Comprehensive Analysis of Copy Number and Allele Status Identifies Multiple Chromosome Defects Underlying Follicular Lymphoma Pathogenesis

Charles W. Ross,² Peter D. Ouillette,¹ Chris M. Saddler,¹ Kerby A. Shedden,³ and Sami N. Malek¹

Abstract

Purpose: Follicular lymphoma (FL) constitutes the second most common non-Hodgkin’s lymphoma in the Western world. The clinical course is variable and only in part explained by known tumor-intrinsic or -extrinsic factors. FL carries the hallmark chromosomal translocation t(14;18), deregulating the expression of Bcl-2, but this is not sufficient to explain either FL biology or clinical behavior.

Experimental Design: We have employed high-density genomic profiling technology using the Affymetrix 50K-XbaI oligonucleotide single nucleotide polymorphism chip platform to interrogate the genomes of 58 fluorescence-activated cell – sorted (FACS) FL specimens for chromosomal copy number changes and 46 specimens for loss of heterozygosity (LOH).

Results: We report (a) previously unknown high-frequency copy-neutral LOH (uniparental disomy) in FL on chromosomes 1p (~50%) and 6p (~30%); (b) that del6q is complex, as reported, with at least two regions of minimal common loss at 6q13-15 and 6q23-24, and that in addition, ~8% of FL specimens contain a homozygous deletion at 6q23.3-24.1 that spans the negative NFκB regulator A20 and the p53 apoptosis effector PERP; (c) that combined analysis of chromosome 17p for LOH, copy number, and p53 mutations shows that most p53 mutations in FL do not involve del17p. Finally, we map high-frequency LOH with and without copy loss on chromosomes 9p, 10q, and 16p and genomic gains on 2p15-16 and 8q24.22-24.3.

Conclusions: This comprehensive description of the pathologic anatomy of the FL genome uncovers novel genetic lesions and should aid with identification of genes relevant to FL biology and clinical behavior.

Follicular lymphoma (FL) is the most common indolent B cell lymphoma and remains incurable with current therapies. FL has a varied clinical course. Genetically, FL is characterized by the hallmark balanced chromosomal translocation t(14;18), leading to deregulated expression of Bcl-2 (1, 2). Translocation t(14;18) is found in about 85% of FL cases, thus predicting the existence of additional tumor-intrinsic and -extrinsic factors that are important in FL biology and clinical behavior (3).

The importance of tumor-extrinsic factors (infiltrating immune cells) on FL biology and outcome was recently shown using array-based expression analysis and tissue array–based immunohistochemistry (infiltrating macrophage content; refs. 4–6).

Conclusions: This comprehensive description of the pathologic anatomy of the FL genome uncovers novel genetic lesions and should aid with identification of genes relevant to FL biology and clinical behavior.
accumulated, but a genome-wide, high-resolution, comprehensive view of all copy number changes or loss of heterozygosity (LOH) is emerging for just a few (17–20).

Given the recent development of high-density technologies to measure genome-wide changes in gene or genome copy number or allele identities in DNA extracted from tumor cells, we reasoned that it would prove promising to interrogate the FL genomes of a large number of samples using single nucleotide polymorphism (SNP) chip technology (21). SNP chip technology has the advantage of allowing for simultaneous LOH detection and copy number estimation, permitting the detection of copy-neutral LOH (22, 23).

In this study, we describe an unbiased, high-density, whole-genome view of LOH and copy number changes in FL. These data may serve as a reference for the detection of FL-associated gene mutations and development of genomics-based clinical assays in the future. Using highly purified FL preparations and paired T cell–derived DNA, we were able to detect novel FL-associated genetic changes, including formerly unknown high-frequency copy-neutral LOH at 1p and 6p, and have established an overview of the pathologic FL genome.

Materials and Methods

Source material and Internal Review Board—Medicine approval. Source material for this study was cryopreserved cell suspensions of variable viability representing low grade FL from 58 adult patients. The 58 samples had been collected between 1990 and 2005 and processed by the clinical flow cytometry laboratory at the University of Michigan. Patient specimen sources included 52 lymph node biopsies, 4 bone marrow aspirates, and 2 peripheral blood samples. Patients ranged in age from 35 to 79 years (median age 55 years), with a male-to-female ratio of 1:1.

