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Brief Report

LYMPHOID NEOPLASIA

High frequency of inactivating tetraspanin *CD37* mutations in diffuse large B-cell lymphoma at immune-privileged sites

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KEY POINTS

- **Loss-of-function mutations in *CD37* occur predominantly in diffuse large B-cell lymphoma at immune-privileged sites.**
- ***CD37*-mutated lymphoma B cells show impaired CD37 cell-surface localization, which may have implications for anti-CD37 therapies.**

Tetraspanin CD37 is predominantly expressed on the cell surface of mature B lymphocytes and is currently being studied as novel therapeutic target for B-cell lymphoma. Recently, we demonstrated that loss of CD37 induces spontaneous B-cell lymphoma in *Cd37*-knockout mice and correlates with inferior survival in patients with diffuse large B-cell lymphoma (DLBCL). Here, *CD37* mutation analysis was performed in a cohort of 137 primary DLBCL samples, including 44 primary immune-privileged site-associated DLBCL (IP-DLBCL) samples originating in the testis or central nervous system. *CD37* mutations were exclusively identified in IP-DLBCL cases (10/44, 23%) but absent in non-IP-DLBCL cases. The aberrations included 10 missense mutations, 1 deletion, and 3 splice-site *CD37* mutations. Modeling and functional analysis of *CD37* missense mutations revealed loss of function by impaired CD37 protein expression at the plasma membrane of human lymphoma B cells. This study provides novel insight into the molecular pathogenesis of IP-DLBCL and indicates that anti-CD37 therapies will be more beneficial for DLBCL patients without *CD37* mutations. (*Blood*. 2019;134(12):946-950)

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of B-cell non-Hodgkin lymphoma. Several genetic aberrations are known to underlie DLBCL development and progression. Recently, we identified that *Cd37*-knockout mice spontaneously develop B-cell lymphoma.[1] Moreover, CD37 protein expression has been reported to be an independent prognostic factor for patient outcome in R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone)-treated DLBCL patients.[2]

CD37 (Tspan-26), a member of the tetraspanin superfamily, is highly expressed on mature B lymphocytes[3] and is currently studied as novel target for chimeric antigen receptor (CAR)-T cell therapy[4] and antibody-based therapies for patients with relapsed/refractory non-Hodgkin lymphoma.[5,6] Tetraspanins control the spatial distribution of membrane proteins through specific interactions with (immune-)receptors and signaling molecules, thereby facilitating intracellular signaling pathways.[7,8] Tetraspanins are associated with different malignancies,[9] but the role of altered tetraspanin function in DLBCL pathogenesis is unclear.

This study evaluated the mutational status of the human *CD37* gene in DLBCL patients, particularly in DLBCL at immune-privileged sites (IP-DLBCL), including primary testis lymphoma (PTL) and primary central nervous system lymphoma (PCNSL). With functional analysis, the effect of identified *CD37* mutations on CD37 protein expression at the cell membrane was studied.

Study design

Cases

The study was conducted with 137 archived primary DLBCL samples, including 44 IP-DLBCL. The discovery cohort included 31 DLBCL samples and 106 DLBCL samples were analyzed in the validation cohort. Samples were collected from multiple Dutch university hospitals (supplemental Table 1, available on the *Blood* Web site).

