Characteristics of biallelic chronic lymphocytic leukemia in a Norwegian population

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04.03.2015

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Abstract

Background: Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with varying prognosis. The best-known prognostic factor today is the mutation status of the IgVh gene. CLL is known to use a restricted specter of the IgVh genes and usually only one IgVh gene is rearranged. In biallelic CLL there are two rearranged IgVh genes. Good knowledge about the frequency and IgVh characteristics of biallelic CLL is missing. A previous study in Norway showed a prevalence of 9 % and higher frequency of unmutated IgVh genes in biallelic cases.

Methods: In this study, all included cases were diagnosed with CLL by flow cytometry and DNA sequencing showed double IgVh rearrangements. In total, 148 patients fulfilled these criteria from January 2004 to March 2013. We did a descriptive study of the mutation status and use of IgVh genes in this group. The mutation status was decided by the average homology to germline of the two IgVh genes.

Results: Among the included patients, 127 patients were proven to have biallelic CLL, 12 cases were biclonal and in nine cases the clonal status was uncertain. In the biallelic patient group, 48 % were assigned as mutated and 52 % as unmutated. Concordant mutation status was seen in 86 %. The most common IgVh families were Vh3 (50 %), Vh4 (22 %) and Vh1 (17 %). The most frequent IgVh genes, together accounting for one third of the patient group, were Vh3-30, Vh4-34, Vh1-69 and Vh3-21. The most prevalent combination was Vh3+Vh4. We found eight recurrent IgVh-IgDh-IgJh rearrangements, with one rearrangement occurring three times and seven rearrangements occurring two times.

Conclusion: Biallelic CLL resembles monoallelic CLL, though there were small differences in use of IgVh genes compared to previous published Norwegian findings. A higher unmutated rate than expected in a population-based study may indicate a poorer prognosis in biallelic CLL, though this has not yet been proved in large-scale studies.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Caucasian population (1). The incidence is approximately 4 in 100 000 person/year (1-5). CLL is characterized by great variation in clinical course. While some patients have an indolent disease not requiring therapy, others succumb within a few months (6, 7). Due to the heterogeneous course of CLL, reliable prognostic markers are important; the best available prognostic markers are the mutation status of the immunoglobulin variable heavy chain gene (IgVh gene), CD38 expression, ZAP70 expression and cytogenetic aberrations (8-12).

In the late 1990s, the IgVh gene was shown to have direct impact on prognosis. It appeared that CLL patients with unmutated IgVh genes had a more aggressive disease and significantly shorter survival than patients with mutated IgVh genes (8, 9). Later, exceptions were identified; the use of the IgVh 3-21 gene was associated with poor survival, regardless of the IgVh gene mutation status (13).

Previously, it was assumed that unmutated CLL cells derived from naïve B cells. However, this was disproved by studies showing that all CLL cells had a phenotype that matched antigen-experienced B cells and resembles memory B cells (14-18).

CLL cells vary in their ability to signal through the B-cell receptor (BCR), and CLL cells with unmutated IgVh genes have more competent BCRs, which could possibly lead to better survival of the CLL cells due to increased stimulation (19, 20). This may explain the good prognosis of mutated CLL, which is associated with less aggressive disease, perhaps due to less stimulation of the cell through signaling (21, 22).

The use of stereotyped BCR in CLL cells is shown in a large proportion of patients (23-27). These studies indicate that CLL cells preferentially express certain IgVh genes displaying stereotyped antigen binding sites and that antigenic stimulation is a driving force in the pathogenesis of CLL. Stereotyped BCR is significantly more common in unmutated CLL (24, 26).

A subgroup of CLL carries multiple IgVh rearrangements. Either the case can be biclonal with two CLL clones where each clone carries one rearranged IgVh gene, or it can be biallelic with only one cell clone, but each cell carries two rearranged IgVh genes.

In biallelic CLL both IgVh alleles of the CLL cell are rearranged. Normally, only one of the IgVh rearrangements is productive. If both are productive, the distinction from biclonal CLL may not be possible. It is not possible to conclude about the functionality of the rearranged genes, unless the analysis is based on RNA.

Biallelic CLL seems to be quite prevalent in Norway than reported from the rest of the western world. A Norwegian study from 2009 found that 18 out of 199 patients (9 %) displayed rearrangements of both IgVh genes (1).
In the present study we identified all patients with biallelic CLL among all CLL cases diagnosed at Oslo University Hospital during 110 months. To the best of our knowledge, there has not been any larger study describing the characteristics of biallelic CLL. We wish to extend our previous knowledge about these CLL cases, and we hope to contribute to the understanding of CLL as a heterogeneous disease.