Subclassification of the FL by the WHO criteria was verified by a hematopathologist (C.W.R.) using histopathologic, cytologic, and immunophenotypic features (24, 25). Thirty-two of the specimens were classified as FL grade 1, and the remainder were grade 2. The immunophenotypic features (24, 25). Thirty-two of the specimens were classified as FL grade 1, and the remainder were grade 2. The research protocol was approved by the University of Michigan Medical Center Institutional Review Board (2005-0338).

Flow cytometry. Aliquots of cryopreserved, postdiagnostic FL cells were washed and recovered by centrifugation and stained with phycoerythrin-conjugated anti-CD20, FITC-conjugated anti-CD3, and APC-conjugated anti-CD45 (BioSource). Propidium iodide was added after the final washing to a concentration of 1 μg/mL to discriminate dead cells, and viable CD45+ CD20+ CD3+ and CD45+ CD20− CD3+ cells were sorted on a high-speed FACS Aria (Becton Dickinson) flow cytometer.

Preparation of sample DNA and hybridization to Affymetrix 50k-XbaI-mapping arrays. Sorted CD20+ or CD3+ cells were digested overnight in 100 mmol/L Tris (pH 8.0), 50 mmol/L EDTA, 50 mmol/L NaCl, 0.5% SDS, and 100 μg/mL of proteinase K (Invitrogen) at 56°C. DNA was extracted using phenol-chloroform and precipitated using ammonium acetate, ethanol, and glycogen. About 250 μg of DNA was digested for 10 h at 37°C, and the samples were subsequently processed according to the manufacturer’s (Affymetrix) instructions. Hybridization of labeled DNA fragments to the 50k-XbaI-mapping arrays was done in an Affymetrix Hybridization Oven 640 for 16 h at 48°C with rotation at 60 rpm. Washing and staining was done on an Affymetrix Fluidics Station 450. Scanning was done using an Affymetrix Scanner 3000 7G with autoloader. Data files were archived and transferred using CAB (windows cabinet) files.

Selected technical characteristics of the 50k-XbaI SNP chips used in this study are a total combined number of ~58,000 SNPs, a median intermarker distance of about 16 kb, a mean intermarker distance of about 47 kb, and an average heterozygosity of 0.29; therefore, on average, about 16,800 SNPs per individual FL genome will be informative for LOH analysis, resulting in an effective median and mean inter-SNP distance for LOH searches of about 50 and 140 kb, respectively.

Genomic DNA sequencing. Primers to amplify exons and adjacent intronic sequences of human p53, IRF4, Blimp1, ID4, and p73 and primers to amplify selected genomic regions containing informative SNPs were designed using the primer 3 program.† Primer sequences can be found in Supplementary Table S3. PCR products were generated using DNA templates from Qiagen Repli-g kit-amplified, fluorescence-activated cell–sorted (FACS) CD20+ or CD3+ cells. Amplifications were done using Taq polymerase. PCR amplicons were prepared for direct sequencing with internal nested sequencing primers using the exonuclease/shrimp alkaline phosphatase method (US3).

Data analysis. SNP chip data outputs from the Affymetrix Chromosome Copy Number Tool software were used to perform LOH analysis. dChipSNP, a freely available software package (26), was used to visualize copy number changes by analyzing Affymetrix CEL files and GDAS software output data. LOH display was done using a software program developed for these studies in our laboratory (LOH tool). The LOH tool is a Java-based genome browser that displays LOH for entire chromosomes across multiple patient samples simultaneously. An executive copy of this software and instructions for use are available on disc by obtaining an academic license from the University of Michigan. In total, 58 patient samples (out of a total of 78 screened samples) could be analyzed for copy changes (with sufficient CD20+ cell-derived DNA available) and 46 for LOH (with sufficient paired CD3+ T cell DNA available) for this study. The remaining samples did not yield sufficient DNA from either CD20+ cells, CD3+ cells, or both for this analysis.

Mutation Surveyor (SoftGenetics LLC) software was used to compare experimental DNA sequences from FL samples against GenBank-derived cDNA sequences or genomic sequences, complemented by visual inspection of sequence chromatograms.