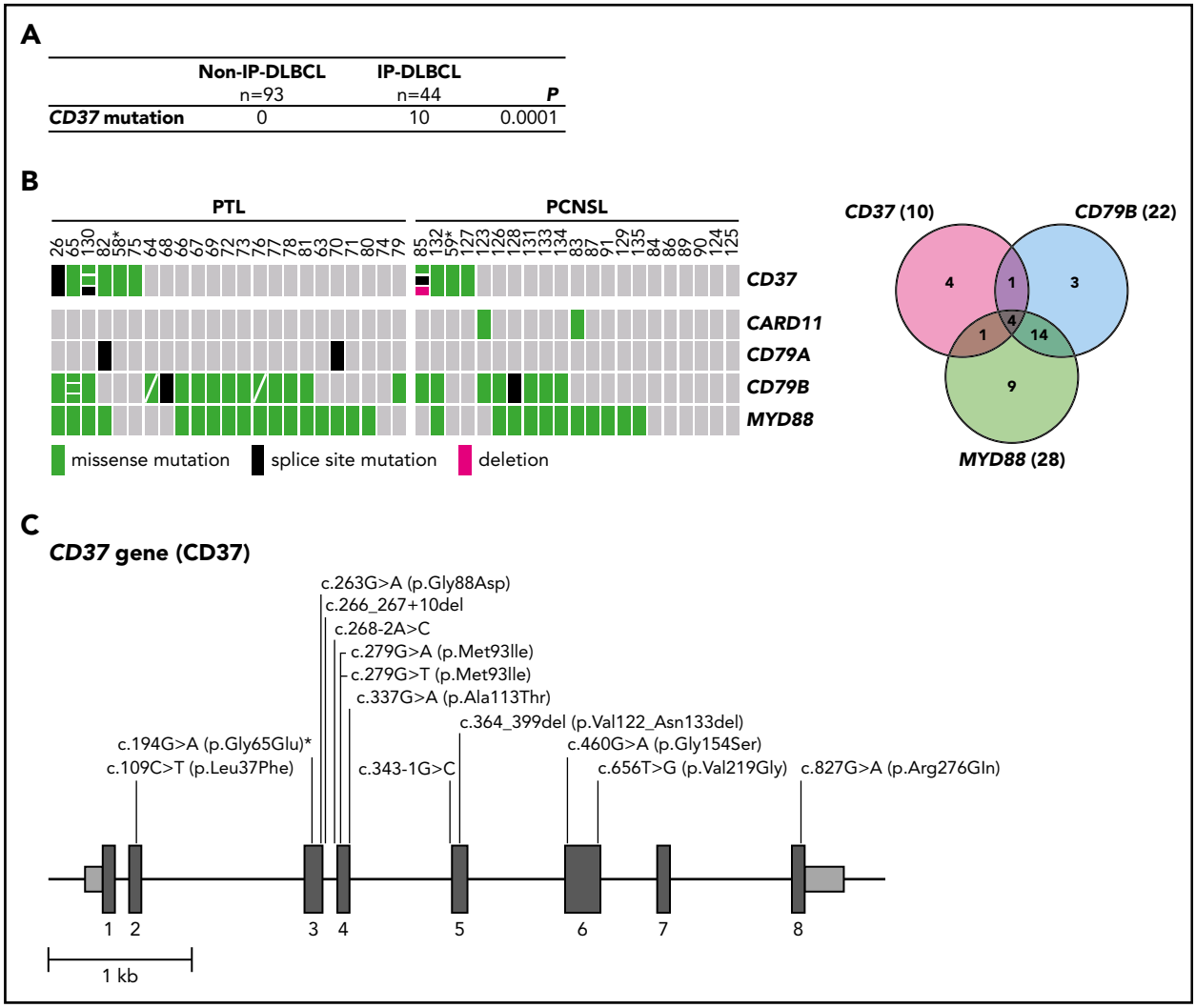

Figure 1. High frequency of mutated *CD37* in IP-DLBCL. (A) Frequency of *CD37* mutations in non-IP-DLBCL vs IP-DLBCL. The total number of lymphoma cases for each group is indicated. Numbers in the *CD37* mutation row indicate the number of lymphoma cases carrying ≥1 *CD37* mutation. *P* values were obtained using the Fisher's exact test. (B) OncoPrint (left) and Venn diagram (right) show the frequencies of *CD37*, *CARD11*, *CD79A*, *CD79B*, and *MYD88* mutations in all IP-DLBCL cases studied (23 PTL and 21 PCSNL). Columns show individual tumors. Asterisk indicates samples from the same patient containing the *CD37* germline mutation. Genetic details per mutation can be found in supplemental Table 2. Green, missense mutation; black, splice-site mutation; magenta, deletion; gray, no mutation. (C) Schematic representation of mutations detected in the *CD37* gene. Dark gray boxes represent the coding sequence of *CD37*, light gray boxes are the noncoding sequence before exon 1 (begin) and after exon 8 (end), and black lines represent introns. Asterisk indicates the *CD37* germline mutation.

Mutation analysis

CD37 mutation analysis of the discovery cohort was performed by amplicon-based sequencing with Ion Torrent and Sanger sequencing. For the validation cohort, single-molecule molecular inversion probe mutation analysis and sequencing with Ion Torrent included the *CD37* gene and hotspot loci of *CARD11*, *CD79A*, *CD79B*, and *MYD88*.

