CD38 Gene Polymorphisms Contribute to Genetic Susceptibility to B-Cell Chronic Lymphocytic Leukemia: Evidence from Two Case-Control Studies in Polish Caucasians

Krzysztof Jamroziak,1 Zofia Szemraj,1 Olga Grzybowska-Izydorczyk,1 Janusz Szemraj,2 Magdalena Bieniasz,3 Barbara Cebula,1 Krzysztof Giannopoulos,5 Ewa Balcerzak,3 Dorota Jesionek-Kupnicka,4 Malgorzata Kowal,6 Aleksandra Kostyra,7 Malgorzata Calbecka,7 Ewa Wawrzyniak,1 Marek Mirowski,3 Radzislaw Kordek,4 and Tadeusz Robak1

Departments of 1Hematology, 2Medical Biochemistry, 3Pharmaceutical Biochemistry, and 4Oncology, Medical University of Lodz, Lodz, Poland; Departments of 5Clinical Immunology and 6Hematology and Bone Marrow Transplantation, Medical University of Lublin, Lublin, Poland; and 7Department of Hematology, Torun City Hospital, Torun, Poland

Abstract

Given the recent findings on the importance of CD38 signaling in the pathogenesis of B-cell chronic lymphocytic leukemia (B-CLL), we hypothesized that single nucleotide polymorphisms (SNP) in the CD38 gene may be related to B-CLL risk. We evaluated two potentially functional CD38 SNPs, intronic rs6449182 (184C>G) and missense rs1800561 (418C>T, Arg140Trp) in two hospital-based case-control studies (study A and validation study B). Genotyping was done using PCR-based assays in a total of 460 Polish Caucasian patients with B-CLL and 503 age-matched and gender-matched controls. We found that frequencies of both variant alleles (rs6449182 G and rs1800561 T) were significantly higher in B-CLL. In study A, logistic regression analysis revealed an association between B-CLL and genotypes rs6449182 G and rs1800561 T alleles (OR, 10.12; 95% CI, 1.3-81.6), and in the combined analysis of both studies. We also observed that rs6449182 G carriers had more advanced clinical stage (P = 0.002) and tended to be younger at diagnosis (P = 0.056). Furthermore, we found higher CD38 transcript levels and higher proportions of CD38-positive cells in carriers of rs6449182 G and rs1800561 T alleles (P < 0.05 for all comparisons). In conclusion, our data show that CD38 SNPs may affect CD38 expression and contribute to the increased risk of B-CLL carcinogenesis. (Cancer Epidemiol Biomarkers Prev 2009;18(3):945–53)

Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is the most common form of leukemia among Caucasians, characterized by the progressive accumulation of distinct monoclonal CD5+/CD19+/CD23+ B lymphocytes in the bone marrow, peripheral blood, and lymphatic organs (1). The prediction of a highly variable clinical course for B-CLL has been recently facilitated by the analysis of novel molecular prognostic factors including CD38 expression, ZAP-70 expression, immunoglobulin heavy chain variable region gene (IgVH) mutational status, and common genomic aberrations assessed by fluorescence in situ hybridization (FISH) assay (1). The etiology of B-CLL, however, is not well understood. Interestingly, a number of observations including significant familial aggregation of B-CLL cases as well as racial differences in its incidence suggest that inherited background plays an important role in B-CLL development (2).

The CD38 molecule is a modern but well-established independent prognostic marker in B-CLL (1). This type II transmembrane glycoprotein is expressed in immature hematopoietic cells, down-regulated by mature cells, and re-expressed at high levels by activated B cells, T cells, natural killer cells, and dendritic cells (3). CD38 is thought to be involved in the regulation of numerous physiologic processes by two, largely independent, mechanisms. First, CD38 functions as a leukocyte plasma membrane signaling receptor, interacting with its ligand CD31/PECAM-1 molecule, expressed by various endothelial and stromal components (3). Second, CD38 is an ectoenzyme catalyzing metabolism of two distinct Ca2+ messengers, cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate (3). The interest in the CD38 protein in the context of B-CLL was initiated by the work of Damle et al. (4), who reported that high CD38 expression in malignant B-CLL cells has a negative...
influence on the disease outcome. Later studies confirmed the negative prognostic role of CD38 expression in B-CLL, although certain controversies regarding the choice of the method of CD38 assessment as well as threshold to distinguish CD38-positive cases remain (5, 6).

