Leukemia Research 39 (2015) 702–708

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

IGHV gene usage and mutational status in follicular lymphoma: Correlations with prognosis and patient age

Ellen Berget[a,b,*], Anders Molven[a,b], Turid Løkeland[c], Lars Helgeland[a,b], Olav Karsten Vintermyr[a,b]

[a] The Gade Laboratory for Pathology, Department of Clinical Medicine, University of Bergen, 5021 Bergen, Norway
[b] Department of Pathology, Haukeland University Hospital, 5021 Bergen, Norway
[c] Department of Oncology, Haukeland University Hospital, 5021 Bergen, Norway

ARTICLE INFO

Article history:
Received 25 November 2014
Received in revised form 10 February 2015
Accepted 7 March 2015
Available online 19 March 2015

Keywords:
Follicular lymphoma
IGHV
Sequence analysis
Somatic hypermutations
Prognosis

ABSTRACT

Follicular lymphoma (FL) is a heterogeneous disease with some patients developing progressively or transformed disease early, whereas others follow an indolent clinical course. We evaluated the prognostic value of *immunoglobulin heavy chain variable* (*IGHV*) gene usage and mutational status in FL patients. One hundred and four *IGH* sequences were obtained in tumour samples from 99 patients. The *IGHV*3 subgroup had the highest usage frequency (57.7%) with *IGHV*3–23 being the most common sequence. Patients with the *IGHV*5 subgroup or *IGHV* sequences from more than one subgroup had significantly less favourable prognosis with an estimated 5-year survival of 62.5 and 50.0%, respectively, as compared with a 5-year survival of 95.1% for patients with other *IGHV* subgroups ($P = 0.013$ and $P < 0.001$, log–rank). The poor survival associated with *IGHV*5 or >1 *IGHV* subgroup usage was an independent prognostic factor in Cox multivariate analysis ($P = 0.005$). *IGHV* genes were unmutated showing >98% homology in 15.2% of cases. Contrasting the situation in chronic lymphocytic leukaemia (CLL), the presence of unmutated sequences did not yield prognostic information, although unmutated sequences were associated with age at diagnosis >60 years ($P = 0.022$, Fisher's exact). In conclusion, our results indicate that analysis of *IGHV* gene usage might aid in predicting prognosis for FL patients.

1. Introduction

Follicular lymphoma (FL) is the second most common B cell lymphoma after diffuse large B cell lymphoma (DLBCL) in Western countries [1]. FL is clinically characterised by a median survival of more than 10 years and a continuous pattern of relapse that often is associated with progression to treatment-resistant disease and sometimes is associated with histological transformation into DLBCL [2,3]. The translocation *t*(14;18)(q32;q21) deregulating *BCL2* expression is considered the primary oncogenic event, but is also considered insufficient for FL development due to the frequent occurrence of this translocation in B cells of healthy human individuals [4–6].

It has long been assumed that FL is derived from the malignant transformation of germinal centre B cells. Morphologically, FLs are composed of tumour cells that appear in a predominantly follicular pattern with cytological and architectural features of normal germinal centres, and that usually express germinal centre B cells' markers such as BCL6 and CD10 [7]. The germinal centre B cell origin has been supported by several previous studies of the *immunoglobulin heavy variable* (*IGHV*) and *light chain* genes demonstrating heavily mutated genes [8–12]. In comparison to normal germinal centre B cells, FLs contain significantly more mutations in the *IGHV* genes, an observation compatible with prolonged expansion in a germinal centre-like environment [12,13].

The analysis of *IGHV* genes of other B cell lymphomas, including chronic lymphocytic leukaemia (CLL), splenic marginal zone lymphoma (SMZL) and mantle cell lymphoma (MCL) has revealed an unexpected heterogeneity in mutational status [14–18]. This heterogeneity has also been related to prognosis particularly in CLL, in which *IGHV* sequence analysis now has become widely used for the purpose of prognostication [19,20]. Furthermore, the increasing use *of IGHV* sequence analysis in CLL patients has

* Corresponding author at: Department of Pathology, Haukeland University Hospital, 5021 Bergen, Norway. Tel.: +47 55 97 31 64; fax: +47 55 97 31 58.
E-mail addresses: ellen.berget@gades.uib.no (E. Berget), anders.molven@gades.uib.no (A. Molven), turid.lokeland@helse-bergen.no (T. Løkeland), lars.helgeland@helse-bergen.no (L. Helgeland), olav.vintermyr@helse-bergen.no (O.K. Vintermyr).

http://dx.doi.org/10.1016/j.leukres.2015.03.003

allowed the recognition of specific *IGHV* genes that are associated with unfavourable prognosis, such as the *IGHV3–21* gene [21–24].

Although most studies have shown that FL is characterised by mutated *IGHV* genes [8–12], a recent investigation has revealed that unmutated genes could also be present in FL [25]. Moreover, there have been only occasional reports on the distribution of *IGHV* subtypes in FL [8,10,12,26], and to our knowledge there has been no study attempting to correlate *IGHV* sequence analysis with clinical outcome. In the present study, the aim was, therefore, to analyse the use of *IGHV* genes and mutational status in relation to clinicopathological characteristics and prognosis in FL.