Quantitative real-time PCR analysis of genomic copy number changes. Primers and TaqMan-based probes for Q-PCR applications were purchased from Applied Biosystems (Primers-on-demand). Primer/probe mixtures included Hs00751717_s1 (PERP, chr.6q), Hs00250581_s1 (BCL11A, chr.2p), Hs01851142_s1 (RAG2, chr.11), Hs01112790_s1 (NHLRC1, chr.6p), Hs01937396_s1 (ZCCHC5, chr. X), Hs00369747_s1 (ACTRT2, chr.1p), Hs00272432_s1 (RSC1A1, chr.1p), and Hs002-52427_s1 (C8orf17, chr.8q). Triplicate amplification reactions included primers/probes, TaqMan 2X Universal PCR Master Mix, No AmpEraseUNG, and 10 ng of unamplified genomic template DNA extracted from CD20+–sorted cells in a 20-μL reaction volume. Reactions were done on an ABI 7900HT machine. The ability of the assay to discriminate one from two genomic allele copies was confirmed using the X chromosome probe set in most reactions and comparing Ct values with the gender of individual cases. Standardization of relative copy number estimates for genomic regions of interest was done using the ΔCt method with the Ct values for the RAG2 locus as reference.

Results

The 50K-SNP chip assay can detect single copy gains or losses. We initially tested the ability of the 50K-SNP chip assay to detect single copy gains and losses in a cohort of CLL patients for which clinical information was available. Using data for the X chromosome for 76 CLL patients, we showed 100% sensitivity and specificity to identify males and females (one versus two copies of the X chromosome; data not shown).

Next, we proceeded to analyze a collection of FACS-sorted FL specimens. We reasoned that FACS sorting of cells would result

† http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
in very highly pure FL cells and would allow for the detection of homozygous losses and single copy gains. In Fig. 1A, we have displayed data using the X chromosome analysis (LOH analysis, left; copy number analysis, right). Female patients (asterisks) were identified as 2N, and female patients with a loss of one X chromosome, as detected by LOH (black double arrows), were identified as 1N. Only 4:56 (7%, red single arrows) of informative samples were not, demonstrating the expected X chromosome copy number, but instead showed 2N and 3N estimates. Analysis of X chromosome copy number estimates for the paired normal T cell DNA in three of the four cases gave expected results (no. 41 lacked T cell data), supporting true copy gains in the tumor cells as described (11).

We next proceeded to test the assay’s ability to detect amplifications using data from the analysis of the T cell receptor \( g \) variable region (\( TRGV \)) on chromosome 7 for all samples (Fig. 1B). SNP rs10487741 is embedded in the \( TRGV \) region and is expected to be deleted in T cells as part of T cell antigen receptor rearrangements that occur during T cell ontogeny. In Fig. 1B, we show copy number estimates at \( TRGV \) and a schema depicting the physical boundaries of the \( TRGV \) locus relative to the position of the informative SNPs of interest. As predicted, copy number estimates for SNP rs10487741 uniformly showed relative gains in CD20\( ^+ \) FL cells (strong red signal) as compared with the \( \alpha/\beta \) TCR-positive and TRGV-rearranged CD3\( ^+ \) T cells that were used as the copy number comparator for this analysis.

As a final semiquantitative assessment to show the ability of the assay to detect mono- and biallelic deletions, we analyzed the \( E \) light-chain locus on chromosome 22. In Fig. 1C, we have displayed a screenshot of copy number estimates for all samples for the IgV-\( E \) locus and flanking SNP positions. Above the screenshot is a schema depicting the physical boundaries of this locus relative to the copy number estimates. As expected, multiple samples (black arrows) showed visible deletions (blue) of various lengths at this locus, presumably reflecting the mono- or biallelic genomic losses following IgV-\( \lambda \) rearrangements.

In summary, these data in aggregate and Q-PCR data presented in Supplementary Table S2 provide confidence that single-copy losses or gains can be detected at high apparent sensitivities and specificities using the assay conditions employed in this study.
Detection of high-frequency copy-neutral LOH at chromosome 1p in FL. Having confirmed that we could reliably detect copy number changes and LOH in these samples, we proceeded to scan the FL genome for novel changes.