Interestingly, recent reports have suggested that CD38 is not only a prognostic marker, but also the key component of the pathogenetic network underlyng B-CLL (7). Given these new data on the role of CD38 in B-CLL evolution, we hypothesized that inherited differences in CD38 function may influence the risk of B-CLL malignant transformation. The CD38 gene is located in the short arm of chromosome 4 (4p15) with its coding sequence organized in eight exons and more than 98% of the gene represented by introns (8). However, little is known on the biological and clinical significance of CD38 single nucleotide polymorphisms (SNP; ref. 8). In this article, we present the results of two independent case-control studies comparing the frequencies of two potentially functional CD38 SNPs, rs6449182 and rs1800561 (Arg140Trp), in B-CLL patients and B-CLL unaffected controls of Polish Caucasian origin.

Patients and Methods

Study Design and Participants. The investigation consisted of two independent case-control hospital-based studies (study A and study B), in which the second study (study B) was done to validate the results of the first study (study A). In study A, 252 consecutive B-CLL patients referred to the Department of Hematology, Medical University of Lodz, Lodz, Poland between January 2000 and February 2005 were enrolled. Among the patients with B-CLL, there were 154 (61%) males and 98 (39%) females with a median age of 66 years (range, 39-89). In all patients, genomic DNA was isolated from peripheral blood at the time of diagnosis and stored in the DNA Bank of the Department of Hematology, Medical University of Lodz. In study B, we enrolled 208 consecutive B-CLL cases were recruited at two hospital hematology centers in Torun (Department of Hematology of Torun City Hospital, n = 105) and Lublin (Department of Hematology and Bone Marrow Transplantation, Medical University of Lublin, n = 103) between June 2001 and December 2007. At both centers, genomic DNA was isolated from peripheral blood at diagnosis and stored in the institutions’ DNA banks. The patient group included 137 (66%) males and 71 (34%) females with a median age of 65 years (range, 40-79).

Control individuals for both studies were randomly selected volunteer cancer-free Polish Caucasian residents of the Lodz city region. The controls were recruited from the same population for both studies because all three participating hospital centers are located in an ethnically homogenous region (Polish Caucasians of Slavic origin) and relatively in the geographic vicinity (the maximal distance between any of the three participating centers is 300 km). Fresh peripheral blood samples (2 mL) were obtained from randomly selected volunteer blood donors at the local blood bank in Lodz (60% of samples), and the remainder were randomly selected among frozen control DNA samples from healthy residents of the Lodz region sampled for previous studies and stored in the DNA Bank of the Department of Hematology, Medical University in Lodz. In study A, there were 249 individuals, including 152 males (61%) and 97 females (39%), with a median age of 64 years (range, 36-85). Additionally, in 106 controls from study A, another peripheral blood sample (2 mL) was collected for the study of CD38 gene expression. For study B, we enrolled 254 individuals including 155 (61%) males and 99 females with a median age of 60 years (36-79). Controls were frequency-matched to cases by age (5-year intervals) and gender. Ethnicity and cancer-free status were self-reported at the time of blood collection. All control samples were obtained between 1999 and 2008.

Reasons for nonparticipation included refusal (23 patients and 63 controls in study A, and 30 patients and 73 controls in study B) and poor quality or low concentration of DNA that prevented PCR amplification (in 40 patients and 39 controls with DNA isolated from blood and in 8 patients with DNA isolated from paraffin blocks). The investigation was in accordance with the Declaration of Helsinki, and was approved by the Bioethical Committee of the Medical University of Lodz. Informed consent for participation in genetic studies in agreement with institutional guidelines was given by all patients and controls.

Selection of CD38 SNPs to Study. SNPs included in this investigation were chosen based on the literature review and their potential functionality. At the time of the study designing the literature search did not reveal any links between CD38 SNPs and B-CLL or other cancer risk. Therefore, we selected the only two CD38 SNPs that were reported as potentially functional in the context of different human pathologies. The SNP rs6449182 (previously referred as 184C>G, 182C>G, or CD38-PeuII polymorphism), is located at the 5’-end of intron 1 in the proximity of the CpG island and retinoid acid–responsive element, and thus in the region potentially involved in the regulation of CD38 gene expression (8). Moreover, rs6449182 polymorphism was found to be associated with peak bone mineral density and postmenopausal bone loss in women, clinical phenotype in patients with systemic lupus erythematosus, and recently with the risk of Richter’s transformation in B-CLL (9-11). The second SNP, rs1800561 (previously referred as 418C>T), located in exon 3 of the CD38 gene, is the only known missense mutation in the CD38 coding region (12). The change from C to T, related with rs1800561 SNP, results in amine acid substitution Arg140Trp that was associated with a 50% decrease of enzymatic CD38 activity as ADP-ribosyl cyclase and cyclic ADP-ribose hydroxylase in vitro, and was postulated to be involved in the genetic predisposition to type II diabetes mellitus (12).

