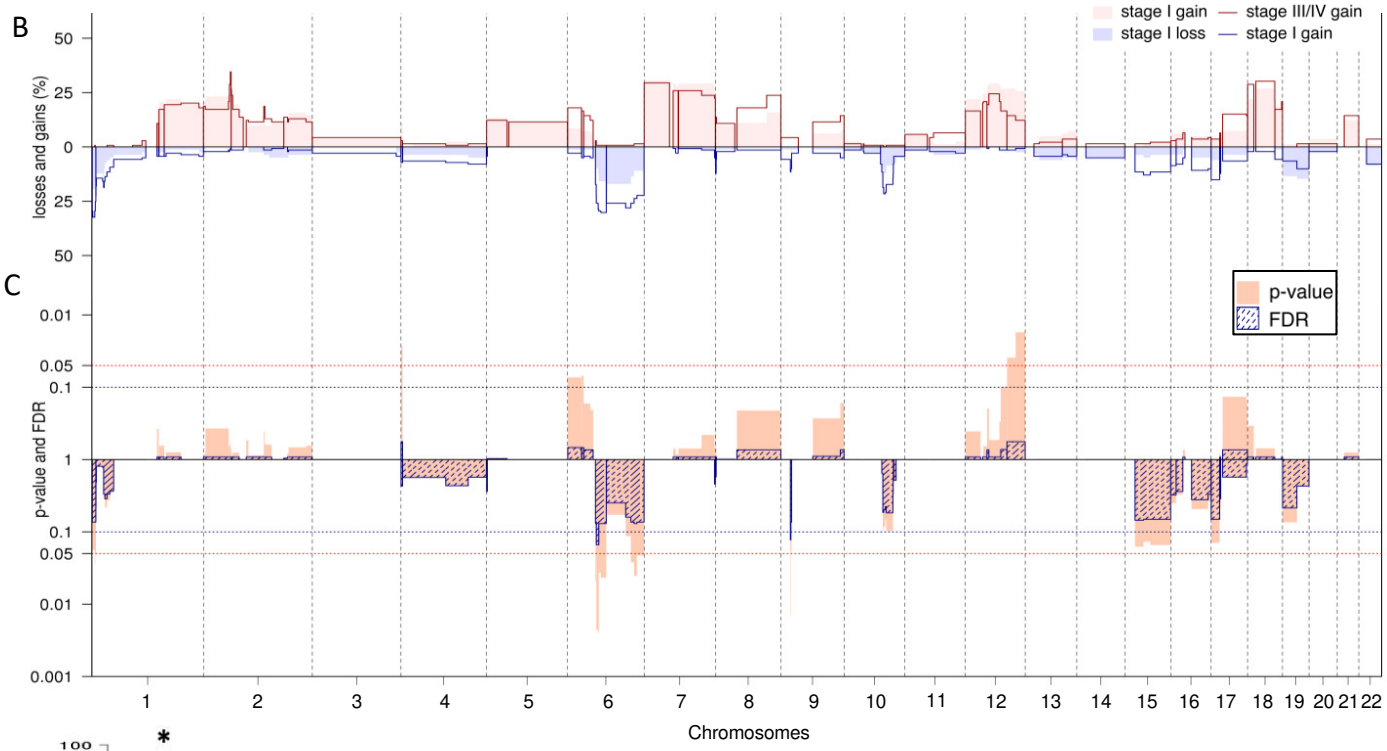


Figure 2

Figure 2. A; Microenvironment, B; mutations and translocations, C; copy number landscape, D; frequency plot of the copy number landscape of stage I versus stage III/IV follicular lymphoma.





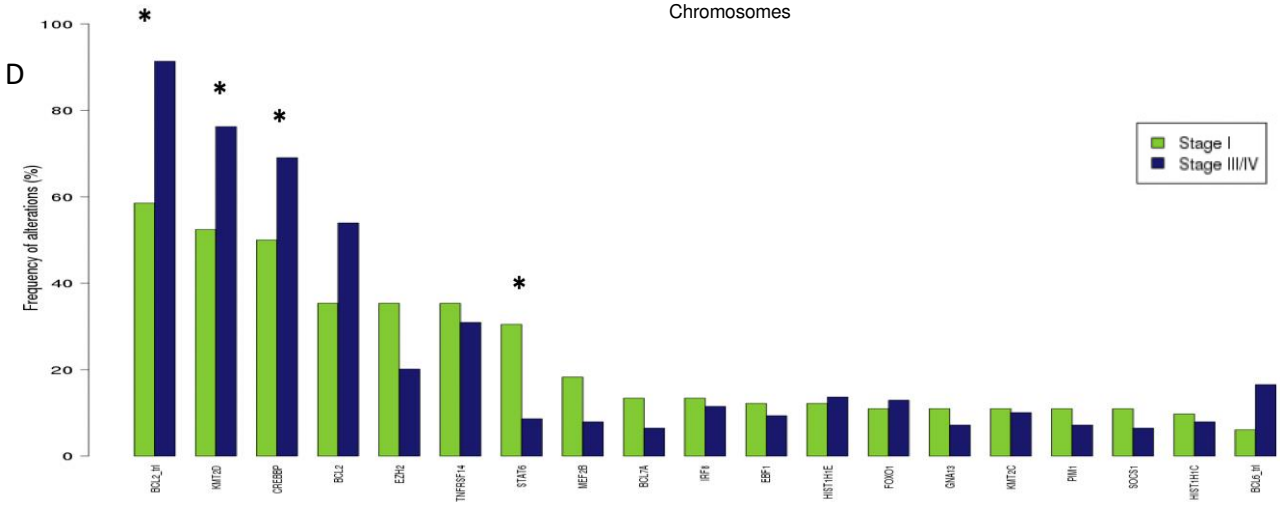


Figure 3

Figure 3. Hierarchic cluster analysis of stage I follicular lymphoma