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The mutational landscape of cold agglutinin disease: *CARD11* and *CXCR4* mutations are correlated with lower hemoglobin levels

To the Editor:

Primary cold agglutinin disease (CAD) is a rare autoimmune hemolytic anemia mediated by monoclonal IgM autoantibodies that bind to the blood group I antigen resulting in erythrocyte agglutination and complement activation. Primary cold agglutinin disease is caused by a unique indolent B-cell lymphoproliferative disorder of the bone marrow. We have previously reported recurrent *KMT2D* and *CARD11* mutations in a small series of CAD patients by whole exome sequencing (WES) of six cases and targeted sequencing of 10 cases.[1] Recently we reported the presence of trisomy 3, 12, or 18 in CAD using cytogenetic microarray and WES. Our group has evaluated the use of bendamustine-rituximab therapy for CAD.[2] Unfortunately, present B-cell directed therapies are not always well tolerated by unfit patients. Therefore, a search for new, low-toxic treatment options is warranted. The goal of this study was to explore genetic changes in CAD that may serve as more specific targets for treatment. In this study, we present comprehensive data on the mutational landscape of CAD in the context of clinical data.

We have analyzed the bone marrow from 18 patients with CAD, included in a recent clinical trial,[2] by WES (Supplementary Information S1). The total number of nonsynonymous mutations detected in each CAD sample ranged from 13 to 62, while the number of all mutations in the sample cohort in the exome regions ranged from 36 to 163 (Figure 1 (A)). Four genes showed nonsynonymous mutations in more than 20% of patients (Table S1): *KMT2D* (12/18; 67%; including splice region mutation), *IGLL5* (8/18, 44% and 11/18, 61% including non-coding region mutations), *CARD11* (6/18, 33%), and *CXCR4* (4/18, 22% and 5/18, 28% including non-coding region mutations). Additionally, several genes showed recurrent nonsynonymous mutations in two or three cases each (11%–17%): *HIST1H1E*, *TMPRSS7*, *CSMD3*, *FAT1*, *FAT4*, *PHLDB1*, *CFTR*, *EP300*, *GRIK2*, *LPIN3*, *LTB*, *MACF1*, *NBEA*, *NEFH*, *PCNX2*, *PKHD1L1*, *RRAGC*, *SLC30A9*, *TMEM132E* and *ZNF618* (Figure 1(B),(C); Table S3). Many of these genes are also mutated in lymphomas. Several other unique gene mutations were also found (Table S3).

All patients with either *CARD11* or *CXCR4* mutations, or both, had concurrent *KMT2D* mutations (Figure 1(C)). Almost all patients with *KMT2D* mutations (10/12, 83%) had at least four other recurrent mutations. Patients with *CARD11* (six cases) or *CXCR4* (five cases) mutations had at least four other recurrent mutations. All *CARD11* mutations are projected to be activating mutations disrupting the coiled-coil domain of *CARD11*.[1] The *CXCR4* functional mutations were detected in four patients (Table S1). These mutations are frameshift mutations located very close to the C-terminal end of the protein at positions 316–330, and are projected to prevent receptor internalization creating a state of prolonged activation. The *IGLL5* gene mutations were located only in the five prime UTR comprising exon 1 and intron 1, with the region affected being less than 600 bp long. (Figure S2).

A difference in hemoglobin levels prior to treatment was found between patients with, and those without mutations in either *CARD11* or *CXCR4*, or both (Figure 1(C), Figure S3, Table S2). The median difference between the lower limit of normal and actual hemoglobin level (adjusted for sex) for patients with *CARD11*, *CXCR4*, or both, mutations was 4.5 g/dl (mean difference 4.7 g/dl) and for patients without *CARD11* or *CXCR4* mutations 1.6 g/dl (mean difference 2.2 g/dl). The difference between the groups was statistically significant ($p = 0.026$; Mann–Whitney 2-tailed *U* test).