2. Materials and methods

2.1. Patients

The 106 patients included in the study had FL grade 1–3B at time of diagnosis, and all had a bone marrow biopsy evaluated for lymphoma involvement at the Department of Pathology, Haukeland University Hospital, Bergen, Norway in the period between February 2003 and July 2011. Patients with transformed FL at diagnosis were excluded. Bone marrow status for 96 cases was reported previously [27]. Diagnoses according to the WHO classification were based on morphological and immunohistochemical assessment of formalin-fixed, paraffin-embedded (FFPE) tumour biopsies from lymph nodes and extranodal sites [7]. Medical records were reviewed to determine age, sex, clinical stage, follicular lymphoma international prognostic index (FLIPI) score, management and disease course. FLIPI score was also determined for the patients with 3B FL, although clinically regarded as equivalent to the diagnosis of DLBCL. The study was approved by the Regional Committee for Ethics in Research (2013/211) and performed in accordance with the Declaration of Helsinki.

2.2. PCR amplification and sequencing

DNA was prepared from FFPE tumour material. In most cases, two 10 μm sections were cut from the paraffin blocks and deparaffinised with Deparaffinisation Solution (Qiagen, Hilden, Germany). In one case, tumour material was successfully dissected from a haematoxylin and eosin stained slide before deparaffinisation. Automated DNA isolation was performed on a QIAsymphony SP pipetting robot (Qiagen) in combination with the QIAsymphony DSP DNA Mini Kit (Qiagen) according to the manufacturer's instructions.

PCR was performed in six separate reactions with the *IGH* framework region 1 (FR1) primers combined with the JH consensus primer as designed for the BIOMED-2 protocol [28]. The BIOMED-2 procedure was adapted for FFPE tissue by using the QIAGEN Multiplex PCR Kit (Qiagen) with HotStarTaq DNA Polymerase as previously described [29]. All reactions were carried out in a total reaction volume of 25 μl containing 2.5 μl of DNA template, 2.5 μl of primer mix (2 μM of each primer), 12.5 μl of the Multiplex buffer and 7.5 μl ddH_2O. PCR conditions included an initial activation step (95 °C, 15 min), 38 cycles consisting of denaturation (95 °C, 45 s), annealing of PCR primers (60 °C, 90 s) and extension (72 °C, 90 s), and a final extension step of 72 °C for 10 min. PCR products were analysed on a 3% agarose gel, visualised with ethidium bromide staining and PCR reactions that showed bands of appropriate size were identified. If several appropriate bands were present in a case, each of the positive PCR reactions was selected for sequencing. For each case, a multiplexed PCR reaction consisting of the six *IGH* FR1 primers and the JH consensus primer was also performed. The multiplexed PCR reaction served as an internal control for the presence of clonal rearrangements.

In preparation for sequencing, the Illustra™ ExoProStar™ 1-Step Kit (GE Healthcare, Little Chalfont Buckinghamshire, UK) was used in order to remove unincorporated primers and nucleotides from the amplification reactions. Direct sequencing was performed in both directions using the same primers as in the PCR amplification and the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). Sequencing reactions were purified by the BigDye® XTerminator™ Purification Kit and subsequently analysed on an ABI Prism 3100 Genetic Analyser (both Applied Biosystems).

Sequence data analysis was done using CLC Main Workbench software (Qiagen). DNA sequence reads were automatically trimmed based on quality scores (0.05 limit) and ambiguity characters (two residues). Manual trimming was performed in a minority of the cases ($n = 4$). When DNA sequence reads from both directions were obtained, they were assembled to contigs (73 sequences). Conflicts, *i.e.* disagreements about residues in a position, were solved by letting the read with best quality decide the nucleotide in the contig. Contigs and single DNA sequence reads were aligned to the closest *IGH* sequences derived from the IMGT database (http://www.imgt.org/IMGT_vquest/vquest) and the percentage of homology determined.

2.3. Statistical analysis

To compare categorical variables χ^2 or Fisher's exact tests were performed. Continuous variables not following the normal distribution were compared between two or more groups using the Mann–Whitney U or Kruskal–Wallis tests. Survival curves of time to death due to lymphoma were estimated using the product-limit procedure (Kaplan–Meier method) with date of histological diagnosis as starting point. Differences between categories were estimated by the log–rank test. Patients who died of other causes than lymphoma were treated as censored observations. Median follow-up time was estimated by the reversed Kaplan–Meier method. Univariate and multivariate analyses of survival were performed with the Cox proportional hazards method. The variables were tested by log–log plot and no non-proportionality was found. To determine their ability to be incorporated in multivariate models, the variables were tested by forward and backward stepwise selection. All results were considered significant if $P \leq 0.05$. SPSS version 21.0 (SPSS Inc, Chicago, IL, USA) was used for all statistical analyses.

3. Results

3.1. Clinical and pathological characteristics

There were 106 patients included with ages ranging from 33 to 94 years (median 61 years). Sixty (56.6%) patients were initially treated with cyclophosphamide, doxorubicin, vincristine and prednisolon (CHOP) or COP. In these patients, Rituximab was added in 55 and radiation therapy was applied as consolidation in 16. There were four patients with grade 3B FL, and all four were

Table 1
Clinicopathological variables and results from univariate survival analysis in the 106 patients.