CD38 SNP Genotyping. Genomic DNA used for CD38 genotyping was isolated from peripheral blood samples in 381 patients and 503 controls, and from
paraffin-embedded marrow biopsy blocks in 79 patients, using methods described elsewhere (13, 14). For the quality control of the DNA isolated from paraffin blocks, we performed CD38 genotyping in 15 randomly selected patients for whom both DNA sources were available, and the results showed 100% concordance.

CD38 rs6449182 (184C>G) SNP was screened by means of PCR-RFLP and PCR-based amplification refractory mutation system (ARMS) using methods previously described by others with small modifications (9, 10). For the PCR-RFLP assay, forward primer 5'-CCGGGTGGTGCTGAGTAGGGAGTC-3' and reverse primer 5'-CTACGCACGAGCCACCACCGAGCAG-3' (IDT, Illinois) were applied (9). The PCR reactions were done in a 20 µL PCR tube containing 50 ng of genomic DNA, 1.5 pmol/L of each primer, 1.5 mmol/L of MgCl₂, 250 nmol/L of dNTPs, and 2 units of Taq polymerase (Promega) (IDT; ref. 10). The PCR reactions were done in a 20 µL PCR tube containing 100 ng of genomic DNA, 5 pmol/L of each primer, 1.6 mmol/L of MgCl₂, 200 nmol/L of dNTPs, and 0.5 units of Taq polymerase (Promega). The cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s followed by an extension step of 72°C for 10 min in a Biometra T Personal thermocycler (Biometra). The 128-bp amplicon was digested with PsuII (Promega) overnight. The presence of the rs6449182 C allele resulted in the digestion of the amplicon to 63 bp and 65 bp products. Digestion products were resolved in 6% polyacrylamide gel stained with ethidium bromide.

Regarding ARMS method of rs6449182 (184C>G) SNP detection, for each sample, two independent PCR reactions were carried out with different forward primers F1 GTTAAAGCTTGCGAGGACGACG-3' or F2 5'-GTAAAGCTTGCGAGGACGACG-3' and a common reverse primer R 5'-CGAGTTCTATTCCGTCCCTGA-3' (IDT; ref. 10). The PCR reactions were done in a 20 µL PCR tube containing 100 ng of genomic DNA, 5 pmol/L of each primer, 1.6 mmol/L of MgCl₂, 250 nmol/L of dNTPs, and 1 unit of Taq polymerase (Promega). The cycling conditions were as follows: initial denaturation at 94°C for 10 min, followed by 10 cycles consisting of 94°C for 30 s, 66°C for 30 s, and 72°C for 30 s, followed by 15 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 30 s, followed by 10 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. PCR reactions were carried out in a thermocycler Biometra T Personal (Biometra). The rs6449182 genotypes were detected following electrophoresis in a 6% polyacrylamide gel and identified as homozygous in case of the presence of product in only one respective tube or as heterozygous if products were shown in both tubes.

The concordance rates between PCR-RFLP and ARMS, assessed after initial genotyping of all enrolled individuals, were 93.3% (448 of 480 concordant genotypes) for CD38 rs6449182 SNP and 97.2% (489 of 503 concordant genotypes) for rs1800561 SNP. The samples with discordant genotypes were re-genotyped by both methods, and in the samples with persistent mismatches, direct sequencing was done as a quality control test. In six out of nine such samples for rs6449182 SNP, and in three out of four samples for rs1800561 SNP, the sequencing and PCR-RFLP were in agreement. Based on these results, we found PCR-RFLP methods to be more accurate than ARMS for the rs6449182 and rs1800561 SNPs genotyping.

**CD38 Gene Expression.** The expression of CD38 and GADPH genes was quantified by a real-time reverse transcription-PCR assay using ABI Prism 7000 Sequence Detection System (Applied Biosystems). Total RNA was extracted from cryopreserved peripheral blood B-CLL samples (n = 103) and fresh normal peripheral blood samples (n = 106) using Trizol (Invitrogen, Life Technologies, Inc.). The median proportion of CD19+/CD5+ cells in B-CLL samples was 83% (range, 65-99%). Total RNA was directly processed to cDNA using SuperScript II RNase Transcriptase System (Invitrogen, Life Technologies). cDNA was amplified in triplicate. Samples were supplemented with 0.3 µmol/L of each forward and reverse primer, a fluorescent probe, and made up to 50 µL using qPCR Mastermix for SYBR Green I (Eurogentec). The following primers were designed using PrimerExpress software (Applied Biosystems) 5'-CAGCAACCTGCTTTTCTAGT-3', and 5'-TTGAGCATCACTAGCCAC-3', 5'-AGCCA-CATCGTGAGACA-3', and 5'-GCCGATTACCGACAATCC-3' specific for CD38 and GADPH mRNA, respectively. Samples were incubated at 50°C for 2 min and 95°C for 10 min, and cycled at 95°C for 30 s, 56°C for 1 min, and 72°C for 1 min for 40 cycles. SYBR Green I
Table 1. Comparison of allele frequencies of two CD38 gene SNPs, rs6449182 and rs1800561, in Polish Caucasian B-CLL patients and controls in two independent case-control studies