Methods

Patients

Our patient cohort includes all patients diagnosed with CLL by flow cytometry at the Department of pathology, Oslo University Hospital between January 2004 and March 2013. In total 1590 patients were diagnosed with CLL in this period. All cases met the diagnostic criteria for CLL according to the iwCLL (28). In 207 cases the sequencing results were inconclusive or sequencing was not performed, making it impossible to assign these cases. Only those with two or more IgVh rearrangements by PCR amplification were included in the study.

Molecular genetics

DNA from whole blood was extracted using Qiagen, All Prep DNA/RNA Micro kit (Qiagen, Hilden, Germany). The rearranged IGH genes were amplified using framework 1 (FR1) VH-family-specific primers and one common JH primer in 2 multiplex PCR reactions as previously described (29). PCR products were analyzed with capillary electrophoresis using Agilent DNA 1000 kit and Agilent Bioanalyzer (Agilent Technologies, Santa Clara, USA). PCR products were subsequently sequenced in 2 directions using the respective VH and JH primers and the Big Dye Terminator v1.1 Cycle Sequencing Kit (Life Technologies Carlsbad, CA, USA) according to the manufacturer's recommendations. The International Immunogenetics Information System web-based software (www.imgt.org) was employed to analyze the rearranged IGH sequences.

Flow cytometry analysis

Until 2011, a 4-colour flow cytometry analysis was used with the following antibody combinations and anti CD19 as the backbone marker: CD45 (clone 2D1, Becton Dickenson (BD), Erembodegem, België), CD19 (clone SJ25C1, BD), CD20 (clone L27, BD), CD22 (clone 4KB128, Dako, Århus, Denmark), CD24 (clone ALB9, Immunotech, Marseille, France), FMC7 (clone FMC7, Dako), CD5 (clone UCHT2, BD), CD23 (clone MHM6, Dako), CD43 (clone L10, Caltag Laboratories, Buckingham, United Kingdom), CD38 (clone HB7, BD), anti-kappa og anti-lambda immunoglobulin light chains (Simultest, BD). The antibodies were labeled to fluoresceine thyocyante (Fitc), phyco-erythrine (Pe), peridinin-chlorophylphosphate cytochrome 5.5 (PercP Cy5.5) and allophycocynanin (APC).

As from 2012, a 8-colour flow cytometry analysis was used with the following antibody combinations labeled with Pacific Blue (PB)/ Krome Orange (KO)/ Fitc/ Pe/PercPCy5.5/ Phyco-erythrone cyanine 7 (PeCy7)/APC/ APC Hilite 7 (APC H7) or APC cyanine 7 (APC Cy7): (1) CD20+CD4/CD45/CD8+Igλ/CD56+ Igκ/CD5/CD19+TCRγδ/CD38 and (2) CD20/CD45/CD23/CD10/CD79b/CD19/CD200/CD43 (30).

A stain, lyse and wash method was used on whole blood and cells were acquired on a Facscalibur or LSR2 (BD). Data-analysis was performed using FlowJo (Tree Star, Ashland, OR, USA).

Results

Patient groups

Single IgVh genes were identified in 1235 of 1383 cases and double IgVh genes were identified in 148 cases. The patients with two rearranged IgVh genes were included in the study, and these cases were divided into three groups.

The first group consisted of 127 definite biallelic CLL, though in four cases the sequencing process failed. This gives a prevalence of 9.2% (127 of 1383 cases) for biallelic CLL. Among the 123 patients with identifiable IgVh genes, the age at the time of examinations showed an average of 68.6 years. There was 82 men and 41 women, i.e. a male-female ratio of 2:1, a higher ratio than previously reported in Norway (1).

The second group contained 12 biclonal cases. These cases were excluded from the biallelic cases, because the mutation analysis showed that the two IgVh genes came from different

subsets of CLL clones. Four of these patients had three IgVh genes, so it is possible they had either two or three subsets of clones.

In the last group were nine cases with double productive IgVh rearrangements. Since only the VDJ part is amplified and sequenced, it is not possible to give a final conclusion about the functionality of the IgVh rearrangements, but we cannot exclude biclonality in these cases. Due to this uncertainty we chose to exclude these nine cases from the biallelic cases. The group of biclonal CLL and the group of double productive rearrangements amounted 1,5 % (21 of 1383 cases). This is somewhat lower than recently published results showing 2,7 % of cases had multiple-productive rearrangements (31).

