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References

1. Ott G, Katzenberger T, Greiner A, et al. The t(11;18)(q21;q21) chromosome translocation is a frequent and specific aberration in low-grade but not high-grade malignant non-Hodgkin's lymphomas of the mucosa-associated lymphoid tissue (MALT-) type. Cancer Res. 1997;57:3944-3948.
2. Rosenwald A, Ott G, Stilgenbauer S, et al. Exclusive detection of the t(11;18)(q21;q21) in extranodal marginal zone B cell lymphomas (MZBL) of MALT type in contrast to other MZBL and extranodal large B cell lymphomas. Am J Pathol. 1999;155:1817-1821.
3. Baens M, Maes B, Steyls A, Geboes K, Marynen P, De Wolf-Peeters C. The product of the t(11;18), an API2-MLT fusion, marks nearly half of gastric MALT type lymphomas without large cell proliferation. Am J Pathol. 2000;156:1433-1439.
4. Dierlamm J, Wlodarska I, Michaux L, et al. Genetic abnormalities in marginal zone B-cell lymphoma. Hematol Oncol. 2000;18:1-13.
5. Remstein ED, James CD, Kurtin PJ. Incidence and subtype specificity of API2-MALT1 fusion translocations in extranodal, nodal, and splenic marginal zone lymphomas. Am J Pathol. 2000;156:1183-1188.
6. Barth TF, Bentz M, Leithauser F, et al. Molecular-cytogenetic comparison of mucosa-associated marginal zone B-cell lymphoma and large B-cell lymphoma arising in the gastro-intestinal tract. Genes Chromosomes Cancer. 2001;31:316-325.
7. Starostik P, Patzner J, Greiner A, et al. Gastric marginal zone B-cell lymphomas of MALT type develop along 2 distinct pathogenetic pathways. Blood. 2002;99:3-9.
8. Liu H, Ruskon-Fourmestraux A, Lavergne-Slove A, et al. Resistance of t(11;18) positive gastric mucosa-associated lymphoid tissue lymphoma to Helicobacter pylori eradication therapy. Lancet. 2001;357:39-40.
9. Peters K, Zettl A, Starostik P, et al. Genetic imbalances in primary gastric diffuse large B-cell lymphomas: comparison of comparative genomic hybridization, microsatellite, and cytogenetic analysis. Diagn Mol Pathol. 2000;9:58-65.
10. Yonezumi M, Suzuki R, Suzuki H, et al. Detection of AP12-MALT1 chimaeric gene in extranodal and nodal marginal zone B-cell lymphoma by reverse transcription polymerase chain reaction (PCR) and genomic long and accurate PCR analyses. Br J Haematol. 2001;115:588-594.
11. Ye H, Dogan A, Liu H, et al. Markedly variable frequencies of t(11;18)(q21;q21) in different MALT lymphomas [abstract]. Blood. 2001;98:767a.

To the editor:

Do B-cell chronic lymphocytic leukemia patients with Ig V_H3-21 genes constitute a new subset of chronic lymphocytic leukemia?

With interest we read the report of Tobin et al[1] describing a new subset of B-cell chronic lymphocytic leukemia (B-CLL). After mutation analysis of rearranged immunoglobulin variable heavy chain (IgV_H) genes, they observed that B-CLL patients with somatically mutated V_H3-21 genes have an unfavorable clinical course similar to B-CLL patients with unmutated immunoglobulin genes.

Their presented data and conclusions need some comments. In several studies,[1-3] as well as in our own unpublished data, a preferential usage of several V_H genes is observed. Data of a selection of frequently used V_H genes are summarized in Table 1. In all studies V_H1-69 is highly represented in B-CLL and is almost exclusively unmutated. But the frequent occurrence of the V_H3-21 gene found by Tobin et al, namely in 13% of all CLL, could not be confirmed by the 3 other studies indicated (Table 1; studies 2-4). This discrepancy of usage of the V_H3-21 gene is statistically significant (χ^2 analysis for V_H3-21 usage: studies 1 versus 2, $P = .013$; studies 1 versus 3, $P = .008$; studies 1 versus 4, $P = .020$). In addition, a statistically significant difference is observed for the overall mutation frequency between studies 1 and 2 (Table 1; χ^2 analysis, $P = .004$).