<table>
<thead>
<tr>
<th>CD38 alleles</th>
<th>Study A</th>
<th>Study B</th>
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<tbody>
<tr>
<td></td>
<td>B-CLL cases (%)</td>
<td>P (HWE)*</td>
</tr>
<tr>
<td>n = 252</td>
<td>n = 249</td>
<td></td>
</tr>
<tr>
<td>rs6449182</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>329 (65.3)</td>
<td>0.38</td>
</tr>
<tr>
<td>G</td>
<td>175 (34.7)</td>
<td></td>
</tr>
<tr>
<td>rs1800561</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>491 (97.4)</td>
<td>0.68</td>
</tr>
<tr>
<td>T</td>
<td>13 (2.6)</td>
<td></td>
</tr>
<tr>
<td>Study B</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 208</td>
<td></td>
</tr>
<tr>
<td>rs6449182</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>261 (62.7)</td>
<td>0.02</td>
</tr>
<tr>
<td>G</td>
<td>155 (37.3)</td>
<td></td>
</tr>
<tr>
<td>rs1800561</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>428 (98.1)</td>
<td>0.78</td>
</tr>
<tr>
<td>T</td>
<td>8 (1.9)</td>
<td></td>
</tr>
</tbody>
</table>

*P value from deviation from the Hardy-Weinberg equilibrium assessed by the permutation test.

**P* values were based on logistic regression analysis.

fluorescence emission was captured, and mRNA was quantified using critical threshold (Ct) value. Analyses were done with ABI Prism 7000 (SDS Software). To compensate for variations in input RNA amounts and reverse transcription efficiency, CD38 expression was normalized to GADPH. Relative gene expression was obtained using the ΔΔCt method (16).

CD38 Protein Expression. Measurement of the proportion of CD38-expressing cells in the CD19+CD5+ population was done in fresh peripheral blood samples taken at diagnosis as routine evaluation of this prognostic variable (4). In brief, the cells were stained with fluorochrome-conjugated anti-CD38, anti-CD19, and anti-CD5 antibodies (all from Becton Dickinson) and the fluorescence was acquired using FACSCalibur (Becton Dickinson) flow cytometer. The proportion of CD38-positive cells in the CD5+/CD19+ gated population was recorded. Data were analyzed using CellQuestPro software (Becton Dickinson). For the purpose of this investigation, the result of baseline CD38 expression were available for a total of 252 patients from studies A and B.

IgVH Mutational Status Analysis. The IgVH mutational status in B-CLL patients was analyzed according to a standardized BIOMED-2 study protocol (17). Genomic DNA was amplified in a single multiplexed PCR reaction consisting of six BIOMED-2 VH framework 1 primers combined with one JH consensus primer 3'-CCAGTGGCAGAGGAGTGCCATTC-5' (17). All reactions were carried out in 50 mL containing 0.5 mg of DNA sample, 10 pmol of each primer (IDT), 200 nmol/L of dNTPs, 1 unit of Taq polmerase (Promega), and 106 PCR buffer II (Applied Biosystems). A Biometra T Personal thermocycler (Biometra) was used as follows: denaturation at 94°C for 15 min, 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and a final cycle for 10 min at 72°C. PCR products were analyzed on a 6% polyacrylamide gel and visualized with ethidium bromide staining. The products were purified using QIAquick PCR purification kit (Qiagen) and sequenced directly using 3' JH consenssus primer in an automated ABI 377 DNA Sequencer using Big-Dye terminators (Applied Biosystems). Mutational status was determined by comparison with the VH germ line sequence using the IMGT database (IMGT/V-Quest tool). Cases with >2% deviation from a germ line VH sequence were identified as mutated, and the remainder as unmutated. The data on IgVH mutational status were available for 71 patients.