Functional analysis of mutant *CD37*

The CD37-Gly88Asp-green fluorescent protein (GFP) and CD37-Gly65Glu-GFP mutant constructs were generated by introducing point mutations c.263G>A or c.194G>A in hCD37-wild-type (WT)-GFP[1] using site-directed mutagenesis. Human BJAB and OCI-Ly8 lymphoma B cells were transfected with the AMAXA Nucleofector or the Neon transfection system and analyzed for CD37 protein expression and subcellular localization using western blot and confocal fluorescence microscopy.

Additional experimental details are provided in supplemental Materials and methods.

Results and discussion

In a discovery cohort of 31 DLBCL cases, including 5 PTL cases, *CD37* mutations were analyzed. *CD37* mutations were identified in 2 PTL cases, 1 harboring a missense mutation (c.263G>A (p.Gly88Asp)) and the other a splice-site mutation (c.343-1G>C), but none were identified in the non-IP-DLBCL samples (n = 26). To confirm this finding, a validation cohort consisting of 39 IP-DLBCL cases (18 PTL and 21 PCNSL) and 67 non-IP-DLBCL cases was analyzed for *CD37* mutations along with *CARD11*, *CD79A*, *CD79B*, and *MYD88* hotspot mutations (Figure 1; supplemental Table 2). In this analysis, we also included the 5 PTL cases from the discovery cohort. Besides confirmation of the 2 initially identified *CD37* mutations, we uncovered 12 additional mutations (variant allele frequency 5.7% to 49%; Figure 1B-C).

