Relevance of the Immunoglobulin $V_{H}$ Somatic Mutation Status in Patients with Chronic Lymphocytic Leukemia Treated with Fludarabine, Cyclophosphamide and Rituximab (FCR) or Related Chemoimmunotherapy Regimens.

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ABSTRACT

Although immunoglobulin V\(_H\) mutation status (IgV\(_H\)-MS) is prognostic in CLL patients treated with alkylating agents or single-agent fludarabine, its significance in the era of chemoimmunotherapy is not known. We determined the IgV\(_H\) somatic mutation status in 177 patients enrolled in a phase II study of fludarabine, cyclophosphamide and rituximab (FCR), and in 127 patients treated with subsequent chemoimmunotherapy protocols. IgV\(_H\)-MS did not impact significantly on the complete remission (CR) rate of patients receiving FCR or related regimens. However, CR duration was significantly shorter in patients with CLL that used unmutated IgV\(_H\) than those whose CLL used mutated IgV\(_H\) (TTP 47% vs 82% at 6 years, p<0.001). In a multivariate model considering all baseline characteristics, IgV\(_H\)-MS emerged as the only determinant of remission duration (hazard ratio 3.8, p<0.001). Our results suggest that post-remission interventions should be targeted towards patients with unmutated IgV\(_H\) status.
INTRODUCTION

Immunoglobulin V_{H} somatic mutation status (IgV_{H}-MS) is an important prognostic marker in patients with chronic lymphocytic leukemia (CLL). In the chemotherapy era, patients with cells that used an unmutated IgV_{H} gene (UM-CLL) had inferior survivals when compared with those that used a mutated IgV_{H} gene (M-CLL)\(^1,2\). However, it was unclear if this was because of inferior treatment response, increased risk of relapse from remission, or both. Furthermore, it is not known if IgV_{H}-MS remains relevant in patients treated with combinations of chemotherapy and monoclonal antibodies (chemoimmunotherapy)\(^3,4\). In order to address these questions, we analyzed the impact of IgV_{H}-MS on the outcome of patients treated with frontline chemoimmunotherapy at our center.

METHODS AND MATERIALS

The U.T. MD Anderson Cancer Center institutional review board approved the studies included in this report and informed consent was obtained in accordance with the Declaration of Helsinki. The main analysis was based on 300 patients treated on the phase II study of the fludarabine, cyclophosphamide & rituximab (FCR) regimen, which had a mature median follow-up of six years. The CR rate was 72\%, and the time to progression (TTP) for complete responders was 85 months; six-year overall survival (OS) for all patients was 77\%\(^3,4\). Subsequent to the FCR study, our center evaluated a number of other chemoimmunotherapy regimens: FCR\(^3\) (FCR with three rituximab doses per cycle, n=56)\(^5\), FCMR (FCR with mitoxantrone, n=24)\(^6\), FCR+GMCSF (n=21), and CFAR (FCR with alemtuzumab, n=26)\(^7\). These protocols were single-arm studies with differences in baseline characteristics; however, they were conducted in CLL patients commencing initial chemotherapy according to uniform criteria\(^8\), and patients underwent response staging in an identical manner\(^8\). Therefore, studies subsequent to FCR were included to provide additional insight into the impact of IgV_{H}-MS on CR achievement. The impact of IgV_{H}-MS
on TTP and OS was not evaluable in these studies due to the short median follow-up (35, 26, 12 and 8 months for FCR3, FCMR, CFAR and FCR+GMCSF, respectively).

For patients treated with FCR, \(IgV_{\mu}r\)-MS was determined retrospectively in 101 patients from pretreatment formalin-fixed, paraffin-embedded bone marrow aspirate clot section samples, and prospectively in 76 patients from fresh peripheral blood or bone marrow samples. The remaining 123 patients had insufficient specimens for analysis. All patients in protocols subsequent to FCR had their \(IgV_{\mu}r\)-MS determined prospectively from fresh tissue. The technique for testing in fresh tissue was according to published methods\(^9\). The technique for paraffin-embedded tissue was as follows: DNA was extracted from paraffin-embedded tissue section using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), and amplified using HotStar Taq DNA Polymerase (Qiagen) and FRC1 primer set, as described previously\(^10\). Amplified monoclonal bands were detected on 1.5% agarose gel, excised, and extracted using the QIAquick Gel Extraction Kit (Qiagen). The purified DNA was subjected to sequence analysis. The mutation status was designated as unmutated (UM-CLL) if we detected fewer than 2.0% mutations (>98.0% homology to germline sequences) or as mutated (M-CLL) if we detected 2.0% or greater mutations (≤98.0% homology to germline sequences) compared with the germline sequences in VBASE2 (www.vbase2.org)\(^11\).