Pathway analysis showed that chromatin modification and chromatin organization were the most affected pathways in CAD patients. Most of the patients (15/18; 83%) had mutations of at least one gene involved in chromatin modification or organization (Figure 1(C) and Figure S3). In addition to *KMT2D* there were multiple other genes mutated in different samples: *ASH1L*, *ARID1B*, *BRD1*, *CACNA1D*, *CREBBP*, *DNMT3A*, *EP300*, *HDAC1*, *HIST1H2BO*, *HIST1H3G*, *JADE3*, *KAT8*, *KDM6A*, *NFKB1*, *PPARD*, *PRDM16*, *SAP18*, *SETD1B*, *SMARCC1* and *TBL1XR1*. Additionally, 14/18 (78%) samples showed mutations in genes involved in regulation of the nuclear factor kappa B (NF-κB) pathway (Figure 1(C),(D); Figure S3). In addition to *CARD11* and *CXCR4*, other genes included were *CASP3*, *COL1A1*, *CREBBP*, *DLL1*, *EP300*, *ERBB3*, *FBXW7*, *FGFR1*, *HDAC1*, *IRAK2*, *LRRC7*, *LTB*, *MTOR*, *NFKB1*, *RBCK1*, *TBL1XR1* and *TLR3*. Only 2/18 patients did not show mutations affecting either chromatin modification or organization, or affecting the NF-κB pathway (CAD-1.34 and CAD-7). Of interest, these two patients had close to normal hemoglobin levels (Figure S3).

Somatic *KMT2D* mutations are found in many lymphoma types, while constitutional *KMT2D* mutations give rise to Kabuki syndrome.