Variables	N	%	HR	95% CI	*P* value (log–rank test)
Sex					
Female	64	60.4	1		
Male	42	39.6	1.092	0.326–3.662	0.886
Age (years)					
≤60	46	43.4	1		
>60	60	56.6	3.823	0.837–17.463	0.063
Morphology					
Grades 1/2	77	72.6	1		
Grades 3A/3B	29	27.4	0.678	0.317–1.451	0.305
Bone marrow					
No involvement	73	68.9	1		
Involvement	33	31.1	1.014	0.304–3.385	0.982
Clinical stage					
I-II	34	32.1	1		
III-IV	72	67.9	1.465	0.877–2.447	0.109
FLIPI score					
Low (0–1)	49	46.2	1		
Intermediate (2)	26	24.5	2.723	0.455–16.318	0.266
High (≥3)	31	29.3	5.492	1.139–26.476	0.016

HR, hazard ratio; CI, confidence interval; FLIPI, follicular lymphoma international prognostic index.

Table 2
Cases of follicular lymphoma with more than one productive *IGH* rearranged sequence.

Case	Closest *VDJ* gene segments			Homology with the closest *IGHV* gene (%)
	V	*D*	*J*	
3	*V*3–23	*D*5–24	*J*4	91.0
	*V*5–51	*D*3–22	*J*4	93.6
7	*V*1–46	*D*6–06	*J*6	98.8
	*V*3–48	*D*4–23	*J*3	74.2
61	*V*1–24	*D*1–26	*J*2	94.8
	*V*3–23	*D*2–02	*J*4	99.5
	*V*4–59	*D*3–03	*J*3	89.8
90	*V*3–23	*D*6–06	*J*5	86.8
	*V*5–51	*D*6–25	*J*4	100.0

among the patients treated with CHOP or R-CHOP. Thirteen (12.3%) patients received chlorambucil. One patient (0.9%) received Rituximab monotherapy. Seventeen (16.0%) patients with stage I or II and grade 1 or 2 FL, were treated with local radiation therapy. Fifteen (14.2%) patients underwent observation only. Median follow-up time was 75 months (range, 62–88). During the follow-up period, 12 patients died as a consequence of their lymphoma disease and eight died of other causes. Clinicopathological characteristics with results from univariate analysis of survival are summarised in Table 1.

3.2. IGHV, IGHD *and* IGHJ *usage*

One hundred and four productive *IGH* rearranged sequences were obtained in samples from 99 patients. Double ($n = 3$) or triple ($n = 1$) productive *IGH* rearranged sequences were obtained in four of these patients (Table 2).

Among the 25 functional *IGHV* genes used, the most frequently encountered were *IGHV*3–23 (12.5%), *IGHV*3–48 (11.5%), *IGHV*3–15 (8.7%), *IGHV*4–59 (7.7%) and *IGHV*5–51 (6.7%, Fig. 1). The use of these specific genes was not correlated with prognosis. The most frequently encountered *IGHV* genes in the cases with more than one productive sequence were *IGHV*3–23 ($n = 3$) and *IGHV*5–51 ($n = 2$, Table 2). The frequencies of *IGHV* subgroups in the 104 productive sequences had the following distribution: *IGHV*1, 19.2%; *IGHV*3, 57.7%; *IGHV*4, 11.5%; *IGHV*5, 7.7% and *IGHV*6, 3.8%. The *IGHV*2 subgroup was not observed in any of the sequences. Twenty three different functional *IGHD* genes were used. The most frequently encountered were *IGHD*3–10 (8.7%), *IGHD*3-03 (7.7%) and *IGHD*3–22 (6.7%). The most frequently expressed *IGHJ* genes were *IGHJ*4 (51.0%), *IGHJ*6 (21.2%) and *IGHJ*3 (17.3%).

No productive *IGHV-D-J* rearrangements could be obtained in the remaining seven patients. In three of these cases, we were unable to establish clonality by the BIOMED-2 framework region one, two and three primers, probably due to the technical limitations related to use of FFPE tissue. Four cases had no material (FFPE tissue or DNA) left after routine diagnostic work was completed.

3.3. IGHV *mutation status*

The percentage of homology to the closest *IGHV* gene ranged from 60.4% to 100.0%, with a mean of 89.4% for the 104 productive sequences.

The means for the *IGHV* subgroups were: *IGHV*1, 95.4%; *IGHV*3, 88.1%; *IGHV*4, 86.3%; *IGHV*5, 91.3% and *IGHV*6, 87.0% (Fig. 2). A Kruskal–Wallis test revealed a statistically significant difference in *IGHV* sequence homology levels across the different *IGHV* subgroups ($P < 0.001$). The *IGHV*1 subgroup recorded the highest median score (95.3%), while the *IGHV*4 subgroup had the lowest (86.6%). Additional testing of the median values with Mann–Whitney U test with Bonferroni adjustment showed