Table 1: Categorization of patient groups.

Total number of patients	1590	
One IgVh rearrangement	1235	
Two IgVh rearrangements	148	
Biallelic CLL		127
Biclonal CLL		12
Double productive IgVh		9
Inconclusive sequence analysis	207	

Mutation status

With biallelic IgVh rearrangements the mutation status is assessed to be the average of the two IgVh rearrangements. If the average homology to germline is less than 98 %, the current case is mutated.

Applying 98 % homology to germline as cutoff, 59 of 123 patients (48 %) were assigned to the mutated subgroup, whereas the remaining 64 patients (52 %) had unmutated CLL. In 47 patients (38 %) both IgVh rearrangements were mutated and in 59 patients (48 %) both were unmutated, hence there were concordant mutation status in 106 patients (86 %). In 17 patients (14 %) there were discordant mutation status; one IgVh rearrangement was mutated and one unmutated. Among the 64 patients with unmutated IgVh genes, 39 patients (61 % of the unmutated cases, 32 % in total) displayed 100 % homology to the corresponding germline IgVh gene sequences. With 100 % homology to germline, the case is said to be “truly unmutated” because it shows no degree of mutation (32). Forty-nine of 82 men (60 %) displayed unmutated IgVh genes, and only 15 of 41 women (37 %).

In four patients with biallelic CLL, the sequencing process failed; hence it was not possible to identify the IgVh genes, and thus the mutation status could not be ascertained.

Table 2: Mutation status and frequency in 123 patients with biallelic CLL.

Mutation status	Number of patients	%
Mutated cases	59	48 %
Unmutated cases	64	52 %
Truly unmutated	39	32 %
Concordant	106	86 %
Discordant	17	14 %

Productive IgVh rearrangements

In the biallelic patient group we assessed the productive IgVh rearrangement in 16 patients. In eight of 16 cases (50 %) both rearrangements were mutated, and in six (38 %) both were unmutated. In the remaining two cases there was discordant mutation status. There were one case with a mutated, productive rearrangement and an unmutated, unproductive rearrangement, and one case with an unmutated productive and one mutated unproductive rearrangement. In the latter case, the unmutated productive rearrangement showed 98 % homology to the germline. This corresponds with similar findings by Langerak et al. (33), who found that in the majority of these cases the productive IgVh rearrangement would show some degree of mutation.

Among the nine cases with two productive IgVh rearrangements, five cases (56 %) were mutated and two cases were unmutated (22 %). In two cases (22 %) there were discordant mutation statuses.

IgVh usage

When all 246 IgVh genes of the 123 patients with biallelic CLL were compared, it showed little variation to previously published results (1, 9, 34). All IgVh families (Vh1-Vh7) were represented. IgVh genes belonging to the Vh3 family were most prevalent accounting for 122 of 246 IgVh genes (50 %). The other most common IgVh families were Vh4 (53 cases, 22 %) and Vh1 (42 cases, 17 %). The following IgVh families were used only in a minority of cases: Vh2 (12 cases, 5 %), Vh5 (10 cases, 4 %), Vh6 (5 cases, 2 %) and Vh7 (2 cases, 0,8 %). Vh3-30 was the most prevalent IgVh gene, found in 25 cases in total (10 %). Other frequently used IgVh genes were Vh4-34 (21 cases, 9 %), Vh1-69 (20 cases, 8 %) and Vh3-21 (11 cases, 4%).

Table 3: Occurrence of IgVh families in 246 identified IgVh genes in 123 patients.

IgVh family	Number of patients	%	% Fais et al. (34)
Vh1	42	17 %	24.1 %
Vh2	12	5 %	1.2 %
Vh3	122	50 %	38.6 %
Vh4	53	22 %	30.1 %
Vh5	10	4 %	2.4 %
Vh6	5	2 %	2.4 %
Vh7	2	0,8 %	1.2 %

Table 4: IgVh genes with the highest frequency

IgVh gene	Number of patients	%	% Tjønnfjord et al. (1)
Vh3-30	26	11 %	10 %
Vh4-34	21	9 %	12.6 %
Vh1-69	18	7 %	9 %
Vh3-21	11	4 %	4 %

Combinations of IgVh genes

The most frequent combination of IgVh families was Vh3+Vh4, which accounted for 33 of 123 cases (27 %), followed by Vh1+Vh3 (25 cases, 20 %), Vh3+Vh3 (22 cases, 18 %) and Vh1+Vh4 (10 cases, 8 %). Some combinations were quite rare despite of the prevalence of these IgVh families. Among these combinations were Vh1+Vh1 (2 cases, 1,6 %), Vh1+Vh2 (1 case, 0,8 %) and Vh4+Vh4 (3 cases, 2,4 %). Vh7 only occurred in combination with Vh3 (2 cases, 1,6 %).