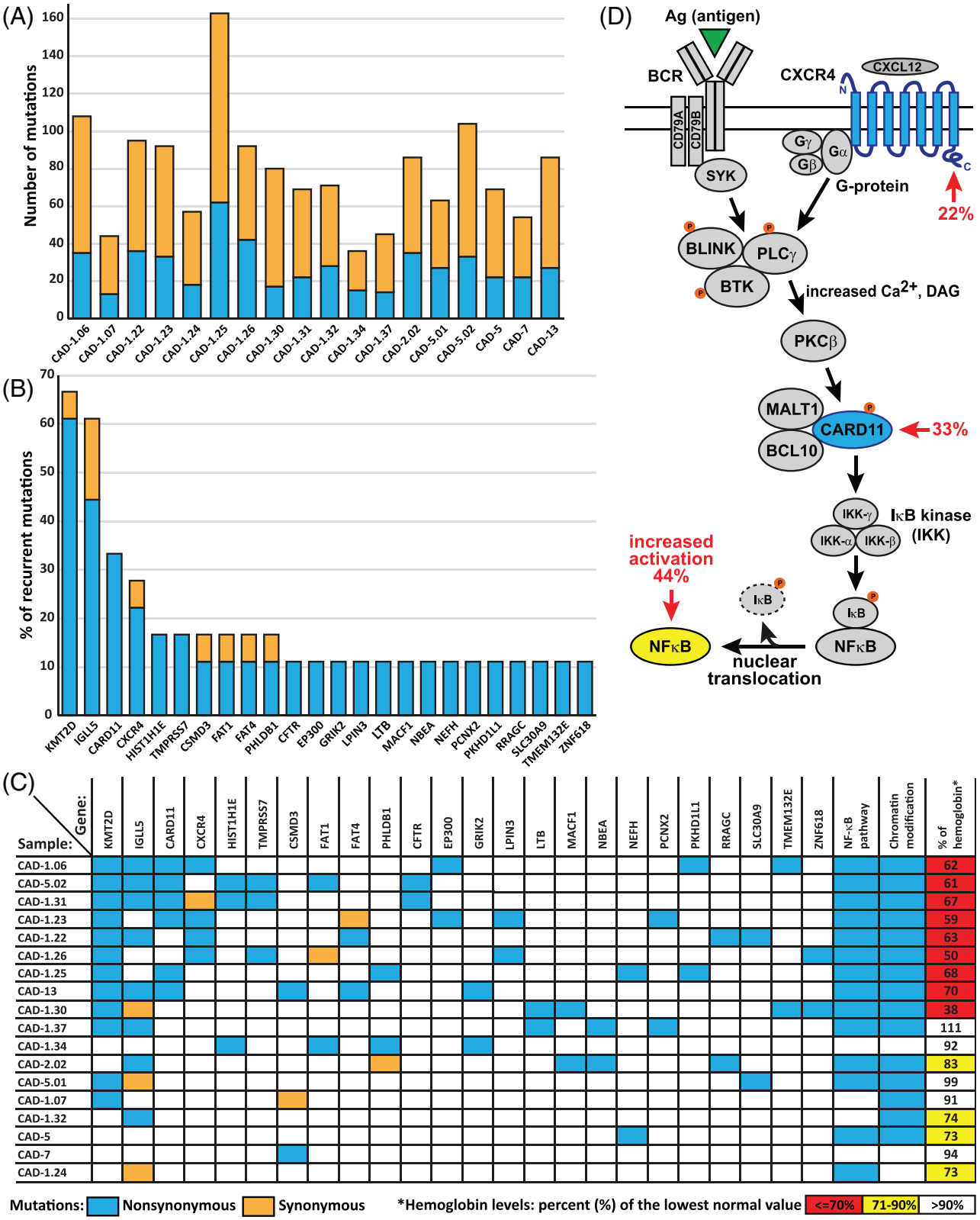

FIGURE 1 The mutational landscape of CAD detected by whole exome sequencing. (A) Number of somatic mutations in individual CAD patients. Mutations are color-coded as nonsynonymous (blue) or synonymous (orange). (B) Recurrent somatic mutations in CAD patients. The y-axis shows the percentage of patients with mutations in genes indicated on x-axis. (C) Overview of recurrently mutated genes and hemoglobin levels for individual CAD patients. The hemoglobin levels are calculated as percent (%) of the lowest normal value adjusted for sex, and are color-coded to indicate to the level of anemia: up to 70% (red), between 71%–90% (yellow) or above 90% (white). Two separate columns indicate whether nonsynonymous mutations were found in genes involved in either chromatin modification/organization or in NF-κB pathway. (B) and (C) Only genes with nonsynonymous mutation found in more than 10% of samples are shown. (D) Schematic representation of signaling cascades leading to activation of nuclear factor kappa B (NF-κB). Indicated are mutations in C-terminal end of CXCR4 (22% cases) and CARD11 (33% cases) that likely result in abnormal activation of NF-κB pathway (overall 44% cases). A more detailed description of NF-κB pathway activation is given in Supplementary Information S1

It is suggested that *KMT2D* is a tumor suppressor gene, and *KMT2D* mutations might act as driver mutations in lymphoma. Interestingly, some of the Kabuki patients develop autoimmune hemolytic anemia (AIHA).[3] Eight of the CAD patients in our study had inactivating *KMT2D* mutations. Three patients had missense mutations in the C-terminal SET domain, and one patient (CAD-13) had mutation affecting a splice site (Supplementary Information S1). These mutations are very likely to inactivate or impair KMT2D activity. Therefore, it is most likely that all detected mutations (12/18; 67%) in the CAD patients significantly reduce KMT2D activity. The presence of *KMT2D* mutations might be exploited for targeted treatment.

Five of six *CARD11* mutations were located in coiled-coil region in exon 6, and therefore are predicted to result in NF-κB activation.[1] The exon 5 mutation in a sixth sample is also located in the coiled-coil region and may be an activating mutation. Both nonsense and frameshift somatic mutations in the C-terminal domain of *CXCR4* have been reported in 27% of Waldenström macroglobulinemia (WM) patients.[4] The location of mutations in WM is very close to the region of mutations detected in CAD samples. Based on the biochemical structure of CXCR4 and the previous functional studies (Supplementary Information S1), *CXCR4* mutations in CAD patients may also prevent receptor internalization creating a state of prolonged activation. In our series, a *CXCR4* mutation always occurred in combination with *KMT2D* mutation. *CXCR4* gene mutations are of potential therapeutic interest as target for CXCR4 inhibitors. CAD patients with *KMT2D* and *CARD11* or *CXCR4* mutations, have all low hemoglobin levels. Further functional studies are required to explain the effect of these mutations upon disease severity.