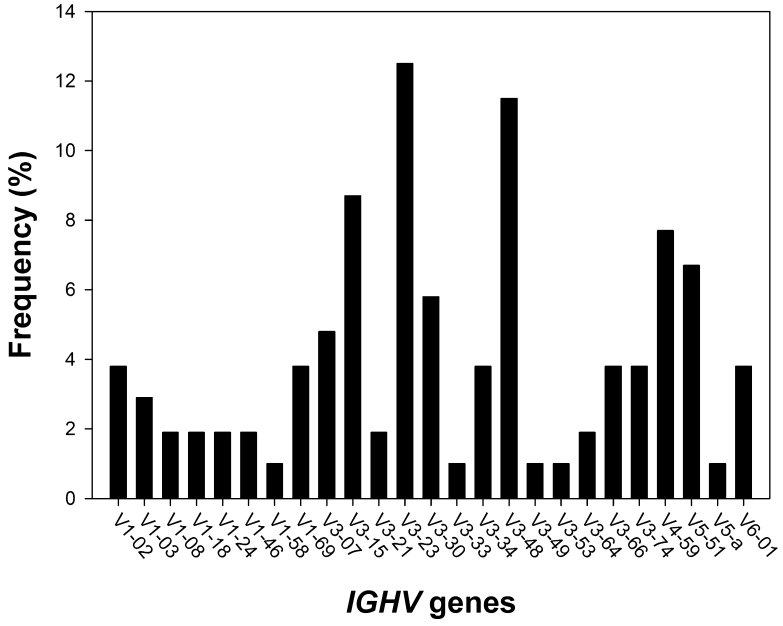

Fig. 1. Usage frequency of *IGHV* genes in follicular lymphoma. Along the X-axis are shown the 25 different *IGHV* sequences detected among 104 productive sequences obtained from 99 patients.

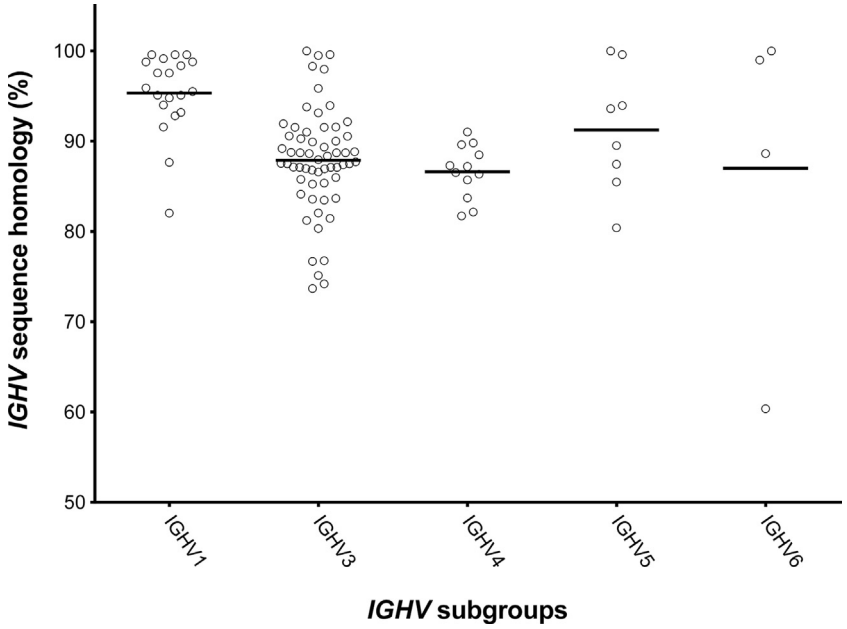

Fig. 2. Homology of *IGHV* genes in follicular lymphoma. The diagram shows percentage of homology according to *IGHV* subgroups in 104 productive sequences obtained from 99 patients. Each observation is indicated by a dot and horizontal lines represent mean percentage of homology for the *IGHV* subgroup.

significant differences between *IGHV*1 and *IGHV*3, and between *IGHV*1 and *IGHV*4 (both $P < 0.001$).

Fifteen productive sequences were unmutated, defined as >98% homology to the closest *IGHV* gene. Three of these sequences showed 100% homology. Unmutated sequences were observed in the *IGHV*1, *IGHV*3, *IGHV*5 and *IGHV*6 subgroups. *IGHV*1 subgroup exhibited the highest number of unmutated sequences. When compared with the *IGHV*3 and the *IGHV*4 subgroup, the difference was statistically significant ($P = 0.004$ and $P = 0.029$, Fisher's exact test).

3.4. *Correlation of* IGHV *subgroup usage with clinical outcome*

The estimated survival at 5 years was 62.5% (SE 21.3%) for patients displaying the *IGHV*5 subgroup and 50.0% (SE 25.0%) for patients exhibiting more than one *IGHV* subgroup, whereas the estimated survival for patients using the other *IGHV* subgroups combined was 95.1% (SE 2.4%). Patients using *IGHV*5 or more than one *IGHV* subgroup had a significantly poorer survival by log–rank test ($P = 0.013$ and $P < 0.001$, respectively) as compared to patients using other *IGHV* subgroups. When the patients using the *IGHV*5 and more than one *IGHV* subgroup were combined in a single group estimated hazard ratio was 7.6 (95% CI, 2.1–26.9, Table 3) with $P < 0.001$ by log–rank test (Fig. 3). Supplementary *t*(14;18)(q32;q21) fluorescence *in situ* hybridisation analysis (*BCL*2) and PCR (*BCL*2 MBR and MCR) was performed in 10 cases with *IGHV*5 or >1 *IGHV* subgroup expression. *BCL*2 translocation was detected in eight supporting the diagnosis of follicular lymphoma (data not shown).

