ATM, CTLA4, MNDA, and *HEM1* in High versus Low CD38–Expressing B-Cell Chronic Lymphocytic Leukemia

Avadhut D. Joshi,[1] Ganapati V. Hegde,[1] John D. Dickinson,[1] Amit K. Mittal,[1] James C. Lynch,[2] James D. Eudy,[1] James O. Armitage,[3] Philip J. Bierman,[3] R. Gregory Bociek,[3] Marcel P. Devetten,[3] Julie M. Vose,[3] and Shantaram S. Joshi[1]

Abstract

Purpose: In B-cell chronic lymphocytic leukemia (CLL), high CD38 expression has been associated with unfavorable clinical course, advanced disease, resistance to therapy, shorter time to first treatment, and shorter survival. However, the genes associated with CLL patient subgroups with high and low CD38 expression and their potential role in disease progression is not known.

Experimental Design: To identify the genes associated with the clinical disparity in CLL patients with high versus low CD38 expression, transcriptional profiles were obtained from CLL cells from 39 different patients using oligonucleotide microarray. Gene expression was also compared between CLL cells and B cells from healthy individuals.

Results: Gene expression analysis identified 76 differentially expressed genes in CD38 high versus low groups. Out of these genes, *HEM1, CTLA4*, and *MNDA* were selected for further studies and their differential expression was confirmed by real-time PCR. HEM1 overexpression was associated with poor outcome, whereas the overexpression of CTLA4 and MNDA was associated with good outcome. Down-regulation of HEM1 expression in patient CLL cells resulted in a significant increase in their susceptibility to fludarabine-mediated killing. In addition, when gene expression patterns in CD38 high and low CLL cells were compared with normal B-cell profiles, ATM expression was found to be significantly lower in CD38 high compared with CD38 low CLL as confirmed by real-time reverse transcription-PCR.

Conclusions: These results identify the possible genes that may be involved in cell proliferation and survival and, thus, determining the clinical behavior of CLL patients expressing high or low CD38.

B-cell chronic lymphocytic leukemia (CLL) is the most common B-cell leukemia in the United States with ~10,000 cases diagnosed every year (1). CLL cells are monoclonal cells expressing CD19, CD5, and CD23 with a phenotype of mature B lymphocytes (2). Clinical heterogeneity and resistance to therapy makes it difficult to effectively treat CLL patients. Rai and Binet staging systems (3, 4) for CLL are useful methods for predicting survival and treatment requirements, but are of limited prognostic value in early stages of the disease (Binet stage A or Rai stage 0-II). Prognostic indicators, such as immunoglobulin V_H (IgVh) gene mutational status, cytogenetic abnormalities, CD38 expression, and ZAP-70 expression have been shown to correlate with clinical outcome (5–8). Among these, the role of the CD38 molecule in CLL biology is becoming more interesting and warrants further understanding of the genes associated with the CD38 expression in the CLL subgroup.

Human CD38, a 45-kDa transmembrane glycoprotein, was initially discovered as a thymocyte differentiation marker (9) and, subsequently, has been found on B cells, natural killer cells, and monocytes (10, 11). CD38 acts as a receptor on B cells and CD38 antibody ligation induces phosphorylation of CD19 and prevents apoptosis of germinal center B cells. Because of a short cytoplasmic tail, the CD38 receptor uses B-cell receptor (BCR) signaling machinery for transmitting signals in the cells (12, 13). In addition, CD38 also plays a role as an ADP ribocyclase ecto-enzyme and as a cell adhesion molecule (14).

CD38 expression is an independent prognostic marker in CLL and several reports have shown that CLL patients expressing high CD38 are associated with an unfavorable clinical course with advanced disease stage, poor responsiveness to chemotherapy, short time to initiation of first treatment, and shorter survival, whereas CLL patients with low CD38

Authors' Affiliations: Departments of [1]Genetics, Cell Biology and Anatomy, [2]Preventive and Societal Medicine; and [3]Internal Medicine-Section of Oncology/Hematology, Center for Research in Leukemia and Lymphoma, University of Nebraska Medical Center, Omaha, Nebraska

Received 2/5/07; revised 6/11/07; accepted 6/27/07.