Through comparison of LOH and copy number estimates, we detected a very high frequency of copy-neutral LOH on chromosome 1p (see Fig. 2, red arrows). Additionally, LOH with copy loss was detected at 1p (see Fig. 2, black arrows). Combined, the frequency of these lesions was 23:46 (50%), the second most frequent genetic lesion ever described in FL. Confirmatory allele copy number measurements by Q-PCR on randomly selected cases using primers/probes embedded in 1p (within ACTRT2), within RAG2 (a reference gene present in two copies in all FL genomes) and on chromosome X (see Materials and Methods and Supplementary Table S2), combined with sequence analysis of informative SNPs in tumor and paired normal DNA (PCR-based LOH analysis, Supplementary Table S1), confirmed uniparental disomy in these cases.

Attempting further subclassification using anatomic criteria, we could identify a small telomeric lesion of about 8.5 Mb in length that was part of all lesions (1p type I) and a second type of lesion of about 25 Mb in length (1p type II). Both lesions are fully contained in cytoband 1p36, deletion of which confers poor outcome in patients with neuroblastoma (27).

Chromosome 1p lesions of types I and II harbor the p73 locus, and we proceeded to sequence all coding exons of p73 in 16 FLs. We did not detect mutations, and due to the lack of fresh FL tissue, we were not able to measure the influence of the LOH/copy loss at p73 on p73 expression.

Finally, 1p LOH was the only genetic lesion significantly overrepresented in FL grade 2, as compared with grade 1 lesions (log odds ratio, 1.37; 95% confidence interval, 0.28-2.46) and co-occurred with copy-neutral LOH at 6p (log odds ratio, 1.9; 95% confidence interval, 0.6-3.1).

Detection of novel high-frequency copy-neutral LOH at chromosome 6p in FL. Focusing our attention on chromosome 6p, we detected novel high-frequency LOH in 14:46 (30%) of cases at 6p that was copy neutral in all but two cases (Fig. 3A). Combined Q-PCR analyses and genomic SNP sequencing confirmed these findings (Supplementary Tables S1 and S2).

Scanning of genes located in these areas with relevance to B cell or cancer physiology identified IRF4 and ID4 as potential candidates for this abnormality, but sequence analysis of all coding exons failed to identify mutations in these genes.

High-density mapping of del(6q) and identification of A20 and PERP as possible del(6q) candidate genes. Loss of material from chromosome 6q has been described in non-Hodgkin lymphoma (NHL), and a number of nonoverlapping minimally deleted regions have been proposed by various investigators over the years (28–30). Various del6q have been associated with negative prognosis in NHL and FL; nothing is known about specific gene aberrations on 6q in FL (15, 31).

We analyzed chromosome 6q by LOH and copy number analysis in all FL samples and detected a total of 22:58 (38%) positive samples (see Fig. 3A and B).

We readily detected at least two nonoverlapping regions of loss at frequencies of 16:58 (28%) at 6q13-15 (MDR1: physical boundaries, 73.69-88.07 Mb) and 13:58 (22%) at 6q23.2-24.2 (MDR2: physical boundaries, 132.45-138.31 Mb). Additional large, recurrent, contiguous regions of loss both between MDR1 and MDR2 and telomeric to MDR2 could be identified, supporting prior reports that multiple genes of importance may reside on 6q.
Scanning the regions of loss for copy estimates indicative of biallelic deletions revealed five cases with presumed homozygous deletion at 6q23.3-24.1 within MDR2 (Fig. 3C). Mean copy number estimates for these cases were 0.34 ± 0.2 (mean ± SD). In comparison, average copy number estimates for the entire FL cohort for all of chromosomes 1, 2, 4, 15, 19, and 20 were 2.01 ± 0.19, respectively. Delineation of a minimal homozygously deleted region uncovered two genes, A20, a well-studied NFκB regulator (32), and PERP, a p53 apoptosis effector (33). Confirmatory copy number Q-PCR analysis using primers/probe with PERP confirmed progressively fewer copy number estimates in reference cases, candidate monoallelic deleted cases, and candidate homozygously deleted cases (Supplementary Table S2).

Sequencing of all coding exons for both genes, A20 and PERP, in ~50 FL cases failed to uncover mutations, but expression studies were hampered by the lack of access to fresh FL tissue.