Several explanations for these different findings can be proposed: there might be a wrong annotation due to closely related germline IgV_H genes. V_H3-48 is the immunoglobulin gene with the highest homology to the V_H3-21, namely, 10 basepair differences between both genes at nucleotide level; but it seems unlikely that, due to mutations at most of these positions, the germline gene is not correctly assigned. Or there might be methodologic differences between the different studies. Each of the studies inevitably excluded samples that failed on the IgV_H mutation analysis, which is interesting information since this might give some insight in the sensitivity of the assays to detect clonal rearrangements. Finally, there might be a different patient population treated at each center or a particular patient group selected for IgV_H sequencing.

Next to the difference in usage of the V_H3-21 gene, the relatively low mutation load observed in the somatically mutated cases described by Tobin et al is striking. Thirteen patients showed a sequence homology to the germline V_H3-21 gene between 92.8% and 98%. Ten of those show a homology between 96% and 98% (Table 1, study 1). The threshold value of 98% to distinguish unmutated from mutated IgV_H genes was originally chosen because polymorphisms, which are quite common in V_H genes, can account for 2% of disparity.[4] But an alternative use of a 96% threshold is currently under debate. Kröber et al,[5] analyzing 300

Table 1. The most frequent V_H genes used in unmutated and mutated B-CLL across 4 studies

Homology to germline V_H gene	Unmutated/mutated cases, by study*			
	No. 1	No. 2	No. 3	No. 4
V_H1-69				
Below 98%	21/0	8/2	5/1	9/1
Below 96%	NM	8/2	5/1	9/1
V_H3-07				
Below 98%	0/4	0/5	1/8	0/5
Below 96%	NM	1/4	1/8	0/5
V_H3-21				
Below 98%	2/13	0/1	0/0	1/1
Below 96%	12/3	0/1	0/0	1/1
V_H3-23				
Below 98%	0/3	0/7	2/1	3/8
Below 96%	NM	1/6	2/1	4/7
V_H3-30				
Below 98%	2/4	3/8	2/0	6/4
Below 96%	NM	4/4	2/0	6/4
Total number of patients	119	74	64	84
Overall frequency of cases, unmutated/mutated cases, %				
Below 98%	58/42	35/65	48/52	45/55
Below 96%	NM	47/53	55/45	57/43
Template type on which analysis was performed	DNA	DNA	RNA	RNA or DNA
Forward primer(s)	V_H1 to V_H6	FR1/2†	V_HL1 to V_HL6	V_H1 to V_H6
Reverse primers(s)	J_H	JH cons†	C_H	J_H

NM indicates not mentioned.

*Study 1 is Tobin et al[1]; study 2, AZ Sint-Jan, Brugge, unpublished data, 2002; study 3, Fais et al[2]; study 4, Hamblin et al.[3]

†Aubin et al.[6]

CLL patients, found that a cutoff value of 96% separates more significantly 2 groups with a different overall survival than the cutoff value of 98%. When a threshold of 96% sequence homology is used for the patients studied by Tobin et al,[1] 12 of 15 B-CLL cases using the V_H3-21 gene are within the unmutated cases group, which is known to have an unfavorable prognosis.

Therefore the definition of a new subset of CLL patients as suggested by Tobin et al seems premature. Multicenter studies of a large number of patients for their IgV_H mutation status and analyses of clinical data are needed to explain the observed differences in V_H-gene usage and define the threshold with the most significant prognostic value.