ZAP-70 Expression. ZAP-70 expression was measured by a flow cytometry method in fixed peripheral blood cells (18). In brief, the samples were stained with ZAP-70 antibody purchased from Biolegend and fluorescence was acquired through FACSCalibur (Becton Dickinson) flow cytometer. Patients were identified as having ZAP-70–positive B-CLL if 20% or more CD5+/CD19+ positive cells expressed ZAP-70. The remaining samples were identified as ZAP-70 negative. Baseline ZAP-70 status was available in 85 patients from studies A and B.

FISH. FISH was done on interphase nuclei of lymphocytes on blood smears prior to the start of the study treatment. Four commercial probes were used (Vysis) including the microsatellite chromosome 12 probe D12Z3 and the unique sequence-specific or region-specific DNA probes: TP53 (17p13.1.1 locus), ATM (11q22.3 locus), and D13S319 (13q14.3 locus). Signals were counted in at least 200 interphase nuclei for each sample. Cutoff levels for the respective gene deletions and for trisomy 12 were defined based on the experiments done on peripheral blood smears from 10 controls. A true deletion was considered to occur if the specimen under study exhibited more than the mean +3 SD of nuclei with only one signal (referring to deletions) or with three signals (referring to trisomy 12). Based on this, the cutoff level was set at 8.4% for 13q14 deletion, 8.8% for 11q22 deletion, 9.6% for 17p13.1 deletion, and 8.4% for 17p13.1 deletion, or with three signals (referring to trisomy 12). Based on this, the cutoff level was set at 8.4% for 13q14 deletion, 8.8% for 11q22 deletion, 9.6% for 17p13.1 deletion, and 8.4% for 17p13.1 deletion, or with three signals (referring to trisomy 12). The results from FISH tests at diagnosis were available for 84 patients.

*8 http://imgt.cines.fr/
**Statistical Analysis.** Accordance with the Hardy-Weinberg equilibrium within each case and control group was estimated by the permutation test using LDA 1.0 software. Linkage disequilibrium between rs6449182 and rs1800561 SNPs was assessed for all cases and controls with likelihood-ratio test by LDA 1.0 software (19). Unconditional logistic regression was used to estimate the odds ratios (OR) with 95% confidence intervals (95% CI) for associations of case-control status with the CD38 genotypes. In addition to the genotype, reference age categories (<50, 50-59, 60-69, and >70 years) and gender, considered to be potential confounders, were also included in the analysis. Differences between specific genotype carriers regarding the presence of prognostic factors at diagnosis including clinical stage (low clinical stage (Rai 0-1) versus advanced stage (Rai II-IV)), IgVH mutational status (unmutated versus mutated), ZAP-70 expression (ZAP-70 positive versus ZAP-70 negative), and FISH genomic aberrations (presence of 17p or/and 11q deletions classified as poor risk versus absent or other abnormalities classified as good risk) were analyzed by χ² test. Age at diagnosis, CD38 gene expression level, and proportion of CD38-positive cells within the B-CLL clone were compared as continuous variables by the Mann-Whitney or the Kruskall-Wallis tests. Statistical analysis was done using SPSS 14.0 (SPSS) statistical package. For all calculations, \( P < 0.05 \) was considered significant.

**Results**

**Associations between CD38 Genotypes and B-CLL Risk.** The allele distributions of the investigated CD38 genetic polymorphisms rs6449182 and rs1800561 in studies A and B, as well as in combined patient and control populations from both studies are summarized in Table 1. Consistent with low rs1800561 T allele frequency, we did not detect any individuals homozygous for this allele. Both SNPs were in accordance with Hardy-Weinberg equilibrium among case and control groups from studies A and B, except for rs6449182 SNP in patients from study B (Table 1). Moreover, we did not find rs6449182 and rs1800561 SNPs to be in a significant linkage disequilibrium (\( D^2 = 0.29, r^2 = 0.0037, P = 0.17 \)).

The allele distributions at both investigated loci differed significantly between patients and controls. In study A, we observed higher frequencies of both variant alleles rs6449182 G (0.347 versus 0.155, \( P = 2 \times 10^{-11} \)) and rs1800561 T (0.026 versus 0.004, \( P = 0.014 \)) in B-CLL cases as compared with controls. These results were validated in study B, which confirmed the overrepresentation of rs6449182 G allele (0.380 versus 0.152, \( P = 3 \times 10^{-13} \)) and rs1800561 T allele (0.019 versus 0.002, \( P = 0.031 \)) in patients with B-CLL (Table 1).