Grant support: Elsa U. Pardee Foundation (Midlands, MI), Chronic Lymphocytic Leukemia Foundation (Houston, TX), and NIH grant P20 RR016469 from the INBRE Program of the National Center for Research Resources.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Requests for reprints: Shantaram S. Joshi, Department of Genetics, Cell Biology and Anatomy, University of Nebraska Medical Center, Omaha, NE 68198-6395. Phone: 402-559-4165; Fax: 402-559-3400; E-mail: ssjoshi@unmc.edu.

doi:10.1158/1078-0432.CCR-07-0283

expression require minimal or no treatment, remain treatment free for a longer time, and have a better survival (5, 15–17), and, when combined with ZAP-70, can predict time to first treatment in CLL patients (18). A recent study by Deaglio et al. (19, 20) suggests that CD38-mediated signaling results in increased proliferation and diminished apoptosis of CLL cells. All these reports confirm the ability of CD38 as a prognostic marker; however, it remains unclear whether CD38 plays a direct role in pathogenesis of CLL or is merely a marker of aggressiveness.

Emerging evidence (19, 20) in CLL biology suggests that CD38 signaling may have a potential role in increased proliferation and decreased apoptosis of CLL cells. Therefore, we investigated to identify the genes associated with CD38 expression in CLL patient subgroups and its correlation with clinical outcome. Transcriptional profiles of CLL cells were obtained from 39 different patients using oligonucleotide microarray. Significance analysis of microarray (21) was used to identify differentially expressed genes in patients with high and low CD38 expression. Among the differentially expressed genes, *HEM1*, *CTLA4*, and *MNDA* were selected for further analyses because of their role in hematopoietic cells (22), cell cycle regulation, immunoregulation (23, 24), and cell proliferation (25). HEM1 was overexpressed and associated with poor clinical outcome, whereas CTLA4 and MNDA were underexpressed and associated with good clinical outcome in the CLL patient subgroup with high CD38 expression compared with low CD38 expression. Targeting of HEM1 using HEM1 antisense oligonucleotide resulted in a higher susceptibility of CLL cells to fludarabine-mediated killing. Finally, gene expression profiles of CD38 high and low CLL cells were compared with profiles of normal human B cells, and the results showed an association with lower ATM expression in the CD38 high CLL subgroup.

Materials and Methods

Patient information. Only untreated patients or patients not treated for the prior 6 months were enrolled in the study, assuming that 6 months would be a long enough time interval to overcome the treatment-induced alterations in gene expression pattern. Indications for treatment included worsening of physical symptoms, anemia, thrombocytopenia, or rapid lymphocyte doubling time. Fresh peripheral blood samples were collected from 39 CLL patients using an institutional review board–approved protocol and informed consent. There were 18 female and 21 male patients with a median age of 67 years. Other details of patient information are provided in Supplementary Table S1.

Isolation and characterization of CLL cells. Monoclonal population of CD5-, CD19-, and CD23-positive circulating B-lymphocytes were defined as CLL cells. Mononuclear cells from blood taken from CLL patients were isolated using lymphocyte separation medium (Accurate Chemical and Scientific Corp.) as described previously (26, 27). The immunophenotypes of the mononuclear cells were determined by flow cytometry using phycoerythrin or FITC-conjugated antibodies CD3-FITC, CD5-PE, CD19-PE, and CD38-PE (BD PharMingen). Cells were also double stained with CD5-PE and CD19-FITC, CD38-PE and CD19-FITC, and CD38-PE and CD3-FITC. The percentage of positive cells for each marker was determined using a Becton Dickinson FACStar plus flow cytometer. To have a homogenous CLL population, only the CLL samples with >90% $CD5^+$ and $CD19^+$ cells were used in the study. Where necessary, we used CD3/CD56 depletion to reduce the contaminating T and natural killer cells using Miltenyi's magnetic bead separation method (27). The purified CLL cells were reanalyzed using flow cytometry to confirm the purity. Similarly, for analyzing CD38 expression on CLL cells, cells double positive for CD19 and CD38 expression were specifically used. Mutations of the immunoglobulin gene variable heavy chain (IgVh) region in CLL cells were determined by PCR as described earlier (27).