Usage of *IGHV*5 was associated with age >60 years at diagnosis, whereas usage of more than one *IGHV* subgroup was not (Supplementary Table S1). Usage of *IGHV*5 or more than one *IGHV* subgroup was not associated with FL grade 3A/3B, clinical stage >III/IV or FLIPI score ≥3. Furthermore, no correlation between usage of *IGHD* or *IGHJ* genes and clinicopathological variables including patient's survival was detected.

3.5. *Correlation of* IGHV *mutation status with clinicopathological variables*

Unmutated *IGHV* sequences with >98% homology to the closest *IGHV* gene was observed more frequently in patients with age >60

Table 3
Univariate survival analysis of *IGHV* subgroup usage and mutation status according to different homology cutoff values in the 99 patients with productive rearrangements.

Variables	N	HR	95% CI	*P* value (log–rank test)
IGHV subgroup				
IGHV1-4, IGHV6	89	1		
IGHV5 or >1 *IGHV*	10	7.555	2.119–26.931	<0.001
IGHV homology				
≤93%	69	1		
>93%	30	2.589	0.827–8.108	0.090
IGHV homology				
≤96%	81	1		
>96%	18	2.198	0.661–7.307	0.187
IGHV homology				
≤98%	84	1		
>98%	15	1.824	0.493–6.743	0.360
IGHV homology				
≤99%	89	1		
>99%	10	1.617	0.353–7.396	0.532

HR, hazard ratio; CI, confidence interval.
The sequences with the highest homology score were included in patients with double or triple productive *IGH* rearranged sequences.

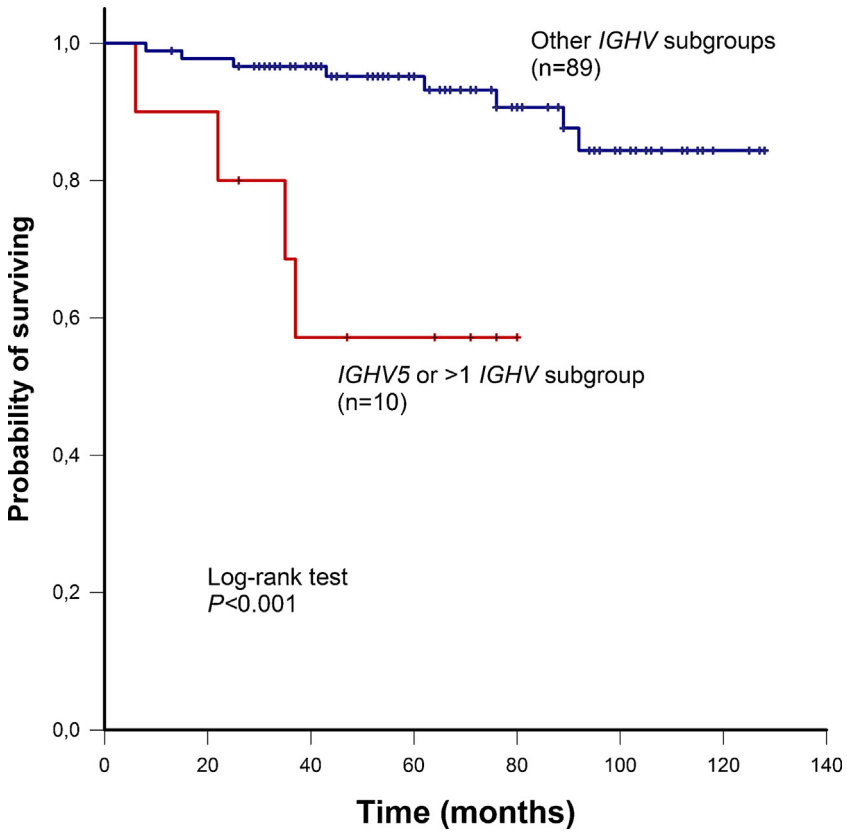

Fig. 3. Survival of follicular lymphoma patients according to *IGHV* subgroup usage. Kaplan–Meier survival curves of 99 patients are shown. The survival of patients using the *IGHV*5 subgroup ($n=6$) or more than one *IGHV* subgroup ($n=4$) were combined into a single group, and compared to the survival of patients using the *IGHV*1–4 or *IGHV*6 subgroups. Statistical difference was calculated by the log-rank test.

years (13 of 58 patients; 22.4%) than in patients aged ≤60 years (two of 41 patients; 4.9%), when sequences with the highest homology scores were included for the patients with double or triple productive rearranged *IGH* sequences. The difference was statistically significant by Fisher's exact test ($P=0.022$). Unmutated sequences were associated with >1 *IGHV* subgroup usage, but not with *IGHV*5 subgroup usage (Supplementary Table S1). There were no significant associations between unmutated *IGHV* genes and the other clinicopathological variables studied.