TTP and OS were calculated from the date of therapy until disease progression and death, respectively. Because most (>70%) patients achieved CR, and in order to obtain as homogenous group of patients as possible, the analysis of the impact of \(IgV_{\mu}r\)-MS on TTP was restricted to complete responders only. Categorical variables were compared using the Chi square or Fisher Exact tests, and continuous variables were compared using the Mann-Whitney test. Survival distributions were compared using the log-rank test and Cox regression, as appropriate. The number of FCR patients analyzed were sufficient to detect with 85% power (at a one-sided significance level of p=0.05) an absolute difference of 20% or more in CR rate favoring patients.
with M-CLL. The 20% threshold was regarded by our treating physicians as being clinically relevant. All other p-values were two-sided.

RESULTS AND DISCUSSION

IgV_{H} mutation status was available in 177 FCR patients, with 59% having UM-CLL and 41% having M-CLL (Table 1). Their baseline characteristics and treatment outcome were not significantly different from that of FCR patients whose IgV_{H}–MS were not determined (p>0.20 for all comparisons, data not shown). Clinical data from 127 patients treated on protocols subsequent to FCR were pooled (Table 1) in order to examine for differences between patients with UM-CLL (69%) and M-CLL (31%). In general, patients with UM-CLL were more likely to have high white cell count, elevated β2-microglobulin (β2m), and/or abnormal conventional karyotypic or adverse fluorescence in-situ hybridization findings (Table 1).

Among FCR patients, CR rates were similar between UM-CLL and M-CLL patients, being 73% and 83%, respectively (p=0.12). The proportions of patients achieving flow-cytometry negative CR (defined as <1% CD5/19 co-expressing cells in the marrow lymphoid gate, with normal light chain ratio) were 57% and 67%, respectively (p=0.21). This result was reproduced in patients treated on protocols subsequent to FCR, where the CR rates were similar between UM-CLL and M-CLL patients both as a group (71% vs 78%, respectively, p=0.46), and within individual regimens (Table 1). Within this group, the proportions of UM-CLL and M-CLL patients achieving flow-cytometry negative CR were 54% and 69%, respectively (p=0.16). Thus, IgV_{H}–MS did not substantially influence the probability of achieving CR.

In the analysis of CR duration in FCR patients, however, UM-CLL patients who entered CR were found to have a significantly inferior TTP compared with M-CLL patients (Figure 1A). At six years, the proportions of complete responders remaining progression free were 47% and 82% for UM-CLL and M-CLL patients, respectively (p<0.001). Among M-CLL patients, those using the VH3-21
gene were at an increased risk of relapse (3 relapses in 6 patients) compared with those using other VH sub-families (7 relapses in 54 patients, p=0.05). This effect of IgV_{i}r-MS on TTP was maintained when the analysis was restricted only to complete responders with confirmed flow-cytometric negativity (Figure 1B). Multivariate analysis was performed to determine if the significance of IgV_{i}r-MS was independent of prognostic factors previously established in the FCR population^4. In this analysis, IgV_{i}r-MS status was strongly and independently significant for TTP (hazard ratio 3.8, p<0.001), whereas established factors (including age, β2m, cytogenetic abnormalities, white cell count, marrow CD38 positivity and interval between diagnosis and treatment) failed to reach statistical significance.

Considering all patients treated with FCR, six-year OS was significantly inferior in UM-CLL compared with M-CLL patients (71% and 82%, respectively, p=0.05). However, on multivariate analysis, IgV_{i}r-MS was not independently associated with inferior OS (p=0.10), whereas advancing age (p=0.001) and high β2m (p=0.006) were significant.

This study demonstrated that the prognostic impact of IgV_{i}r-MS in patients receiving chemoimmunotherapy regimens was not related to differences in CR rates, but was due to aggressive relapse kinetics in patients with UM-CLL. The reason for this effect is uncertain, and may be related to differential responses to B-cell receptor signalling^12. This data is highly relevant to clinical investigators as it suggests that studies of post-remission interventions (eg. maintenance therapy, early relapse detection strategies, use of novel agents in remission, etc.) should be targeted towards those patients with UM-CLL, as these are the patients at the highest risk of early relapse. Patients with M-CLL using the VH3-21 gene should be managed as if they have UM-CLL.