Note, NF-κB signaling is important for lymphoma development, due to its role in lymphocyte survival and proliferation. In normal B cells, the NF-κB pathway is transiently activated in response to antigen stimulation. However, during lymphoma development acquired genetic mutations might cause constitutional activation of the NF-κB pathway.[5] In CAD, we found activating mutations that are expected to cause constitutive activation of this pathway. Note, CARD11 plays an important role in NF-κB activation through the B cell receptor (BCR), and CXCR4 signaling intercrosses with BCR signaling (Figure 1(D)).[6] Therefore, activating mutations in *CARD11* and *CXCR4*, as the ones we found in CAD samples, might cause constitutional NF-κB activation. The latter might enable cold-agglutinin producing B cells to survive and proliferate.

The *IGLL5* mutations are seen in lymphomas (Supplementary Information S1) however, the function of IGLL5 in CAD requires more study. The *MYD88* L265P mutation was absent in 27 CAD patients in our previous study, but was found in a minority of patients by another group. In this series, one unique patient (CAD-1.32) displayed the *MYD88* L265P mutation. This patient did not have any other recurrent somatic gene mutations that are common in CAD, except for *IGLL5* mutations (Figure 1(C)). Trisomy 3, 12 and 18, frequently seen in CAD, was also absent in this case. Further, *IGHV3-7* clonal rearrangement was found with an additional minor *IGHV4-34* clone (0.2% of reads; unpublished data). Flow cytometry assessment revealed two IGK-restricted B cell populations, one CD5 positive and the other CD5 negative. Therefore, it seems likely that this patient may have a composite lymphoproliferative disorder, possibly lymphoplasmacytic lymphoma and CAD, the latter represented by the minor clone. This is supported by the observation of absence of hemolytic anemia at relapse of the lymphoplasmacytic lymphoma in this patient; relapse occurred 7 years after initial treatment with bendamustine and rituximab[2] had resulted in complete remission with absence of hemolytic anemia. The clonal B cell population was CD5 negative.

In conclusion, CAD showed a relatively low mutational burden, but with recurrent gene mutations. Current study confirms and expands our previous findings[1] in a larger series of CAD patients. The most common recurrent mutations were in genes known to be involved in lymphoma development. The CAD patients with a *KMT2D* mutation associated with *CARD11* or *CXCR4* mutations, or both, had lower hemoglobin levels at diagnosis compared to patients with absence of *KMT2D* mutation or patients with *KMT2D* mutation without *CARD11* or *CXCR4* mutations. Both *CARD11* and *CXCR4* mutations in CAD are expected to be activating mutations, likely activating the NF-κB pathway. Gene mutations observed affect the NF-κB pathway as well as chromatin modification or organization. This is the most comprehensive study of gene mutations in CAD as of yet, and identifies possible new avenues for targeted treatment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

Agnieszka Małecka, Gunhild Trøen, Jan Delabie, Anne Tierens, Sigbjørn Berentsen and Geir E. Tjønnfjord designed the study. Agnieszka Małecka, Gunhild Trøen, Ingunn Østlie and Jędrzej Małecki performed the analyses. Gunhild Trøen, Jan Delabie, Anne Tierens, Sigbjørn Berentsen and Geir E. Tjønnfjord supervised the study. Jan Delabie, Anne Tierens, Ulla Randen, Sigbjørn Berentsen and Geir E. Tjønnfjord reviewed the diagnostic patient samples and collected the clinical data. Agnieszka Małecka, Gunhild Trøen, Jan Delabie and Jędrzej Małecki prepared the manuscript. All authors have critically read the manuscript.

ETHICS STATEMENT

The patients included in this study were enrolled in a clinical trial (NCT02689986).[2] The study was approved by the Regional Committee for Medical and Health Research Ethics of Southeast Norway (2012/131/REK).

PATIENT CONSENT STATEMENT

Written informed consent was procured by using consent forms approved by the Regional Committee for Medical and Health Research Ethics of Southeast Norway.

DATA AVAILABILITY STATEMENT

In accordance with Norwegian legislation and the ethic approval of the study, all sensitive data are stored in protected databases at Oslo University Hospital. On request, the data will be made available for other institutions. However additional ethic approval might be required before sharing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.