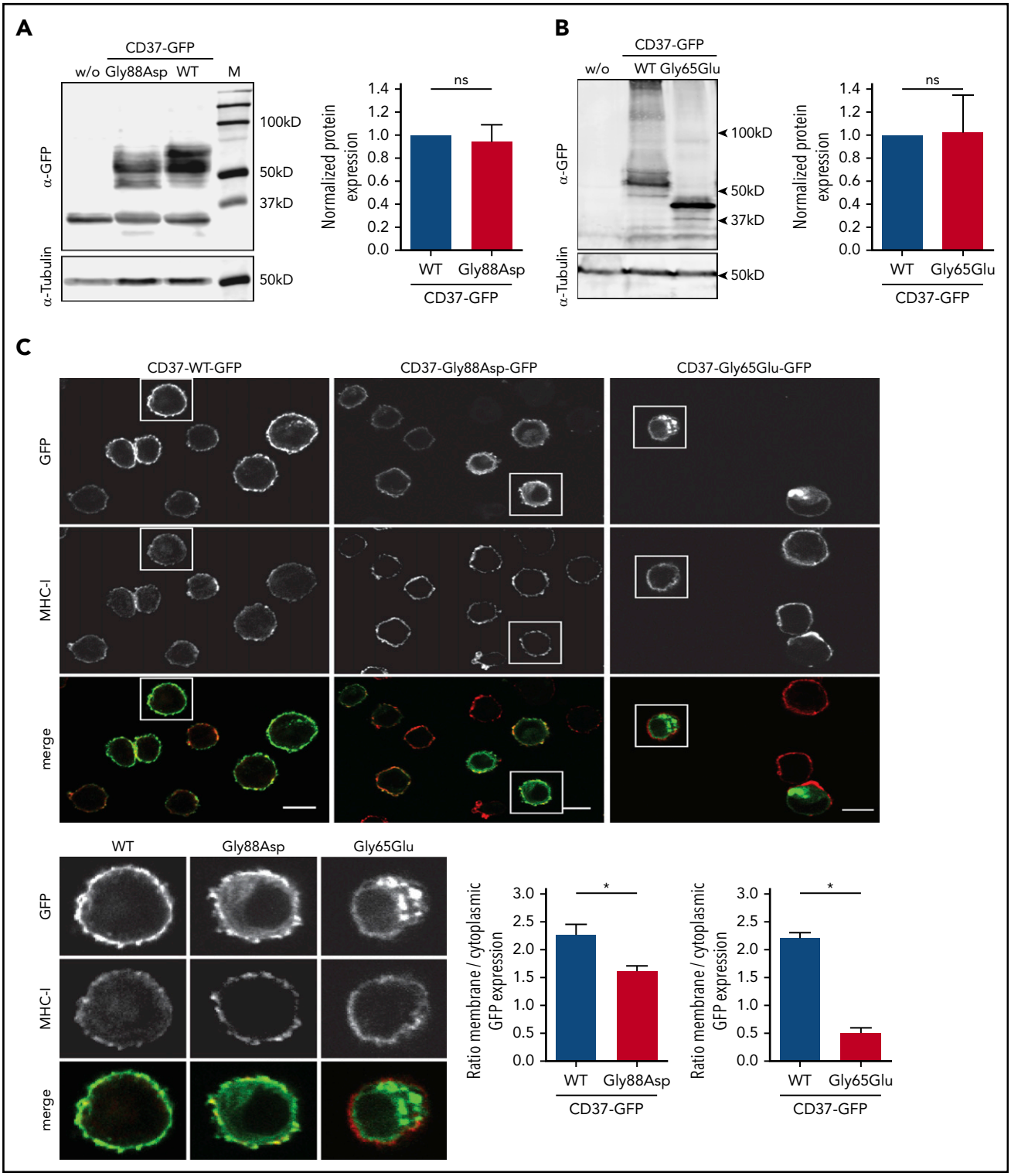

Figure 2. Mutations in *CD37* cause aberrant CD37 glycosylation and localization. Western blot analysis of CD37 protein expression in BJAB lymphoma B cells transfected with CD37-WT-GFP, CD37-Gly88Asp-GFP (A), or CD37-Gly65Glu-GFP (B). Blots were probed with α-GFP to detect CD37-GFP expression (50-75 kDa; upper blot). α-Tubulin was used as a loading control (lower blot). Protein expression level of CD37-mutant-GFP was normalized to CD37-WT-GFP for each experiment (n = 3). ns, not significant ($P = .73$ [left], $P = .96$ [right]), paired *t* test. (C) Confocal microscopy images of BJAB cells expressing CD37-WT-GFP and CD37-Gly88Asp-GFP or CD37-Gly65Glu-GFP (green) costained for MHC-I (red) to identify the plasma membrane. Overview images (top) and single-cell images (bottom left) show representative cells of 3 independent experiments for both CD37-WT-GFP and CD37-mutant-GFP. Scale bar, 10 μm. Ratio between membrane and cytoplasmic GFP expression was quantified from 10 representative cells of 3 independent experiments. *$P = .023$ (left), $P = .011$ (right), paired *t* test. All data represent mean ± standard error of the mean.

Intriguingly, all *CD37* mutations were exclusively present in IP-DLBCL cases (n = 10/44, 23%); and none were present in the non-IP-DLBCL cases (n = 0/93; Fisher's exact test, $P = .0001$; Figure 1A). Mutation rates of frequently affected genes *CARD11* (5%), *CD79A* (5%), *CD79B* (50%), and *MYD88* (64%) in our cohort (Figure 1B; supplemental Table 2) were in line with other DLBCL studies.[10-13] Whole-genome and exome sequencing studies of DLBCL report low mutation frequencies of *CD37*, ranging from no mutations to 8%.[11,14-17]

The *CD37* mutations identified in the validation cohort were detected in 8 IP-DLBCL cases and included 9 missense mutations (c.109C>T (p.Leu37Phe), c.194G>A (p.Gly65Glu) [twice], c.279G>A (p.Met93Ile), c.279G>T (p.Met93Ile), c.460G>A, (p.Gly154Ser), c.656T>G (p.Val219Gly), c.337G>A (p.Ala113Thr), and c.827G>A (p.Arg276Gln)), 1 in-frame deletion (c.364_399del (p.Val122_Asn133del)), and 2 splice-site mutations (c.266_267+10del, c.268-2A>C) (Figure 1C; pathogenicity scores are listed in supplemental Table 2). Within 1 patient, missense mutation c.194G>A (p.Gly65Glu) was detected in 2 primary lymphoma specimens as well as healthy control tissue, but not described as a single-nucleotide polymorphism, indicating the presence of a *CD37* germline mutation. To the best of our knowledge, this is the first reported *CD37* germline mutation in humans.