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Author Contributions

K.I.L. performed the $IgV_{H}$ mutation determination in paraffin-embedded tissue and wrote the paper. C.S.T. analyzed the data and co-authored the manuscript. M.J.K. and L.V.A. designed the study and provided supervision and advice in data analysis and manuscript preparation. T.J.K. and L.R. performed the $IgV_{H}$ mutation determination in fresh tissue and provided advice and oversight in manuscript preparation. K.R.C. performed the sample size analysis and provided statistical oversight. E.S. and L.L.B. assisted in the sample preparation and provided technical advice. W.G.W., S.O., A.F., S.F. and H.K. contributed and verified the accuracy of patient data and provided advice and oversight on data analysis and manuscript preparation. S.L. collected the patient data and coordinated the verification of data integrity. The authors have no relevant conflicts of interest to disclose.
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### Table 1: Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>FCR STUDY (n=177)</th>
<th>SUBSEQUENT PROTOCOLS (n=127)</th>
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<tr>
<td><strong>Age in years, median (range)</strong></td>
<td>UM M</td>
<td>UM M</td>
</tr>
<tr>
<td></td>
<td>58 (24-82) 58 (35-76)</td>
<td>58 (36-82) 57 (27-76)</td>
</tr>
<tr>
<td><strong>Male gender, n (%)</strong></td>
<td>UM M</td>
<td>UM M</td>
</tr>
<tr>
<td></td>
<td>80 (76) 58 (78)</td>
<td>68 (78) 26 (65)</td>
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<tr>
<td><strong>More than 2 years from diagnosis, n (%)</strong></td>
<td>UM M</td>
<td>UM M</td>
</tr>
<tr>
<td></td>
<td>50 (48) 42 (58)</td>
<td>35 (40) 19 (48)</td>
</tr>
<tr>
<td><strong>Rai stages 3-4, n (%)</strong></td>
<td>UM M</td>
<td>UM M</td>
</tr>
<tr>
<td></td>
<td>36 (34) 28 (39)</td>
<td>23 (26) 13 (33)</td>
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<tr>
<td><strong>White cell count ≥ 150 x 10^9/L, n (%)</strong></td>
<td>UM M</td>
<td>UM M</td>
</tr>
<tr>
<td></td>
<td>25 (24) 9 (13)</td>
<td>22 (25) 6 (15)</td>
</tr>
<tr>
<td><strong>β2m ≥ 2 x upper limit normal, n (%)</strong></td>
<td>UM M</td>
<td>UM M</td>
</tr>
<tr>
<td></td>
<td>51 (49) 22 (31)</td>
<td>37 (43) 13 (33)</td>
</tr>
<tr>
<td><strong>Abnormal karyotype, n (%)</strong></td>
<td>UM M</td>
<td>UM M</td>
</tr>
<tr>
<td></td>
<td>24 (32) 7 (15) 54 patients</td>
<td>30 (42) 9 (30) 25 patients</td>
</tr>
<tr>
<td><strong>FISH deletion of 11q or 17p, n (%)</strong></td>
<td>UM M</td>
<td>UM M</td>
</tr>
<tr>
<td></td>
<td>- - 177 patients</td>
<td>30 (41) 2 (7) 23 patients</td>
</tr>
<tr>
<td><strong>Complete response, n (%)</strong></td>
<td>FCR, UM FCR, M</td>
<td>All Reg, UM All Reg, M</td>
</tr>
<tr>
<td></td>
<td>77 (73) 60 (83)</td>
<td>62 (71) 31 (78)</td>
</tr>
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* p<0.08  * p<0.02  * p<0.05  * p<0.001  * p=0.12  * p=0.46  * p=0.54  * p=0.24  * p=0.23  * p=1.00

**Table 1: Patient characteristics.** Unless specified otherwise, all comparisons between categories were not statistically significant. UM, unmutated IgVH; M, mutated IgVH; β2m, β2-microglobulin; FCR, fludarabine, cyclophosphamide & rituximab; FCR3, FCR with thrice weekly rituximab in first week of each cycle; FCMR, FCR & mitoxantrone; FCR+GM, FCR & GM-CSF; CFAR, FCR & alemtuzumab.
**Figure 1: Duration of complete remission.** In the FCR cohort, patients with CLL cells that used mutated $\text{i}gV_\mu$ (M-CLL) had significantly longer complete remission duration than did patients with CLL cells that used unmutated $\text{i}gV_\mu$ (UM-CLL), despite similar proportions achieving complete remission. This effect was present both in the total population of complete responders (figure 1A) and in complete responders with negative marrow flow-cytometry (figure 1B).
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