Isolation of normal human B lymphocytes. B cells were selected using a negative selection principle. Using the B-cell Isolation Kit II (Miltenyi Biotech), human naive B cells were isolated by depletion of non–B cells (negative selection). Non–B cells were magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies, as primary labeling reagent, and antibiotin monoclonal antibodies were conjugated to magnetic microbeads as secondary labeling reagent. The magnetically labeled non–B cells were depleted by retaining them on a MACS column in the magnetic field of a MACS separator, whereas the unlabeled B cells pass through the column (28). The purity of the isolated B lymphocytes was tested by flow cytometry using anti-CD19 antibodies. B lymphocytes with >95% purity were used for these studies.

RNA extraction and DNA microarray analysis. Total RNA was extracted from CLL cells using TRIzol reagent (Invitrogen) per the manufacturer's instructions and RNA quality was assessed as recently published (29). Gene expression profiling was done by using a custom oligonucleotide microarray chip consisting of 50mer oligonucleotides representing 9,986 different genes (MWG Biotech, Human 10K oligo set A). The cDNAs were generated from the RNA obtained from CLL cells and Stratagene reference RNA and labeled with Cy-5 and Cy-3 dye, respectively, hybridized as previously described. Similarly, cDNA was generated from RNA from normal human donor B cells and Stratagene reference RNA and labeled with Cy-5 and Cy-3 dye, respectively, and hybridized. Hybridized slides were scanned for the microarray images using an Axon 4000B scanner (Axon Instruments). Fluorescence ratios for array elements were extracted by using Genepix 5.1 software. The microarray data files of all CLL patients obtained from Genepix 5.1 software were collated and further analyzed using BRB analysis tools. Lowess intensity–dependent normalization was used to adjust for differences in labeling intensities of the Cy3 and Cy5 dyes. Differential gene expression levels between samples were determined using significance analysis of microarray analyses.

Quantitative reverse transcription-PCR and real-time PCR. Differentially expressed genes identified by the microarray analyses were confirmed using semiquantitative reverse transcription-PCR, as previously described (30). First-strand cDNA template was generated from 5 μg of total RNA from each CLL sample as described previously (27). Specific transcripts were amplified with gene-specific forward and reverse primers (Supplementary Table S2) using a step-cycle program using Taq Polymerase (Invitrogen), as described earlier. PCR products were visualized on a 1% agarose gel stained with ethidium bromide. Expression of glyceraldehyde-3-phosphate dehydrogenase was used to normalize HEM1, CTLA4, and MNDA expression. Absorbance of each band was measured by Image Quant Imaging Software (Bio-Rad). The standard curve was generated with serial dilutions of PCR products, and the relative expression of HEM1, CTLA4, and MNDA with glyceraldehyde-3-phosphate dehydrogenase was determined as previously described (30). Real-time PCR was done to confirm the results of quantitative PCR. SYBR green real-time PCR assay was done in 20 μL PCR mixture volume consisting of 2× SYBR green PCR Master Mix (Roche), 200 nmol/L of primer mix, and 2 μL of cDNA template. Respective C_t values were used for further analysis.