The association between CD38 SNPs and B-CLL risk is presented in Table 2. Initially, we introduced reference age categories and gender in the logistic regression model, but none of them were statistically significant in either study (\( P > 0.05 \)), thus, crude ORs for the respective genotypes are shown in the text and in Table 2. In study A, regarding rs6449182 SNP, we observed an association between B-CLL and heterozygous CG genotype with an OR of 3.57 (95% CI, 2.41-5.29). Interestingly, the B-CLL risk was further elevated with rs6449182 GG homozygous

<table>
<thead>
<tr>
<th>genotype</th>
<th>A</th>
<th>B</th>
<th>A &amp; B</th>
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<tbody>
<tr>
<td>rs6449182</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>104 (41.3)</td>
<td>111 (44.1)</td>
<td>215 (44.0)</td>
</tr>
<tr>
<td>CG</td>
<td>121 (48.0)</td>
<td>108 (40.9)</td>
<td>229 (48.0)</td>
</tr>
<tr>
<td>GG</td>
<td>27 (10.7)</td>
<td>7 (2.8)</td>
<td>34 (6.9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>rs1800561</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>CC</td>
<td>239 (94.8)</td>
<td>247 (99.2)</td>
<td>486 (97.1)</td>
</tr>
<tr>
<td>CT</td>
<td>13 (5.2)</td>
<td>2 (0.8)</td>
<td>15 (3.0)</td>
</tr>
<tr>
<td>TT</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
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\( \text{Crude ORs with their 95% CI are shown as two potential confounders (reference age group and gender included in the initial logistic regression model) were not statistically significant and did not alter risk} \)

\( \text{P-values were based on logistic regression analysis.} \)
genotype (OR, 5.22; 95% CI, 2.36-11.53), suggesting an allele-dose effect (Table 2). Concerning the second studied polymorphism, rs1800561, heterozygous CT genotype was related to elevated risk of B-CLL (OR, 6.57; 95% CI, 1.47-29.25) compared with wild-type CC genotype. Similar associations were found in study B. Compared with the CC genotype of rs6449182 SNP, CG and GG genotypes were related to the elevated risk of B-CLL (OR, 4.00; 95% CI, 2.68-6.00 and OR, 12.84; 95% CI, 4.26-38.69, respectively). Furthermore, CT rs1800561 genotype was associated with elevated risk of B-CLL compared with CC genotype (OR, 9.94; 95% CI 1.24-79.81). The associations observed in study A and study B remain significant in the combined analysis of both studies with elevated risk of B-CLL related to rs6449182 CG (OR, 3.72; 95% CI, 2.8-4.9), rs6449182 GG (OR, 7.51; 95% CI, 4.0-14.2), and rs1800561 CT genotypes (OR, 7.96; 95% CI, 2.4-28.9; Table 2).

**Functional Effects of CD38 Genotypes.** In search for functional effects of studied polymorphisms, we investigated whether these SNPs influence CD38 expression levels in B-CLL cases and controls. The CD38 gene expression levels in cryopreserved mononuclear cell samples from 103 B-CLL patients enrolled to study A were estimated by quantitative reverse transcription-PCR assay and analyzed in association with CD38 genotypes. Interestingly, both polymorphisms were associated with an increased CD38 gene expression (Fig. 1A and C). Median relative CD38 expression was higher in rs6449182 G allele carriers than in noncarriers (0.23 versus 0.13, \( P = 2 \times 10^{-10} \)). Moreover, the CD38 transcript level was the highest in samples from rs6449182 GG homozygous patients (Fig. 1A). Regarding rs1800561 SNP, we also found significantly elevated CD38 expression levels in patients harboring T allele compared with CC genotype carriers (median, 0.64 versus 0.20; \( P = 0.018 \)).

To validate these findings at the protein level, we analyzed the percentage of CD38-positive cells that were recorded as prognostic markers at the time of B-CLL diagnosis. The results of baseline CD38 flow cytometry tests were available for 252 subjects pooled from studies A and B. Consistent with the observed transcript levels, we found that the proportion of CD38-positive B-CLL cells (CD19+/CD5+/CD38+) was higher in