The estimated survival at 5 years was 80.0% (SE 10.3%) for patients carrying unmutated *IGHV* genes and 93.6% (SE 2.8%) for patients with mutated *IGHV*. The difference in survival was not significant by log–rank test. Moreover, no significant impact on survival was observed according to different homology cutoff values (Table 3). The estimated hazard ratio was 2.6 (95% CI, 0.8–8.1, $P=0.090$) when a cutoff of >93% homology was used.

3.6. Multivariate survival analysis

The combination of *IGHV*5 and >1 *IGHV* subgroup usage remained a significant factor in a multivariate analysis when the variables age >60 years, clinical stage >III/IV, FLIPI score ≥3 and *IGHV* homology >93% were included (Table 4).

Table 4
Cox multivariate analysis of prognostic factors in the 99 patients with productive *IGH* rearranged sequences.

Variables	HR	95% CI	*P* value
Age >60 years	2.830	0.614–13.033	0.182
Clinical stages III/IV	4.143	0.447–38.398	0.211
FLIPI score ≥3	1.942	0.535–7.042	0.313
*IGHV*5 or >1 *IGHV* subgroup	5.621	1.484–21.290	0.005
IGHV homology >93%	2.185	0.630–7.571	0.218

HR, hazard ratio; CI, confidence interval; FLIPI, follicular lymphoma international prognostic index.

4. Discussion

Our analysis showed that the usage of *IGHV* genes used in follicular lymphomas mostly resembled the repertoire of normal peripheral blood B cells [30,31]. Thus, the *IGHV*3–23 gene was the most frequently used *IGHV* gene. However, some discrepancies were observed. The *IGHV*3–15, *IGHV*3–48 and the *IGHV*5–51 genes, which are not among the most frequently employed *IGHV* genes in normal B cells, were frequently expressed in our patient cohort. Conversely, the *IGHV*3-30-3 and the *IGHV*4–39 genes that are common in normal B cells were not represented in our patients.

The *IGHV*3–23 was the most frequently used gene also in a previous study of 30 FL samples [12]. In that study, however, *IGHV*3–15, *IGHV*3–48 and *IGHV*5–51 were not commonly expressed, contrasting our results. In another study of 26 FLs, the *IGHV*1–18 and the *IGHV*3–48 were the most frequently encountered genes [32]. Although relative frequencies of individual *IGHV* genes diverge among series, the frequencies of *IGHV* subgroups tend to be more similar for FL [8,12,26,32,33]. In agreement with previous studies, *IGHV*3 was the most frequently used subgroup, followed by the *IGHV*1 and *IGHV*4 subgroups.

A novel finding in our study was that usage of *IGHV* subgroups was related to survival. Unfavourable prognosis was observed for two patient groups; the patients using the *IGHV*5 subgroup and

the patients using more than one *IGHV* subgroup. Whether these patient groups were compared separately or combined, survival was significantly different from that of patients using other *IGHV* subgroups. The poor survival associated with subgroup usage was also of independent prognostic importance in a multivariate model. Similarly, the *IGHV*3–21 gene has been correlated with poor clinical outcome in studies of CLL [21,22]. The *IGHV*3–21 gene was expressed in only two of our FL patients, and an association with prognosis could; therefore, not be evaluated. Other *IGHV* genes or subgroups have, to our knowledge, not been associated with adverse prognosis, neither in CLL nor in other B cell neoplasms.

The *IGHV* genes were commonly mutated in our study, as also observed in previous studies of FL [10,12,32,34]. Unexpectedly, however, unmutated *IGHV* genes were detected in 15.2% (15/99) of the patients from whom productive rearrangements were obtained. Three of these patients even had truly unmutated *IGHV* genes showing 100% homology. Unmutated *IGHV* genes have previously been observed in a minority of other germinal or post-germinal centre-associated lymphomas, such as DLBCL and Burkitt lymphoma [35–39], but to our knowledge not in FL. Due to the unexpected high frequency of unmutated *IGHV* genes in our patients, the morphology of these cases were re-evaluated. Morphological and immunophenotypic characteristics of FL were confirmed in all cases. Nine showed FL grades one and two, five grades 3A and one grade 3B.

We were unable to show any significant difference in survival for patients carrying unmutated *IGHV* genes as compared with patients carrying mutated sequences, despite the fact that unmutated *IGHV* genes were seen more frequently in patients with age >60 years. The reason for the association with age is unclear. The 98% homology cutoff value for assigning cases to the unmutated or mutated subgroup has been widely used for prognostication in CLL. Recently, the best cutoff identity of *IGHV* genes to predict survival was shown to be 97% in MCL [40]. Cutoffs of 98 or 97% may not necessarily be appropriate for FL. We were, however, not able to detect any significant differences in survival according to different homology cutoff values in our FL patients (Table 3).

Although no differences in outcome were detected, the observation of unmutated and truly unmutated *IGHV* genes indicates that a subset of FL cases could be derived from pre-germinal centre B cells. A more naïve cell of origin has previously also been suggested for the unmutated cases of CLL and MCL. [15,40] On the other hand, mutational status appeared to be related to the use of different *IGHV* subgroups with the *IGHV*1 subgroup showing highest number of unmutated sequences. Such biased *IGHV* usage could also reflect selection and activation by a restricted set of antigens, superantigens, or both [41]. Accordingly, the definition of a cell of origin for a subset of FL cases may be facing the same challenges already encountered for CLL [42].