Down-regulation of* HEM1 *in CLL cells. Freshly isolated CLL cells (10×10^6) from patients, along with 2.5 μmol/L HEM1 antisense oligonucleotide (5′-TCTTTGACATCTGCTTACC-3′) or an irrelevant antisense (5′-TATGCTGTGCCGGGGTCTTCGGGC-3′) to human herpes simplex virus genome or cells treated without oligonucleotides as control were cultured for 72 h in 37°C incubator with 5% CO_2. Cells

were harvested, and RNA was isolated and processed for RT-PCR as described above.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. To determine the susceptibility of HEM1 down-regulated CLL cells to fludarabine, freshly isolated cells (50×10^3) were cultured in RF-10 medium in triplicate wells with and without HEM1 antisense or control oligonucleotides and 0.5 μmol/L fludarabine (Fludarabine desphosphate, F-2773, Sigma Chemical), and were incubated at 37°C with 5% CO_2 for 72 h. To avoid the nonspecific toxicity of fludarabine, only 75% of IC_{50} drug concentration (0.5 μmol/L) was used in this assay. The viability of both the control and treated cells were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described (31).

Statistical analysis. To determine the clinical outcome, time to treatment was used as a measure where the time to treatment was defined as the time in months for initiation of the first treatment cycle since diagnosis in CLL patients. The Kaplan-Meier method was used to estimate the time to treatment distribution. Relative gene expression levels obtained from quantitative PCR were used as a measure of expression of CTLA4, HEM1, and MNDA. The expression levels were dichotomized at their median value and the log-rank test was used to compare the time to treatment between the below median and above median groups. The log-rank test was also used to assess the relationship of CD38 expression to time to treatment. In addition, where appropriate, Student's *t* test was used to determine the significance.

Results

Clinical outcome and gene expression in high versus low CD38–expressing CLL patients. To determine the relationship between CD38 expression and clinical outcome of CLL patients, time to treatment was compared with CD38 expression levels. Patients having >30% CD38-positive cells were considered as the high CD38 expression group, and those with <30% CD38-positive cells were considered as the low CD38 expression group. Time to treatment distribution between patients with high and low CD38 expression was determined by the Kaplan-Meier curve (Fig. 1A). Patients with high CD38 expression showed significantly ($P = 0.0003$) shorter time (30 months) to treatment compared with the patients with low CD38 expression (69 months). Furthermore, CD38 expression was correlated with immunoglobulin IgVh mutation status, a reliable known prognostic marker for CLL. CD38 expression of CLL patients with unmutated IgVh was significantly higher ($55.8 \pm 7.5\%$) compared with mutated IgVh ($16.35 \pm 3.8\%$) as shown in Fig. 1B. These results suggest that high CD38 expression is associated with poor clinical outcome and low CD38 expression is associated with good clinical outcome and, thus, confirms the validity of CD38 expression as a prognostic indicator.

To understand the molecular basis of CD38 expression and signaling, differentially expressed genes between high and low CD38–expressing CLL cells were compared using oligonucleotide microarray analysis. The expression of genes was analyzed by BRB analysis tools. The global expression profiles of CLL cells expressing high CD38 were compared with low CD38 using significance analysis of microarray and identified 76 differentially expressed genes in high and low CD38–expressing CLL patient subgroups. The results are shown as a supervised cluster (Fig. 1C). Of the 76 differentially expressed genes, genes such as *HEM1*, *CTLA4*, and *MNDA* with known relevant immunohematologic functions were selected to dissect the molecular basis of clinical disparity in CLL patients with high versus low CD38 expression (22, 24, 25).