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**Figure 1.** Influence of CD38 gene rs6449182 and rs1800561 SNPs on CD38 gene and protein expression. **A.** Relative CD38 gene expression quantified by real-time reverse transcription-PCR in B-CLL cells from 103 patients stratified by CD38 rs6449182 SNP; \( P \) values derived from the Kruskall-Wallis test. **B.** Percentage of CD38-expressing cells in B-CLL clone measured by flow cytometry at diagnosis in 252 patients with B-CLL stratified by CD38 rs6449182 SNP; \( P \) values derived from the Kruskall-Wallis test. **C.** Relative CD38 gene expression quantified by real-time reverse transcription-PCR in B-CLL cells from 103 patients stratified by rs1800561 CT SNP; \( P \) values derived from the Mann-Whitney test. **D.** Percentage of CD38-positive cells in B-CLL clone at diagnosis measured by flow cytometry in 252 patients with B-CLL, stratified by rs1800561 SNP; \( P \) values derived from the Mann-Whitney test.
rs6449182 G allele carriers compared with noncarriers (median, 13% versus 2%; \( P = 0.0012 \)), and that this proportion was the highest in patients with the homozygous GG genotype (Fig. 1B). For rs1800561 SNP, we also observed that samples from CT allele carriers had a higher percentage of CD38-positive B-CLL cells as compared with CC genotype carriers (34% versus 7%, \( P = 0.010 \); Fig. 1D).

Furthermore, we analyzed CD38 mRNA levels in blood mononuclear cells from 106 normal controls from study A. Distribution of rs6449182 genotypes in this group was as follows: 69, CC genotype; 34, CG genotype, and 3, GG genotype. Interestingly, we found that CD38 expression was also increased in rs6449182 G allele carriers compared with noncarriers, although the differences were not as pronounced as in B-CLL samples (0.20 versus 0.16, \( P = 0.029 \)). No comparison could be done for the rs1800561 polymorphism as all 106 patients carried the wild-type CC genotype.

**Influence of CD38 Genotypes on Patients’ Characteristics.** We also analyzed whether CD38 polymorphisms may explain some of the clinical heterogeneity of B-CLL cases at diagnosis. We found that the rs6449182 G allele tended to associate with younger age of B-CLL manifestation (Fig. 2). The patients’ median age at presentation was 66, 63, and 57 years in carriers of rs6449182 CC, CG, and GG genotypes, respectively (\( P = 0.056 \)). In contrast, no association with age at diagnosis was observed for rs1800561 genotypes. Next, we investigated whether CD38 SNPs were associated with different prognostic factors for B-CLL recorded at diagnosis. In both studies, there were 244 (53%) patients with low clinical stage (Rai 0-I) and 216 (47%) patients with advanced clinical stage (Rai II-IV). We found that the rs6449182 SNP G allele was associated with clinical stage at diagnosis (\( P = 0.002 \)). Importantly, only 33.7% of wild-type rs6449182 CC genotype carriers had advanced stage at diagnosis compared with 60.4% of patients homozygous for alternative G allele (Table 3). Any associations between clinical stage and rs1800561 polymorphism could be observed (Table 3). Regarding modern prognosticators including IgVH mutational status, FISH, and ZAP-70, no significant differences between carriers of different rs6449182 and rs1800561 genotypes were detected (data not shown). However, the results were available in low numbers of patients in both studies (IgVH mutational status in 71 patients, ZAP-70 in 85 patients, and FISH in 84 patients), thus these analyses were likely underpowered.

**Discussion**

Given the recent findings on the importance of CD38 in B-CLL, it is conceivable that polymorphisms of the molecules involved in the CD38 signaling pathway might serve as determinants of B-CLL predisposition. In this investigation, we found that the associations between B-CLL risk and two CD38 SNPs, rs6449182 and rs1800561, were consistently observed in two independent case-control studies as well as in the combined analysis of both studies.

The biological mechanisms underlying these associations are unknown. Interestingly, we found that CD38

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**Table 3. Frequencies of different CD38 genotypes among 460 patients with B-CLL from studies A and B stratified according to B-CLL clinical stage at diagnosis**

<table>
<thead>
<tr>
<th>CD38 genotype</th>
<th>All B-CLL patients, ( n = 460 ) (100%)</th>
<th>Patients with low clinical stage, ( n = 244 ) (53.0%)</th>
<th>Patients with advanced stage, ( n = 216 ) (47.0%)</th>
<th>( P ) (^{1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs6449182 SNP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>178 (100%)</td>
<td>118 (66.3)</td>
<td>60 (33.7)</td>
<td>0.002</td>
</tr>
<tr>
<td>CG</td>
<td>234 (100%)</td>
<td>107 (45.7)</td>
<td>127 (54.3)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>48 (100%)</td>
<td>19 (39.6)</td>
<td>29 (60.4)</td>
<td></td>
</tr>
<tr>
<td>rs1800561 SNP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>439 (100%)</td>
<td>234 (52.3)</td>
<td>205 (46.7)</td>
<td>0.56</td>
</tr>
<tr>
<td>CT</td>
<td>21 (100%)</td>
<td>12 (57.1)</td>
<td>9 (42.9)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

\(^{1}\)Low clinical stage refers to stages 0 and I, and advanced clinical stage refers to stages II, II, and IV according to Rai classification.

