To the editor:

Glycosylation of V region genes in follicular lymphoma as a result of the somatic hypermutation mechanism

We read with interest the report by Zhu et al., who examined the generation of potential N-glycosylation sites in the clonogenic immunoglobulin (Ig) heavy chain (VH) genes of patients with follicular lymphoma (FL). Using their own and a number of sequences derived from the published literature, these authors have investigated the frequency of potential N-glycosylation sites introduced into functional VH genes as a consequence of somatic mutation. FL cells were compared with normal memory B cells or plasma cells matched for similar levels of mutation. Strikingly, novel sites were detected in almost 80% of patients with FL.

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References


Collagen IV levels are an excellent surrogate for hepatic fibrosis in hemochromatosis, and, indeed, we have found that there is a slight excess of individuals with elevated collagen IV levels in homozygotes for the C282Y mutation, even among those without manifest liver disease. Figure 1 shows the relationship of serum collagen IV levels to TNFα promoter genotype in homozygotes for the C282Y mutation. Clearly, there is no major difference between these measurements of liver damage in the population that we have studied. Table 1 compares the serum collagen, aspartate transaminase (AST), and ferritin levels in homozygotes for the C282Y mutation with different TNFα promoter genotypes.

TNFα promoter polymorphisms may be a risk factor for liver damage; if so, the effect is so small that it cannot be shown even with a group of more than 100 patients homozygous for the C282Y mutation. There must be other, more powerful, influences on the expression of the HFE mutations. So far our attempt to find such polymorphisms in the coding regions or promoters of HFE, calreticulin, B2-microglobulin, transferrin, transferrin receptor-2, DMT1 (nRamp2), ferroportin, transferrin receptor-1, IRP-1, IRP-2, hepcidin, the ferritin light and heavy chains, and ceruloplasmin have been unsuccessful. The search for genetic and environmental factors that influence the hemochromatosis phenotype must go on.

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compared with 9% in the normal B-cell population (P < .001), whereas diffuse large B-cell lymphoma (DLCL) showed an intermediate frequency (41%). Multiple myeloma (MM) and the mutated subset of B-cell chronic lymphocytic leukemia (B-CLL) showed frequencies similar to those of normal cells in 8% and 13% of patients, respectively. The authors concluded that N-glycosylation of the V\textsubscript{H} region may be a common event in FL and in a subset of DLCL, with the novel sites predominantly located in complementarity-determining regions (CDRs), the major target of the somatic hypermutation machinery. Sequences of nonfunctional V\textsubscript{H} genes contained few sites, and for positive selection for novel glycosylation sites in functional FL V\textsubscript{H} sequences. Moreover, the authors suggested that the added carbohydrate in the V\textsubscript{H} region might contribute to interaction with antigenic elements in the germinal center (GC) environment, and that this common feature of FL may be critical for tumor behavior.

We conducted a similar analysis for novel glycosylation sites in our published series of V\textsubscript{H} and V\textsubscript{\alpha} sequences from FL and MM patients. Novel glycosylation sites were identified in 4 out of 10 FL V\textsubscript{H} genes; there were 2 novel sites in the CDR2 and one each in the FWR1 and FWR2 regions. In MM, V\textsubscript{\alpha} and V\textsubscript{H} genes, novel glycosylation sites were detected in only 2 out of 17 cases (11.7%); both sites were located in V\textsubscript{\alpha} CDR1 region. Our findings are generally in accordance with those by Zhu et al regarding MM. However, markedly different results were obtained from analysis of FL V\textsubscript{\alpha} genes; Zhu et al reported an overall incidence of almost 59% (10/17 cases; Table 1 of their article), whereas no novel glycosylation sites were identified in our published sequences.