Association of divergent expression of HEM1, CTLA4, and MNDA with disease progression in CLL patients. HEM1 is one of the significantly overexpressed genes in CLL cells expressing high CD38 compared with low CD38 in the microarray analysis. Expression of HEM1 is limited to hematopoietic cells and is highly conserved from invertebrates to mammals. HEM1 is located at the 12q13.1 region (22), a frequent target for cytogenetic abnormalities in hematologic malignancies including CLL. Therefore, the overexpression of HEM1 in high CD38 group compared with low CD38 group was further confirmed by semiquantitative real-time RT-PCR (Fig. 2A and B), thus validating the microarray data. Further, to determine whether expression levels of HEM1 correlate with disease progression, the log-rank test was applied across the relative expression levels of HEM1 obtained from quantitative PCR analyses. HEM1 expression directly correlated with the disease progression, implying that higher expression levels of HEM1 was associated with shorter time to treatment (Fig. 2C). Taken together, our results suggest that HEM1 is overexpressed in CLL cells expressing high CD38, and is associated with poor clinical outcome. Further, to test whether HEM1 overexpression is associated with resistance to therapy in CLL patients, freshly isolated CLL cells from patients were treated with HEM1 antisense oligonucleotide *in vitro* to down-regulate the overexpressed HEM1 followed by treatment with fludarabine, a standard chemotherapy agent used in CLL. The expression levels of HEM1 transcripts were analyzed by RT-PCR, and the viability of the cells were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The levels of HEM1 transcripts were significantly lower in the cells treated with HEM1 antisense compared with untreated cells (Fig. 2D). Further, HEM1 antisense treatment significantly increased fludarabine-mediated killing of CLL cells ($P = 0.007$) compared with either cells treated with only fludarabine or irrelevant antisense as controls (Fig. 2E). This suggests that down-regulation of overexpressed HEM1 in CLL cells expressing high CD38 may make the CLL cells more susceptible for chemotherapy, and HEM1 might be one of the genes associated with poor clinical outcome.

CTLA4 is one of the significantly underexpressed genes in CLL cells expressing high CD38 compared with low CD38 in the microarray analysis. CTLA4 is a member of the immunoglobulin superfamily and is a costimulatory molecule expressed by activated T cells. CTLA4 transmits an inhibitory signal to the T cells and thus helps in regulation of immune response (23). Recently, Komaczewska et al. (32) showed that the proportion of CTLA4 was significantly higher in leukemia B cells compared with their normal counterpart, and the expression of CTLA4 correlated with the number of CLL cells in G_0-G_1 phase. Moreover, CTLA4 may be involved in the cell cycle machinery and is thought to prolong the progression through the G_1 phase of the cell cycle (24). Therefore, underexpression of CTLA4 in CD38 high CLL cells compared with low CD38 cells was confirmed by semiquantitative RT-PCR and real-time PCR (Fig. 2F and G), thus confirming the microarray results. Further, to analyze whether expression levels of CTLA4 correlate with disease progression, the log-rank test was applied across the relative expression levels of CTLA4 obtained from quantitative PCR. CTLA4 expression inversely correlated with the disease