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References

1. Tobin G, Thunberg U, Johnson A, et al. Somatically mutated Ig V_H3-21 genes characterize a new subset of chronic lymphocytic leukemia. Blood. 2002;99: 2262-2264.
2. Fais F, Ghiotto F, Hashimoto S, et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. J Clin Invest. 1998;102:1515-1525.
3. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V_H genes are associated with a more aggressive form of chronic lymphocytic leukemia [see comments]. Blood. 1999;94:1848-1854.
4. Matsuda F, Shin EK, Nagaoka H, et al. Structure and physical map of 64 variable segments in the 3'0.8-megabase region of the human immunoglobulin heavy-chain locus. Nat Genet. 1993;3:88-94.
5. Kröber A, Bühler A, Kienle D, et al. Analysis of VDJ rearrangement structure and VH mutation status in chronic lymphocytic leukemia [abstract]. Blood 2001;98:358a.
6. Aubin J, Davi F, Nguyen-Salomon F, et al. Description of a novel FR1 IgH PCR strategy and its comparison with three other strategies for the detection of clonality in B cell malignancies. Leukemia. 1995;9:471-479.

Response:

IgV_H3-21 gene usage in chronic lymphocytic leukemia

We have recently reported a restricted usage of the V_H3-21 gene in mutated B-cell chronic lymphocytic leukemia (CLL), where the V_H3-21–utilizing cases displayed distinctive characteristics such as clonal expression of λ light chains and shorter average length of the complementarity determining region (CDR) 3.[1] Interestingly, this subset of mutated V_H3-21 cases also showed an inferior survival compared with the remaining mutated CLL cases. We have therefore suggested that mutated V_H3-21 cases might constitute a new entity of CLL.

In their letter Nollet et al have compared our findings with 2 prior studies on V_H gene usage and their own unpublished results, and they found it remarkable that we have shown a significantly higher number of V_H3-21–utilizing CLL cases compared with the other studies.[1-4] Nollet et al have speculated on the basis of the divergent results. First, they have addressed the correctness of the V_H gene alignment. We have used 3 different germline databases, and it is not likely that we have aligned them incorrectly, especially since all 3 databases indicate V_H3-21 gene usage and the closest related gene, V_H3-48, displays 7-bp differences to the V_H3-21 gene. Second, Nollet et al have suggested that methodological differences could account for the disagreement between the studies. Certainly, different primer systems may have varied capability to amplify different V_H genes, but we find it rather unlikely that this would explain the diverse results. Third, they have argued that the selection of patients could differ between the studies. We believe that the selection of patient material is the most probable factor explaining the different findings, since none of the referred studies are population-based. Prior studies have reported different frequencies of the mutated and unmutated subsets, and the median survival for these 2 groups has varied, thus indicating that different patient populations have been selected for V_H gene analysis.[1-5] In addition, we cannot rule out that the difference in frequency of V_H3-21 usage compared with previous studies reflects a different genetic background or environmental factors in the Scandinavian population/countries.

Nollet et al have also brought up the discussion about the appropriate cutoff level to distinguish mutated and unmutated CLL. We have chosen the mutation border of 98% to eliminate the possibility of polymorphic sites in accordance with previous CLL studies.[3-5] Kröber et al have recently shown that a cutoff level at 97% gave the best separation of the 2 subsets regarding overall survival.[6] Using this threshold, some of our mutated V_H3-21 cases would indeed be interpreted as unmutated. But the true biologic level that distinguishes somatically mutated V_H genes from unmutated ones is currently unknown and may also differ for different V_H genes. Most of our mutated V_H3-21 cases have a low frequency of mutations, but almost half of the mutations were found in hypermutation hotspot regions (RGYW/WRCY), indicating that they were introduced by the somatic hypermutation mechanism rather than representing V_H3-21 gene polymorphisms. To solve this issue, future studies are warranted to analyze the germline V_H genes in cases that display a low number of somatic hypermutations and compare the germline sequence with the sequence of the clonal rearrangement.