Table 5: Frequency of combinations of IgVh families

Combinations	Number of patients	%
Vh3 + Vh4	33	27 %
Vh1 + Vh3	25	20 %
Vh3 + Vh3	22	18 %
Vh1 + Vh4	10	8 %

IgVh-IgDh-IgJh rearrangements

In 53 patients with biallelic CLL one or two IgVh-IgDh-IgJh rearrangements were identified. Ninety-seven sequences could be identified in total. It was a segregation of six gene family sequences; Vh3-Dh3-Jh4 was the most frequent with 12 cases (12 %) followed by Vh4-Dh3-J4 and Vh1-Dh3-Jh6 with five cases each (each 5 %). Three combinations each counted four cases (each 4 %); Vh3-Dh3-Jh5, Vh3-Dh6-Jh4 and Vh3-Dh3-Jh6. Thirty-one sequences (32

%) occurred one time only. Ten and four sequences occurred two and three times, respectively.

In total there were 88 different combinations of IgVh-IgDh-IgJh arrangements. Eighty of 88 (91 %) arrangements only occurred in one case, seven arrangements (8 %) occurred in two cases and one arrangement (1 %) presented itself in three cases.

The latter arrangement was Vh1-69-Dh3-3-Jh-6. The arrangements that occurred two times were as following; Vh1-69-Dh2-2-Jh-6, Vh2-5-Dh4-4-Jh4, Vh3-30-Dh3-10-Jh4, Vh3-30-Dh3-22-Jh4, Vh3-48-Dh3-3-Jh6, Vh5-51-Dh3-3-Jh6 and Vh4-61-Dh3-16-Jh4.

Table 6: Recurrent IgVh-IgDh-IgJh rearrangements.

IgVh	IgDh	IgJh
1-69	3-3	6
1-69	2-2	6
3-48	3-3	6
2-5	4-4	4
3-30	3-10	4
3-30	3-22	4
5-51	3-3	6
4-61	3-16	4

Discussion

The primary aim of this study was to evaluate the characteristics of the IgVh genes in biallelic CLL. We did this by assembling the CLL patients with two rearranged IgVh genes, and based on DNA sequencing we could distinguish between three groups: Group I was the definite biallelic cases, group II was the biclonal cases with two evident clones, and group III was the cases with two productive IgVh rearrangements.

In the biallelic group the mean age was 68.6 years, probably due to an exclusion of older patients because of lack of treatment purposes in this group (1). Despite the possible exclusion of elderly, our material should be representative for CLL patients in Norway.

We confirm our previous findings of a high frequency of biallelic CLL in Norway; 9 % compared to 9,2% in the present study. This is likely a correct measure of the prevalence in Norway (1). Previous reports on the frequency of double rearranged IgVh genes show great variation, ranging from 1,4 % (28 of 1939 patients) in a European study (32), 2,3% (172 of 7424 patients) in the largest performed CLL study (35), and 14 % in a recent study from the United States (36). The reason for this discrepancy is unknown. It could be either due to

genetic differences, an unknown environmental factor or differences in technical conditions and choice of patients groups. A study done by the ERIC review board found that double IgVh rearrangements were more prevalent when using DNA (14,8%) than cDNA (3,8%) (33). Previously DNA was used in Norway, but in recent years was changed to cDNA, hence there are IgVh gene sequence analyzes performed on both DNA and cDNA in our material.

Of the biallelic cases 48 % were assigned as mutated and 52 % as unmutated. Of the unmutated cases 61 % were "truly unmutated". This corresponds with previous findings (8, 9, 32). Previously reported in Norway the overall unmutated rate was 31 %, while in biallelic cases it was 61 % (1). Our study was population-based, however, corresponding with results from tertiary care centers (8, 9). This may indicate a higher rate of unmutated cases in biallelic CLL and possibly more aggressive disease.