To determine the effect of splice-site mutation c.343-1G>C, complementary DNA of this DLBCL sample was sequenced, which revealed an 11-bp deletion (supplemental Figure 1). This resulted in a frameshift in the *CD37* mRNA sequence, leading to a completely altered protein after transmembrane domain 3. Based on previous studies on tetraspanin *CD81* in B cells,[18] this mutation is predicted to result in a nonfunctional CD37 protein, since the essential extracellular domain 2 is truncated.[19] Next, the *CD37* missense mutations and in-frame deletion were mapped onto the reported crystal structure of tetraspanin CD81[20] (supplemental Figure 2). Deletion c.364_399del (p.Val122_Asn133del) disrupts an important structural element of extracellular domain 2, namely the A helix, and is therefore also predicted to result in a nonfunctional CD37 protein.[19]

To further assess whether the observed missense mutations resulted in CD37 loss of function, we introduced 2 of the identified *CD37* mutations, c.263G>A (p.Gly88Asp) and c.194G>A (p.Gly65Glu), in human lymphoma B cells using a GFP expression vector. WT CD37 protein appeared as 50- to 75-kDa bands on western blot (Figure 2A-B; supplemental Figure 3A) due to heavy glycosylation. Protein isoforms with the highest glycosylation state (±70 kDa) were less pronounced in lymphoma B cells expressing mutant CD37-Gly88Asp-GFP (Figure 2A), and CD37-Gly65Glu-GFP showed a lower-molecular-weight band on western blot indicating a glycosylation defect (Figure 2B). Total CD37-GFP protein levels of both mutants were similar to CD37-WT-GFP (Figure 2A-B).

Next, the subcellular localization of the CD37 mutants was determined using confocal microscopy. Lymphoma B cells expressing the CD37-GFP mutants had significantly lower cell membrane expression and more retention of intracellular CD37 protein than cells expressing CD37-WT-GFP (Figure 2C; supplemental Figure 3B). Moreover, CD37-Gly88Asp had a dominant-negative effect on the membrane expression of CD37-WT protein (supplemental Figure 4). These data indicate that these missense mutations result in defective glycosylation and trafficking of CD37 in human lymphoma B cells, which corresponds to the reported role of glycosylation as a sorting signal for protein transport to the plasma membrane.[21] Moreover, modification of cell-surface proteins by defective glycosylation frequently contributes to cancer development by enhancing invasive growth and tumor cell migration.[22]

We previously showed that loss of CD37 results in increased interleukin-6 signaling and STAT3 activation,[1] which are both known to be involved in the pathogenesis of hematological malignancies.[23] *MYD88* mutations also stimulate cell survival by promoting JAK/STAT3 signaling and interleukin-6 secretion, suggesting a similar phenotype.[24] As proposed for *MYD88* and *CD79B* mutations in IP-DLBCL,[12] mutations in *CD37* could therefore provide a survival advantage in the otherwise stimulus-poor environment of immune-privileged sites.

Seven out of 9 evaluable cases with a *CD37* mutation did not express CD37 protein (supplemental Figure 5 and supplemental Table 1). Since immunohistochemistry does not distinguish between cell-surface and cytosolic expression, the importance of CD37 in DLBCL biology[1,2] is likely underestimated. Moreover, the recent identification of loss of CD19 expression as a mechanism underlying disease relapse upon CD19 CAR-T cell therapy[25] emphasizes the need to investigate CD37 surface expression in hematologic malignancies prior to the use of novel CD37 CAR-T cell therapies.

In conclusion, inactivating *CD37* mutations occur predominantly in IP-DLBCL and lead to aberrant CD37 cell-surface expression. This study provides novel molecular insight into DLBCL at immune-privileged sites and indicates that CD37-based therapies are more likely to be beneficial for DLBCL patients that harbor no *CD37* mutations.

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Authorship

Contribution: S.E., C.M.d.W., M.v.d.B., S.v.D., B.S., and A.B.v.S. designed the study; S.E., C.M.d.W., M.v.d.B., S.v.D., and A.B.v.S. wrote the manuscript; M.v.d.B., C.J.H., W.S., M.G.M.R., D.d.J., J.S.P.V., A.H.G.C., and B.S. collected DLBCL tissue and corresponding pathological information; S.E., C.M.d.W., M.v.d.B., E.J., A.v.d.S., S.v.D., P.J.T.A.G., A.E., R.A.L.d.G., and M.B. performed mutation analysis; S.E., F.A., L.J., and S.v.D. performed functional in vitro experiments; V.N.

performed CD37 modeling studies; and J.H.v.K., S.v.D., B.S., and A.B.v.S. supervised the work.

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Footnotes

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