Despite the V_H gene mutation status, we still believe that the V_H3-21–utilizing cases represent an additional entity, especially since they display certain features with short and in some cases identical CDR3 structure and a highly preferential $V_λ$2-14 usage. We have now identified 24 cases with V_H3-21 usage (out of 215 analyzed CLL cases), where 17 were mutated and 7 unmutated according to the 98% cutoff level. The V_H3-21 CLL cases from our published study originated from the central and northern counties in Sweden, but we have now also found V_H3-21–utilizing CLL cases from both Finland and southern Sweden. In addition, a German research group has also identified V_H3-21–positive cases with similar genotypic findings that showed an inferior outcome irrespective of their mutational status (S. Stilgenbauer, personal communication, April 3, 2002). But it will be important to study larger numbers of V_H3-21–positive cases to confirm our findings and to fully evaluate the prognostic impact of V_H3-21 gene usage in CLL. Additionally, multicenter and population-based studies are needed to investigate the clinical significance of V_H gene usage in general in CLL.

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References

1. Tobin G, Thunberg U, Johnson A, et al. Somatically mutated Ig V_H3-21 genes characterize a new subset of chronic lymphocytic leukemia. Blood. 2002;99: 2262-2264.
2. Fais F, Ghiotto F, Hashimoto S, et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. J Clin Invest. 1998;102:1515-1525.
3. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood. 1999;94:1848-1854.
4. 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;94:1840-1847.
5. Maloum K, Davi F, Merle-Beral H, et al. Expression of unmutated VH genes is a detrimental prognostic factor in chronic lymphocytic leukemia. Blood. 2000;96: 377-379.
6. Kröber A, Seiler T, Benner A, et al. V_H mutation status, CD38 expression level, genomic aberrations, and survival in chronic lymphocytic leukemia. Blood. In press.

To the editor:

Mutation analysis of transferrin-receptor 2 in patients with atypical hemochromatosis

Most cases of hereditary hemochromatosis are due to a single nucleotide mutation in the hemochromatosis gene *(HFE)* that results in a Cys to Tyr conversion at amino acid 282 (Cys282Tyr) in the protein.[1] Sequencing revealed a second mutation (His63Asp) in the HFE protein, but the penetrance of this mutation is much lower compared to Cys282Tyr. Although a candidate early in the search for the hemochromatosis gene, the transferrin receptor (TFR) was not found to be mutated in hemochromatosis. Several years ago, we discovered a second human transferrin receptor termed transferrin receptor 2 (TFR2).[2] Recently, a hemochromatosis pedigree was discovered in Sicily, where a non–sense mutation at position 250 in the *TFR2* gene was found.[3] Affected individuals lacked the Cys282Tyr *HFE* mutation. The carrier frequency of this mutation is 0.9% among a cohort of southern and central Europeans.[4] Roetto and colleagues recently reported 2 new mutations (at exon 2, 84-88insC, resulting in Glu60Xaa; and at exon 4, Met172Lys) in iron overload patients having what has been termed hemochromatosis type 3.[5] A fourth inactivating mutation of *TFR2* (a 4–amino-acid loss Ala-Val-Ala-Gln at 594-597) has recently been reported.[6]

Here, we investigated the genomic DNA from individuals having atypical hemochromatosis with the aim to look for a correlation between mutations of the *TFR2* gene and an altered iron phenotype. We also asked whether differences in penetrance of the Cys282Tyr mutation were associated with mutations in *TFR2*. The study included several selected cohorts: (1) Sibling pairs homozygous for *HFE* Cys282Tyr with a discordant phenotype. The most common discordance between homozygote siblings was serum ferritin concentration. Many of these patients, however, also exhibited significant differences in liver fibrosis and aminotransferase levels (11 patients); (2) Non-Cys282Tyr *HFE* homozygotes with evidence of iron excess (7 patients), which included patients having evidence of iron overload but lacking the Cys282Tyr mutation (3 patients), a patient normal at the 282 position but homozygous for His63Asp (1 pt), and patients with iron overload but heterozygous for Cys282Tyr (3 patients); (3) Homozygous (Cys282Tyr) relatives of probands identified in a blood bank screen (because of elevated transferrin saturation) who have evidence of morbidity (3 patients); and (4) Cys282Tyr homozygotes under 30 years of age with iron overload (9 patients). In addition, we investigated samples from 10 healthy individuals as controls. Values for serum iron, transferrin saturation, and serum ferritin were available from all individuals.