FL can be considered as the neoplastic counterpart of a certain stage in the intra-germinal center maturation of B cells. At this stage, normal B cells that are specific for antigen (with surface Ig [sIg] exhibiting an affinity above the germ line potential) are subject to positive selection. Sequence analysis of rearranged V\textsubscript{H} and V\textsubscript{\alpha} genes of FL cells carried out by our group demonstrated that whereas rearranged V\textsubscript{H} genes were hypermutated in their majority, with clustering of mutations in the CDRs, the rearranged V\textsubscript{\alpha} genes of the same neoplastic clones varied significantly with regard to their mutational profile; indeed, very few or no mutations were observed in a significant number of cases (5/10; 50%) of the analyzed V\textsubscript{\alpha} sequences. A similar pattern is obtained from the study by Zhu et al; although the V\textsubscript{\alpha} genes of that study were, on average, more heavily mutated than the corresponding genes in our study, the median “mutation load” in V\textsubscript{\alpha} sequences was still significantly smaller than in the same cohort’s V\textsubscript{H} genes (median homology to germ line was 89% for V\textsubscript{H} genes versus 96% for V\textsubscript{\alpha} genes). The fact that clonogenic V\textsubscript{\alpha} genes are less frequently mutated indicates that the somatic hypermutation machinery might have ceased to operate in the V\textsubscript{\alpha} locus at the time when neoplastic transformation had occurred. Perhaps this is a reflection of the generally smaller potential contribution of V\textsubscript{\alpha} genes (compared with V\textsubscript{H} genes) in antigen selection of the clonogenic B cells in FL. Similar conclusions regarding the role of Ig light chains in antigen recognition (evidenced by the mutation status of the corresponding V region genes) have been reached from single-cell studies in the normal peripheral B-cell repertoire, indicating a more limited mutational load both in the expressed as well as nonfunctional V\textsubscript{\alpha} genes compared with their partner V\textsubscript{H} genes in IgM+/CD19− B cells. In conclusion, the study of Zhu et al, as well as analysis of our previously published series, refocuses the interest of antigen selection in human lymphomagenesis to the potential contribution of Ig V region glycosylation at novel sites as a result of active somatic hypermutation. However, until these results are accepted as definite, further confirmatory studies in larger numbers of patients are awaited.

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References


Response:

High incidence of novel N-glycosylation sites in the immunoglobulin variable region genes of follicular lymphoma

Our finding of a high frequency of potential glycosylation sites in the variable regions of cases of follicular lymphoma (FL) has implications for the understanding of the pathogenesis of this tumor. The presence of oligosaccharides in the binding site of the B-cell receptor of FL cells may influence interactions with stromal elements in the germinal center and could contribute to tumor growth or survival. Clearly the data on 70 cases in our paper need to be extended, and Belessi et al also make this point. The sites are largely acquired by somatic mutation and can be found in either V\textsubscript{H} or V\textsubscript{\alpha}. One problem in extending the data is that generally only V\textsubscript{H} sequences are available from the databases, leaving open the question of the contribution of sites in V\textsubscript{\alpha}. Our paper included matched V\textsubscript{H} and V\textsubscript{\alpha} sequences from 17 cases from this laboratory, and we have now added a further 11 matched cases, all of which had sites. In summary, we now have 26 of 28 cases with sites in V\textsubscript{H} and 14 of 28 with sites in V\textsubscript{\alpha}, and all our cases had at least one site. An interesting exception was a case initially diagnosed as FL, but with no sites evident. We asked a pathologist (Professor Dennis Wright) to give a second opinion, and he made the diagnosis of mantle cell lymphoma, subsequently confirmed by staining for cyclin D1. While this does not yet allow us to conclude that all cases of FL will have sites, it does indicate that diagnostic criteria
must be met. Disturbingly, Belessi et al have added 10 cases, and only 4 of these have sites, all of which were in V_{H}. While this is a small number, it does not fit with our observations, and we await further findings from our cases and from the community.