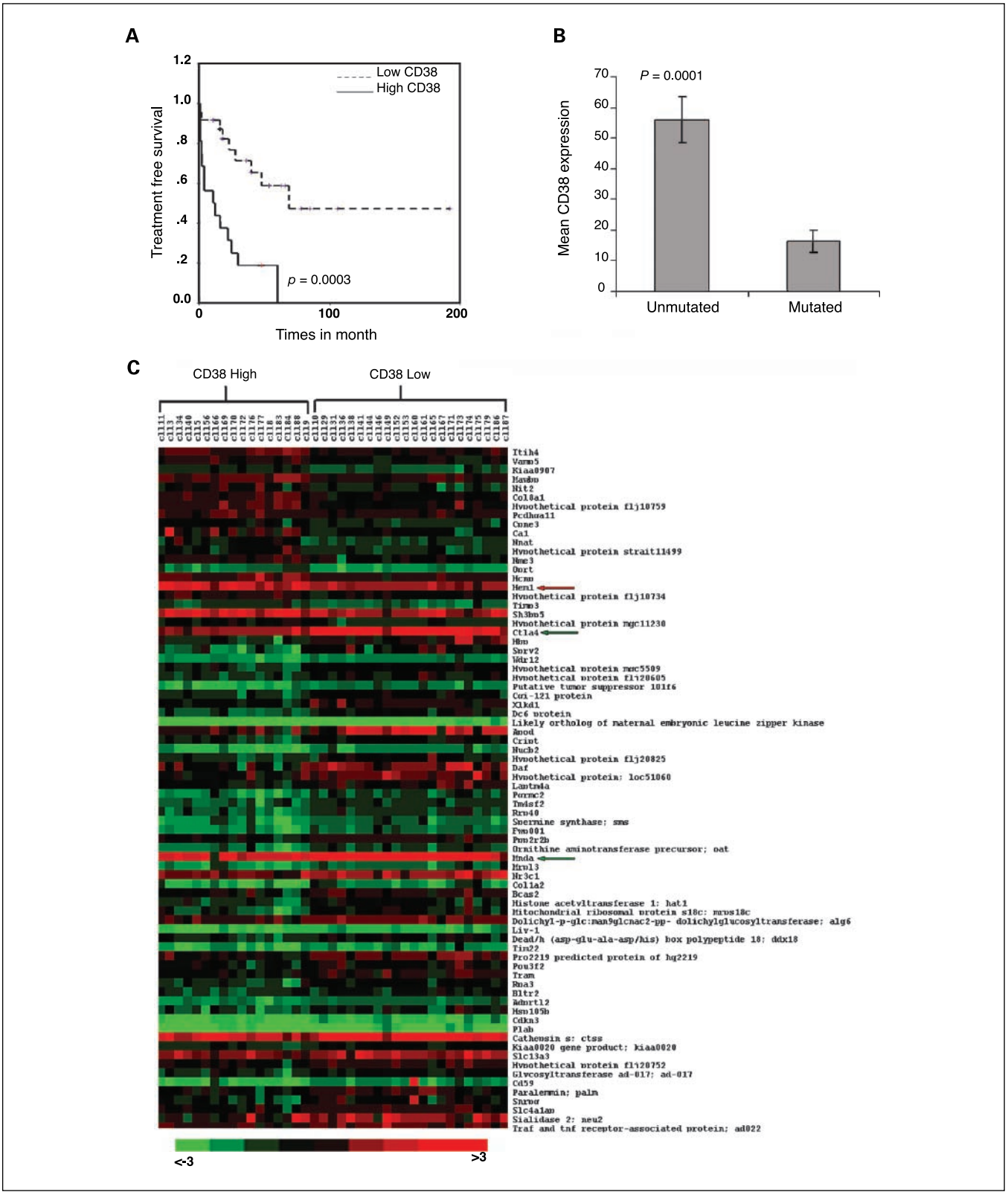

Fig. 1. Disease progression and underlying gene expression in CLL patients with high versus low CD38 expression. *A,* probability of disease progression in CLL as indicated by time to treatment: Kaplan-Meier curve comparing time periods from diagnosis to initiation of chemotherapy in CLL patients expressing high CD38 to patients expressing low CD38. Statistical analysis was done using the log-rank test. *B,* comparison of CD38 expression across mutated and unmutated CLL. Mean CD38 expression in CLL patients with mutated immunoglobulin chain was significantly lower than mean CD38 expression in CLL patients with unmutated immunoglobulin heavy chain ($P = 0.0001$). *C,* supervised cluster analysis demonstrating differentially expressed genes in high CD38 – expressing ($n = 19$) and low CD38 – expressing ($n = 20$) CLL subgroups. The expression data are presented as a matrix: rows, individual genes; columns, individual samples. Color scale at the bottom, the relative level of gene expression. *Arrows,* location and expression levels of the three genes, namely *HEM1, CTLA*, and *MINDA*.