Genomic DNA was analyzed by polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP) as previously described.[7] Nineteen primer pairs were designed using sequence information from the Genbank for *TFR2* (GI 3135305) from the complete sequence for chromosome 7q22. Each PCR reaction contained 20 ng DNA, 10 pmol of each of the primers, 2 nmol of each of the deoxynucleoside triphosphates (dNTPs), 0.5 units *Taq* DNA polymerase, and 3 μCi (11.1×10^4 Bq) α-[^{32}P] deoxycytidine triphosphate (dCTP) in 20 μL of the specified buffer with 1.5 mM $MgCl_2$. The PCR cycles were 30 seconds for denaturing at 94°C, 40 seconds for annealing at 60°C, and 60 seconds for extension at 72°C (35 cycles). The samples were separated on a 6% nondenaturating polyacrylamide mutation detection enhancement (MDE) gel. DNA purified from mutant candidate bands showing altered migration through SSCP analysis was directly sequenced by the ABI PRISM dye terminator cycle sequencing reaction (Perkin Elmer, Foster, CA).

Analysis of exon 10 of *TFR2* revealed an aberrantly shifted band (Figure 1A) in a sample from an individual in group A, pedigrees in which a discordance existed in phenotype between Cys282Tyr homozygous sibling pairs. Direct nucleotide sequencing found a G>A transversion at nt 1391, resulting in a substitution at codon 455 of Gln (mutant) for an Arg (normal sequence). The

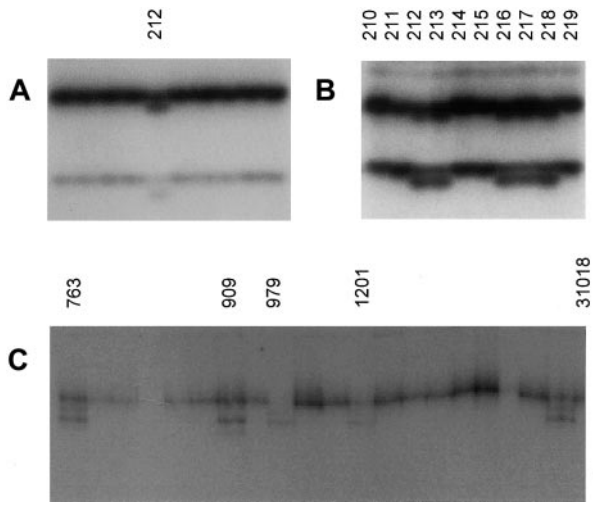

Figure 1. Polymerase chain reaction–single strand conformation polymorphism analysis of exons 10 and 18 in atypical hemochromatosis. (A) Sample no. 212 (mother of proband) had an aberrantly shifted band in exon 10. Direct nucleotide sequencing found a G>A transversion resulting in a substitution at codon 455 of Gln (aberrant) for an Arg (normal sequence). (B) Four of the genomic DNA samples (nos. 213, 216-218) from the children of proband no. 212 showed, by PCR-SSCP, the same DNA-migration pattern as sample no. 212. Direct DNA sequencing of these samples revealed Arg455Gln. (C) Evidence for polymorphism in the 3′ untranslated region of exon 18 of TRF2. Samples 763, 909, 979, 1201, and 31018 showed the same polymorphic pattern by SSCP. Direct sequencing of these samples identified a change of G>C at nucleotide 154513 (GI3135305).