Concordant mutation status was seen in 86 % among the biallelic cases. A previous report states 93 % (33). A problem enlightened by the same study is whether a case is mutated or unmutated when there are double IgVh rearrangements and discordant mutation status. To decide on the mutation status in these cases the productive IgVh rearrangement is considered decisive (33). In the present study the mutation status was decided by the average homology to germline of the two IgVh rearrangements. When a case carries a mutated productive and an unmutated unproductive rearrangement, it should be regarded as mutated according to Langerak et al. (33). We had one such case. Though, in our study it is categorized as unmutated due to the average of the two IgVh rearrangements. A recent study found no significant difference in survival between monoallelic and biallelic CLL (36). Their results showed that having at least one mutated IgVh rearrangement improves on prognosis, hence their conclusion was that a case with one mutated and one unmutated IgVh rearrangement should be regarded as mutated. In contrast, Langerak et al. (33) states that with double productive rearrangements and discordant mutation status or with an unmutated productive and a mutated unproductive rearrangement, it is impossible to make a prognostication. Both of these cases are reported as rare, as they also were in our study.

Here we present three different ways to interpret the mutation status of biallelic CLL. Further research is needed to provide better knowledge about the implications of discordant mutation status and survival. If discordant biallelic cases are most similar to mutated monoallelic cases with respect to survival, it might be best to consider these cases as mutated, as proposed by Heyman et al. (36). Of course, it is still important to take into account exceptions like Vh3-21 and Vh1-69, which has been linked to poor prognosis and unmutated CLL, respectively (13, 37).

The present study confirms previous findings on IgVh usage in CLL (1, 9, 34). The Vh3 family was the most prevalent IgVh family (50 %), followed by Vh4 (22 %) and Vh1 (17 %). The most frequent IgVh genes were Vh3-30, Vh4-34 and Vh1-69. This correlates with previous findings in Norway (1). There were some differences, such as former results showing a high frequency of Vh3-7 and Vh3-23, in contrast to our study, where Vh3-7 accounted for 9 cases (3.7 %) and Vh3-23 were used only in 7 cases (2,8 %). Along with previous reports, there is an overuse of Vh1-69 compared with normal CD5+ B-cells (9, 37). In our study Vh3-21 was the forth most frequent IgVh gene with 11 cases (4 %). This is the same prevalence as previously reported from Norway (1). Varying frequencies of Vh3-21 usage have been reported. A Swedish study showed a frequency of 11.7 % in comparison to 2,9 % in a Mediterranean study (38, 39). Considering the small geographic differences between Norway and Sweden, and comparing their prevalence of Vh3-21 with ours (11 % vs. 4 %), we could possibly expect more equal findings, though they used a more selected study-group than us. The combination of IgVh families correlates with previous findings, though there has been reported a low frequency on Vh3+Vh3 (31), while this was one of the most prevalent combinations in our study (22 cases, 18%). Vh4 and Vh1 were among the most common IgVh families in our material, nevertheless the combinations of Vh1+Vh1 (1.6 %) and Vh4+Vh4 (2.4 %) were infrequent.

Ninety-seven sequences of the IgVh-IgDh-IgJh rearrangements were identified and eight arrangements were recurrent. In recent studies, the use of the same IgVh-IgDh-IgJh germline genes have been analyzed in addition to the IgDh reading frame and if the HCDR3 amino acid identity was > 60 % (26). Forty-eight subsets of stereotyped HCDR3 have been reported (24). We did not have enough data beyond the use of IgVh-IgDh-IgJh rearrangements to fulfill the criteria proposed by Messmer et al. (26) to compare our results with previous findings on stereotyped BCR. Nevertheless, there are 51 different IgVh genes, 25 different IgDh genes and 6 different IgJh genes, and though these genes are not used completely randomly by normal B-cells or by CLL cells, the probability is 1 in 7650 (1/51 x 1/25 x 1/6) to get a specific combination of IgVh-IgDh-IgJh (25), so our findings cannot be considered as coincidental.

In summary, we confirm a high prevalence of biallelic CLL in Norway. A higher proportion of unmutated cases than previously reported in Norway on monoallelic cases may indicate more aggressive disease in biallelic CLL, but our study did not include survival data to support this notion. Biallelic CLL resembles monoallelic CLL on IgVh usage, though there were small differences.

Acknowledgments

I would like to thank Geir E. Tjønnfjord, prof. MD PhD, for valuable guidance. I also send my thanks to Anne Tierens MD PhD, who contributed with the comments on molecular genetics and flow cytometry analysis in the Methods section.

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