We also observed a somewhat higher frequency of *IGHV*5 subgroup usage in our FLs (7.7%) than that reported for normal peripheral blood B cells, *i.e.* 1.4% for CD5+ and 2.9% for CD5− B cells [30]. These differences suggest an overrepresentation of *IGHV*5 genes in FL. Given the poorer survival of *IGHV*5-positive patients, it is tempting to speculate that specific antigens may be responsible for a more aggressive clinical course in this subset of FL patients.

In summary, the *IGHV*3 subgroup and in particular the *IGHV*3–23 were the most frequently used in FL. Usage of the *IGHV*5 and more than one *IGHV* subgroup were associated with shorter survival, and was an independent risk factor in multivariate analysis. Although validation in larger series is warranted, the survival data indicate that *IGHV* sequence analysis could aid in predicting prognosis of FL patients. Finally, heterogeneity in mutational status was noted as also unmutated *IGHV* genes were present. This could raise the possibility that a subset of FLs might be derived from pre-germinal centre B cells.

Conflicts of interest statement

There are no conflicts of interest to declare.

Acknowledgements

The study has been funded by a fellowship from the University of Bergen, and grants provided by the Western Norway Regional Health Authority and Dr. Med F. G. Gades Legat.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2015.03.003.

References

[1] Roman E, Smith AG. Epidemiology of lymphomas. Histopathology 2011;58(1):4–14.
[2] Solal-Celigny P, Roy P, Colombat P, et al. Follicular lymphoma international prognostic index. Blood 2004;104(5):1258–65.
[3] Federico M, Bellei M, Marcheselli L, et al. Follicular lymphoma international prognostic index 2: a new prognostic index for follicular lymphoma developed by the international follicular lymphoma prognostic factor project. J Clin Oncol 2009;27(27):4555–62.
[4] Liu YF, Hernandez AM, Shibata D, et al. BCL2 translocation frequency rises with age in humans. Proc Natl Acad Sci USA 1994;91(19):8910–4.
[5] Limpens J, Stad R, Vos C, et al. Lymphoma-associated translocation *t*(14;18) in blood B cells of normal individuals. Blood 1995;85(9):2528–36.
[6] Dolken G, Illerhaus G, Hirt C, et al. BCL-2/J(H) rearrangements in circulating B cells of healthy blood donors and patients with nonmalignant diseases. J Clin Oncol 1996;14(4):1333–44.
[7] Swerdlow SH, Campo E, Harris NL, et al., editors. WHO classification of tumours of haematopoietic and lymphoid tissues. 4th ed Lyon: IARC Press; 2008.
[8] Bahler DW, Campbell MJ, Hart S, et al. Ig VH gene expression among human follicular lymphoma. Blood 1991;78(6):1561–8.
[9] Zhu D, Hawkins RE, Hamblin TJ, et al. Clonal history of a human follicular lymphoma as revealed in the immunoglobulin variable region genes. Br J Haematol 1994;86(3):505–12.
[10] Stamatopoulos K, Kosmas C, Papadaki T, et al. Follicular lymphoma immunoglobulin kappa light chains are affected by the antigen selection process, but to a lesser degree than their partner heavy chains. Br J Haematol 1997;96(1):132–46.
[11] Ottensmeier Ch, Fau-Thompsett AR, Thompsett Ar, Fau-Zhu D, Zhu D, Fau-Wilkins BS, et al. Analysis of VH genes in follicular and diffuse lymphoma shows ongoing somatic mutation and multiple isotype transcripts in early diseases with changes during disease progression. Blood 1998;91(11):4292–9.
[12] Aarts WM, Bende RJ, Steenbergen EJ, et al. Variable heavy chain gene analysis of follicular lymphomas: correlation between heavy chain isotype expression and somatic mutation load. Blood 2000;95(9):2922–9.
[13] Pascual V, Liu YJ, Magalski A, et al. Analysis of somatic mutation in five B cell subsets of human tonsil. J Exp Med 1994;180(1):329–39.
[14] Damle RN, Wasil T, Fais F, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 1999;94(6):1840–7.
[15] Hamblin TJ, Davis Z, Gardiner A, et al. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 1999;94(6):1848–54.
[16] Algara P, Mateo MS, Sanchez-Beato M, et al. Analysis of the IgV(H) somatic mutations in splenic marginal zone lymphoma defines a group of unmutated cases with frequent 7q deletion and adverse clinical course. Blood 2002;99(4):1299–304.
[17] Bahler DW, Pindzola JA, Swerdlow SH. Splenic marginal zone lymphomas appear to originate from different B cell types. Am J Pathol 2002;161(1):81–8.
[18] Camacho FI, Algara P, Rodriguez A, et al. Molecular heterogeneity in MCL defined by the use of specific VH genes and the frequency of somatic mutations. Blood 2003;101(10):4042–6.
[19] Ghia P, Stamatopoulos K, Belessi C, et al. ERIC recommendations on IGHV gene mutational status analysis in chronic lymphocytic leukemia. Leukemia 2007;21(1):1–3.
[20] Langerak AW, Davi F, Ghia P, et al. Immunoglobulin sequence analysis and prognostication in CLL: guidelines from the ERIC review board for reliable interpretation of problematic cases. Leukemia 2011;25(6):979–84.
[21] Tobin G, Thunberg U, Johnson A, et al. Somatically mutated Ig V(H)3–21 genes characterize a new subset of chronic lymphocytic leukemia. Blood 2002;99(6):2262–4.
[22] Thorselius M, Krober A, Murray F, et al. Strikingly homologous immunoglobulin gene rearrangements and poor outcome in V(H)3–21-using chronic