Blimp1, found mutated or underexpressed in subsets of diffuse large B cell lymphoma (34, 35) and located outside of MDR1 or MDR2 in FL, was not found to be mutated in our FL cohort.

Detection of genetic changes in FL on chromosomes 16p, 9p, and 10q. Completing the genome-wide survey for LOH and copy number changes, we identified three additional regions of interest: chromosome 16p LOH (Supplementary Fig. S1) in 12:46 (26%) of cases (mostly copy neutral), chromosome 9p LOH (Supplementary Fig. S2) in 9:46 (20%) of cases (copy neutral or with copy loss) spanning the CDKN2A and CDKN2B gene loci, and chromosome 10q LOH and copy loss (Supplementary Fig. S2), in which the PTEN locus was included in 7:46 (15%) of cases.

FL-associated 10q lesions co-occurred with 6q-MDR2 lesions (log odds ratio, 2.4, 95% confidence interval, 0.7-4.1).
Most p53 mutations in FL are not associated with structural changes at 17p. CLL patients with del17p by fluorescence in situ hybridization have a very poor prognosis, with an average survival of about 2.5 years (9). Given the importance of this cytogenetic abnormality in CLL, we proceeded to analyze the 17p regions in our FL cohort.

In Fig. 4A, we have displayed LOH calls on 17p. Three patients showed LOH of about 7 to 18 Mb length at this chromosomal position, a frequency of 3:58, or about 5%. Unlike the situation in CLL, where del17p invariably deletes one p53 allele, one of the three FL cases (case 65) did not involve the p53 locus. In Fig. 4B, we display matching copy number data and, in Fig. 4C, a p53 mutation analysis.

It is commonly assumed that the del17p indicates that p53 is mutated on the retained allele. We decided to test this relationship directly and found 6:58 (10%) patients with mutations in p53 (exons 5-9), including 4 patients with missense mutations and 2 patients with frameshifts.

Interestingly, del17p did not predict for mutated p53, as only 1:6 cases (FL 67) had both a del17p involving the p53 gene and a p53 mutation on the retained allele. Most p53 mutations occurred without notable chromosomal alterations at 17p.

Finally, p53 mutations co-occurred with 6q-MDR2 lesions, 8q24 gains, and 16p LOH (log odds ratios, 2.3, 2.3, and 2.0; 95% confidence intervals, 0.5-4.2, 0.5-4.2 and 0.2-3.8, respectively).

The non-Hodgkin's lymphoma-associated amplification on chromosome 2p15-16 invariably includes BCL11A and REL. Hodgkin's lymphoma and NHL, including FL, are known to harbor a genomic amplification on chromosome 2p15-16 in 10% to 40% of samples (36, 37). In Fig. 5A and B, we display copy number estimates for chromosome 2p15-16 for all 58 samples. As can be seen, 9:58 (16%) of samples showed an amplification of varying length, and a minimally amplified region could be derived spanning ~600 kb (physical position, 60.5-61.1 Mb on chr.2). These copy gains were confirmed by Q-PCR with primers/probe for BCL11A (Supplementary Table S2). Interestingly, even this relatively small consensus region contained both BCL11A and REL, providing support to the hypothesis that both genes may play a pathogenetic role in FL (38).

Delineation of a minimal region of amplification on chromosome 8q. A genome-wide review of copy number data showed frequent low-level amplifications of all or parts of chromosome 8 in 10:58 (17%) of the specimens (see Fig. 6). Further...
analysis narrowed a region of minimal amplification to 8q24.22-8q24.23 (physical boundaries 134.43-144 Mb), thereby excluding the Myc locus, which has previously been suspected as being the primary target of 8q amplifications. These copy gains were confirmed by Q-PCR with primers/probe for C8orf17 (Supplementary Table S2). Scanning of 8q24.22-8q24.23 identified a minimum of four genes that have previously been implicated in tumor biology, including the potassium channel KCNK9 (39), the NFκB-activating protein NIBP (40), the protein tyrosine kinase PTK2/FAK (41), and the protein tyrosine phosphatase PTP4A3 (42).