\(^{1}\) \( P \) values were based on \( \chi^2 \) test.

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gene and protein expression are elevated in B-CLL cells from carriers of rs6449182 G and rs1800561 T variant alleles. Moreover, a recent report showed that in vitro stimulation of B-CLL cells with interleukin-2 results in more significant up-regulation of surface CD38 in cells from carriers of rs6449182 G allele than from wild-type rs6449182 CC genotype carriers (11). Therefore, these findings may suggest that stronger activation of the CD38 pathway contributes to the risk of B-CLL malignant transformation in patients with variant alleles. This is in line with growing evidence of the involvement of CD38 signaling in B-CLL pathogenesis. It was shown that signals through CD38 receptors induce proliferation and increase survival of B-CLL cells (20). Moreover, stromal and nurse-like cells, which contribute to B-CLL clone survival, express high levels of CD31/PECAM-1, the ligand of CD38 (21). Therefore, the risk of B-CLL carcinogenesis may be influenced by the inherited alterations of the receptor cross-talk between CD38, CD31/PECAM-1, CD100, plexin B, and ZAP-70 that is thought to sustain B-CLL growth (22, 23). However, the mechanisms linking rs6449182 and rs1800561 SNPs to altered CD38 expression are not clear. The rs6449182 polymorphisms is noncoding, although its localization in the regulatory region at the 5’-end of intron 1 in proximity to the CpG island and retinoid acid–responsive element may potentially affect gene expression (8). Regarding rs1800561 SNP, Arg140Trp substitution may alter the protein functional characteristics. Interestingly, this SNP was associated with 50% decrease in CD38 activity as ADP-ribosyl cyclase and cyclic ADP-ribose hydroxylase in vitro, although its consequences for CD38 receptor function were not studied (11). Furthermore, potential linkage disequilibrium between rs6449182 and rs1800561 SNPs, and a functional variant(s) located in or outside the CD38 locus may be responsible for altered gene expression and association with B-CLL.

In contrast to the results of our study, a recent article by Aydin et al. (11) showed overlapping distribution of rs6449182 SNP alleles in 248 Italian patients with B-CLL and in 232 controls. The reasons for the discrepancies between these two investigations are not clear, but some potential explanations may be proposed. Importantly, both studies showed significant association between rs6449182 G allele and advanced B-CLL clinical stage. However, in our study, there were 53% patients with advanced stage (Rai II-IV or Binet B-C) compared with only 29% in the study by Aydin et al. (11); thus, this difference in patient characteristics may partially explain the lower frequency of rs6449182 G in the latter study. Furthermore, it is not unlikely that association between B-CLL and CD38 SNPs may be modified by well-known functional SNPs and haplotypes of its ligand CD31/PECAM-1 (24). Moreover, some interethnic differences may also be of importance. Interestingly, both studies are consistent in describing more aggressive B-CLL phenotypes in carriers of the rs6449182 G allele. Besides the association between rs6449182 G and advanced clinical stage, we found earlier disease onset in these patients, whereas Aydin et al. (11) reported association with poor-risk molecular features and elevated risk of transformation to high-grade lymphoma (Richter’s transformation). Therefore, it could be hypothesized that among the carriers of the rs6449182 G allele, B-CLL develops earlier and has a more dramatic clinical course. In light of these findings, monoclonal B-cell lymphocytosis, a frequent premalignant condition progressing to B-CLL at the rate of 1% per year, seems to be an interesting area for further studies on the influence of CD38 SNPs (25).

The major strengths of our investigation are the design, which has enabled the results to be confirmed in a validation study, and functional findings that are in line with the observed disease associations. However, some limitations of the study should also be mentioned. The consequences of rs1800561 SNPs seem to be somehow different compared with rs6449182 SNP, as it did not associate with age of disease presentation and clinical stage. However, the study could be underpowered to detect such differences due to the low frequency of the variant T allele. It should also be underlined that we applied the candidate gene approach, and focused on only two SNPs with functional effects as suggested by previous reports. Therefore, we cannot rule out the possibility that CD38 SNPs other than those included in our study may be related to B-CLL risk.

In conclusion, in this study, we found some evidence that CD38 SNPs rs6449182 and rs1800561 affects CD38 expression in B-CLL cells and contributes to B-CLL predisposition.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
CD38 Gene Polymorphisms Contribute to Genetic Susceptibility to B-Cell Chronic Lymphocytic Leukemia: Evidence from Two Case-Control Studies in Polish Caucasians


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