lymphocytic leukemia patients independent of geographic origin and mutational status. Blood 2006;107(7):2889–94.
[23] Bomben R, Dal Bo M, Capello D, et al. Molecular and clinical features of chronic lymphocytic leukaemia with stereotyped B cell receptors: results from an Italian multicentre study. Br J Haematol 2009;144(4):492–506.
[24] Oscier D, Wade R, Davis Z, et al. Prognostic factors identified three risk groups in the LRF CLL4 trial, independent of treatment allocation. Haematol Hematol J 2010;95(10):1705–12.
[25] Wartenberg M, Vasil P, zum Bueschenfelde CM, et al. Somatic hypermutation analysis in follicular lymphoma provides evidence suggesting bidirectional cell migration between lymph node and bone marrow during disease progression and relapse. Haematologica 2013;98(9):1433–41.
[26] Bende RJ, Aarts WM, Riedl RG, et al. Among B cell non-Hodgkin's lymphomas, MALT lymphomas express a unique antibody repertoire with frequent rheumatoid factor reactivity. J Exp Med 2005;201(8):1229–41.
[27] Berget E, Helgeland L, Liseth K, et al. Prognostic value of bone marrow involvement by clonal immunoglobulin gene rearrangements in follicular lymphoma. J Clin Pathol 2014;67(12):1072–7.
[28] van Dongen JJM, Langerak AW, Bruggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 concerted action BMH4-CT98-3936. Leukemia 2003;17(12):2257–317.
[29] Berget E, Helgeland L, Molven A, et al. Detection of clonality in follicular lymphoma using formalin-fixed, paraffin-embedded tissue samples and BIOMED-2 immunoglobulin primers. J Clin Pathol 2011;64(1):37–41.
[30] Brezinschek HP, Foster SJ, Brezinschek RI, et al. Analysis of the human V-H gene repertoire—differential effects of selection and somatic hypermutation on human peripheral CD5(+)/IgM(+) and CD5(-)/IgM(+) B cells. J Clin Invest 1997;99(10):2488–501.
[31] Brezinschek HP, Brezinschek RI, Dorner T, et al. Similar characteristics of the CDR3 of V(H)1-69/DP-10 rearrangements in normal human peripheral blood and chronic lymphocytic leukaemia B cells. Br J Haematol 1998;102(2):516–21.
[32] Catherwood MA, Gonzalez D, Patton C, et al. Improved clonality assessment in germinal centre/post-germinal centre non-Hodgkin's lymphomas with high rates of somatic hypermutation. J Clin Pathol 2007;60(5):524–8.
[33] Rosenquist R, Lindstrom A, Holmberg D, et al. V-H gene family utilization in different B cell lymphoma subgroups. Eur J Haematol 1999;62(2):123–8.
[34] Bahler DW, Levy R. Clonal evolution of a follicular lymphoma: evidence for antigen selection. Proc Natl Acad Sci USA 1992;89(15):6770–4.
[35] Stiernholm N, Kuzniar B, Berinstein NL. Absence of immunoglobulin variable region hypermutation in a large cell lymphoma after *in vivo* and *in vitro* propagation. Blood 1992;80(3):738–43.
[36] Hsu FJ, Levy R. Preferential use of the VH4 Ig gene family by diffuse large-cell lymphoma. Blood 1995;86(8):3072–82.
[37] Delecluse HJ, Hummel M, Marafioti T, et al. Common and HIV-related diffuse large B cell lymphomas differ in their immunoglobulin gene mutation pattern. J Pathol 1999;188(2):133–8.
[38] Lossos IS, Okada CY, Tibshirani R, et al. Molecular analysis of immunoglobulin genes in diffuse large B cell lymphomas. Blood 2000;95(5):1797–803.
[39] Baptista MJ, Calpe E, Fernandez E, et al. Analysis of the IGHV region in Burkitt's lymphomas supports a germinal centre origin and a role for superantigens in lymphomagenesis. Leuk Res 2014;38(4):509–15.
[40] Navarro A, Clot G, Royo C, et al. Molecular subsets of mantle cell lymphoma defined by the IGHV mutational status and SOX11 expression have distinct biologic and clinical features. Cancer Res 2012;72(20):5307–16.
[41] Kuppers R. Mechanisms of B cell lymphoma pathogenesis. Nat Rev Cancer 2005;5(4):251–62.
[42] Zenz T, Mertens D, Kuppers R, et al. From pathogenesis to treatment of chronic lymphocytic leukaemia. Nat Rev Cancer 2010;10(1):37–50.