Amplifications of der(18)t(14;18) do not involve Bcl-2. The genetic hallmark lesion of FL is the translocation t(14;18), juxtaposing the Bcl-2 gene and the immunoglobulin heavy-chain locus enhancer elements. Fine mapping of the breakpoints showed that the part of chromosome 18 that did not contain Bcl-2 was present in three copies as der(18)t(14;18) (11), as previously reported (43), whereas the remainder of this chromosome was disomic (Supplementary Fig. S3A and B). Trisomy 18 (black arrows) was detected as well.

This analysis suggests (a) that chromosome 18 may harbor an additional, amplified gene with relevance in FL biology located on der(18)t(14;18) other than Bcl-2; and (b) that a significant fraction of additional FL cases harbor trisomy 18, suggesting a mechanism to explain increased Bcl-2 expression independent of t(14;18) (43).

Discussion

In this study we are presenting high-density genomic profiling data, including LOH and copy number changes, for a series of FL specimens. These data have implications for basic as well as clinical research in FL; they are novel in nature, and have not been reported for this important malignancy.

We have described novel, high-frequency regions of LOH and copy loss as well as copy-neutral LOH (uniparental disomy) on multiple chromosomes in the FL genome. Copy-neutral LOH is not detectable using either conventional cytogenetics or array-CGH and, therefore, has not been previously described in FL.

The occurrence of alterations at 1p36 in about half of all FL may be of clinical significance because a similar region has been

Fig. 5. The lymphoma-associated amplification on chromosome 2p15-16 invariably includes BCL11A and REL. A, genomic copy number display for parts of chromosome 2. The physical positions of BCL11A and REL are indicated. Arrows, samples with amplifications. The estimated copy numbers for all SNP positions for FL 53 are displayed below the chromosome 2 display; red line, 2N state. B, genomic copy number display for a subregion of chromosome 2p15-16, with a schema of the BCL11A and REL loci in relation to individual SNP positions.
shown to confer poor prognosis for children with neuroblastoma (27). It certainly makes analysis of the impact of this lesion on FL outcome a research priority.

Chromosome 6p LOH spans the IRF4 locus. This may be significant to FL pathogenesis because others have recently shown that the transcriptional regulator IRF4 is necessary for B cell germinal center exit (44).

Deletion 6q has been reported to confer negative prognostic impact on patients with NHL, but a critical gene has not been identified in any of the studied regions.

Analysis of a novel homozygously deleted region at 6q23.3-24.1 identifies A20 and PERP as two novel genes of potential interest in FL biology.

A20 is a negative regulator of NFκB and Toll-like receptor signaling (32). Studies in multiple cellular and murine systems suggest that reduction in A20 function results in sustained NFκB signaling, a molecular feature of many NHL subtypes (45). A20 knock-out animals develop a lethal inflammatory syndrome and die shortly after birth, precluding possible observation of tumor proneness (46).

PERP, a gene induced by p53, executes part of the p53 apoptosis program. Radiation-induced apoptosis in thymocytes or neuronal cells is impaired in PERP-/- mice (47). Down-regulation of PERP has been implicated in the poor outcome of patients with various cancers (48, 49). Both genes are attractive targets for future studies on well-preserved FL tissue.

The data presented on the well-known amplification on chromosome 2p15-16 show that BCL11A and REL are always coamplified. BCL11A is a Krueppel zinc finger transcription factor essential for pre-B cell development, with restricted expression in tissues; it should be investigated further for the role it assumes in malignant B cell physiology (9, 50).

The analysis of a minimally amplified region on chromosome 8q24 excludes Myc as the critically relevant gene and suggests a short list of genes that all have proven roles in cancer cell properties or cell proliferation.

Combined analysis of chromosome arm 17p for LOH, copy loss, and p53 mutations shows that most p53 mutations in FL are not associated with chromosomal alterations at 17p, and that the development of clinically useful tests based on genomic profiling should include p53 sequence analysis.

In summary, this large genomic profiling study extends knowledge about the FL genome and suggests basic and clinical research implications. The high-resolution mapping data highlight novel chromosomal regions of potential importance to FL biology and candidate genes worthy of further analysis. The clinical importance of these findings awaits testing of various genetic FL subgroups for prognostic power.

References


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