Belessi et al then focus on events occurring in V_{K}, where they have sequence data on 10 cases. They make the point that levels of somatic mutation are lower in V_{K} than in V_{H}, an observation that we confirm in our set of sequences, which had 89% and 96% homology for V_{H} and V_{K}, respectively. This discrepancy has been reported in normal B cells and in other B-cell malignancies, eg, in Burkitt lymphoma. In relation to acquisition of glycosylation sites, it is obvious that they will be less likely to accumulate in V_{K}, and, in fact, 5 of 10 of the cases analyzed by Belessi et al were close to germline sequence. However, a low mutational rate may be sufficient since, in one of our cases with 99% homology in V_{K}, a site had been acquired. Belessi et al speculate on the reason for the differential mutational rate in V_{H} and V_{L}, and they suggest that “the somatic hypermutation machinery might have ceased to operate in the V_{K} locus at the time when neoplastic transformation had occurred.” In our view this is unlikely, given that intraclonal events occurring in V_{K} sequences in FL (Zhu et al) have sequence data on 10 cases. They make the point that levels of somatic mutation are lower in V_{K} than in V_{H}, an observation that we confirm in our set of sequences, which had 89% and 96% homology for V_{H} and V_{K}, respectively. This discrepancy has been reported in normal B cells and in other B-cell malignancies, eg, in Burkitt lymphoma. In relation to acquisition of glycosylation sites, it is obvious that they will be less likely to accumulate in V_{K}, and, in fact, 5 of 10 of the cases analyzed by Belessi et al were close to germline sequence. However, a low mutational rate may be sufficient since, in one of our cases with 99% homology in V_{K}, a site had been acquired. Belessi et al speculate on the reason for the differential mutational rate in V_{H} and V_{L}, and they suggest that “the somatic hypermutation machinery might have ceased to operate in the V_{K} locus at the time when neoplastic transformation had occurred.” In our view this is unlikely, given that intraclonal variation continues to accumulate in V_{K} sequences in FL (Zhu et al) and our unpublished observations, May 2002. An alternative explanation for the discrepancy in mutational level may be a lower frequency of sequence motifs that surround mutational hotspots, or a difference in the elements outside the coding region that influence somatic mutation. The role of germline V_{L} sequence in antigen recognition has been assumed to be less than that of V_{H}, due to the lack of D-segment genes. The relatively low level of somatic mutation would support this lesser role. However, crystallographic analysis clearly implicates V_{L} in antigen recognition, and the degree of involvement is likely to vary for different antibodies. Generalizations about the immensely variable structures of the binding site of antibodies may be difficult, but the presence of oligosaccharides, largely avoided by normal B cells, could be telling us something about B-cell tumors. Since there may be consequences for new treatment options, we await data from more matched sequences of FL with considerable interest.

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References


To the editor:

TT virus does not contaminate first-generation recombinant factor VIII concentrate

Recently, Azzi et al suggested TT virus (TTV) occurs in first-generation recombinant factor VIII concentrate (rFVIII) products with the stabilizer, human serum albumin (HSA) as its source. As these results differ from those of earlier studies, and Baxter’s internal findings, we reinvestigated 11 lots of rFVIII (Recombinate), and 13 lots of HSA. Lyophilized rFVIII was reconstituted with distilled water, or HSA used directly. One part of each sample was extracted directly and the other spiked with approximately 300 genome equivalents of TTV (derived from a TTV-positive plasma sample) before extraction, to control for inhibition of the polymerase chain reaction (PCR).

DNA was extracted from 200-μL samples (Blood Kit; Qiagen, Hilden, Germany) and the solution in the quantity described (6 μL) subjected to either nested PCR (primer sets untranslated region [UTR] A and B as described1,4), or single-stage PCR (primers T801 and T935, used previously for Baxter’s internal investigations5), using a thermally activated DNA polymerase (HotStarTaq, Qiagen). All primer sets are specific for the non-coding region (NCR, also UTR) of the TTV genome.

Both the single-stage and nested PCR reactions were done in 50 μL containing 1 U polymerase, 200 μM dNTP, and 50 pmol each of forward and reverse primer. The samples were overlaid with mineral oil, incubated for 14 minutes at 94°C and amplified for 45 cycles (single-stage PCR) or 35 cycles followed by 25 cycles (nested PCR) in a TRIO-Thermoblock (Biometra, Göttingen, Germany) with the following cycle profile: 30 seconds at 94°C, 30 seconds at 55°C, 60 seconds at 72°C with a final elongation at 72°C for 1 minute. The PCR products were analyzed using a 3.5% low-melting agarose gel stained with ethidium bromide.

Amplification of a positive control sample (DNA extracted from human TTV-positive plasma) with primer pair T801/T935 resulted in a PCR product of 199 bp as expected, with the UTR A primers of 143 bp, and the UTR B primers of 141 bp.

All rFVIII samples were negative with each primer set evaluated. To control for false-negative results caused by inhibition of PCR by the sample matrix, all samples were extracted again and PCR-tested after spiking with a TTV control. All these samples showed positive PCR signals, verifying the results from the unspiked rFVIII samples. Positive samples (TTV-positive human plasma) and negative controls (buffer) also included with each PCR reaction again verified the experimental set-up used.

Additionally, 13 lots of HSA were tested as described above. All samples were negative, with positive results for the same samples after spiking with a low amount of TTV-positive human plasma.

When TTV was discovered in 1997 and suggested to be associated with posttransfusion hepatitis, naturally patients, regulatory bodies, and the plasma products industry were concerned. Further investigations revealed a low viral load in plasma and high prevalence of TTV in up to 82% of plasma donations, and
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