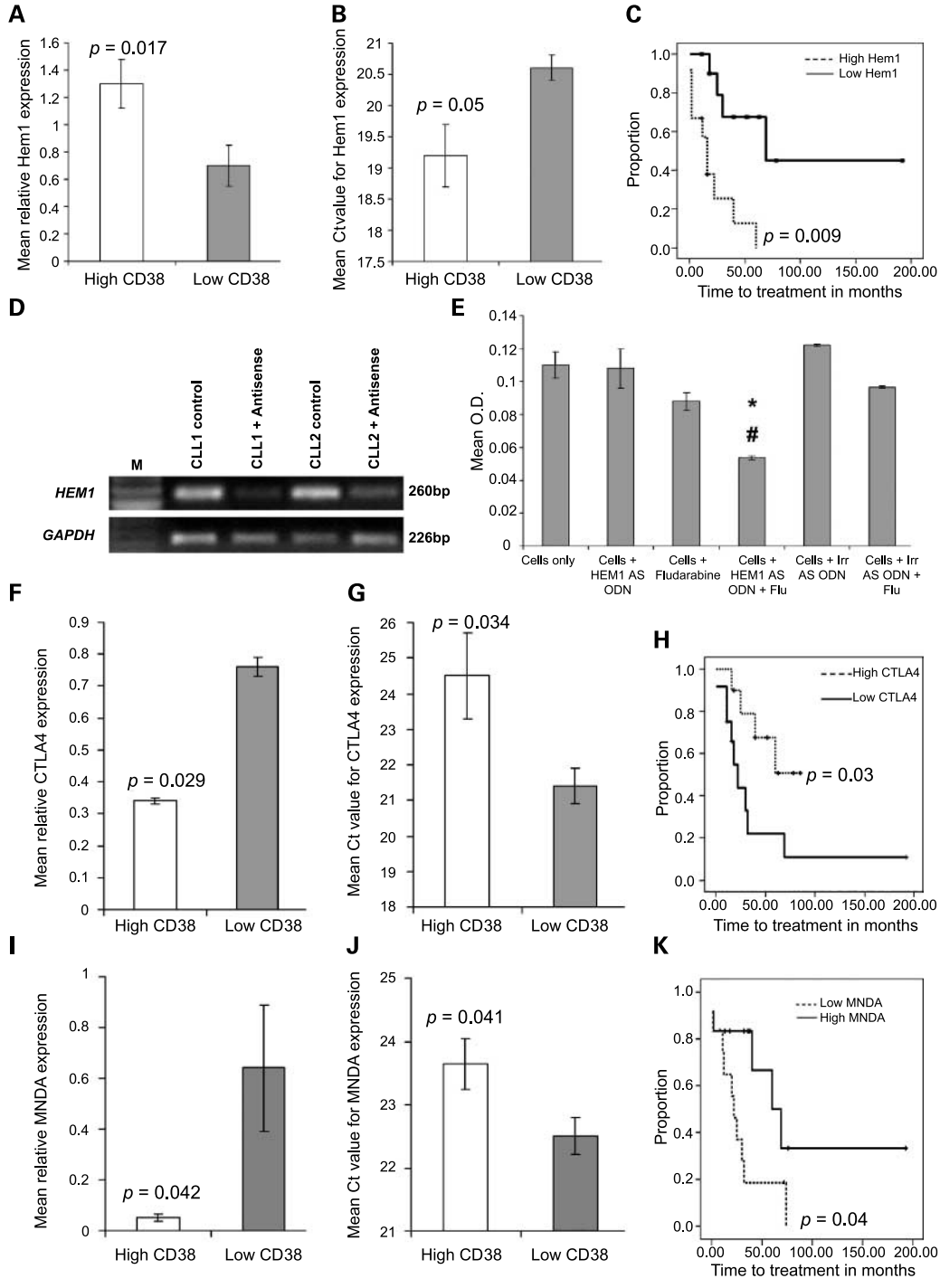

Fig. 2. Confirmation of differential expression of HEM1 in CD38 high and low CLL cells. *A,* mean HEM1 expression for each high and low CD38− expressing group. *B,* confirmation of microarray data using real-time PCR demonstrating significantly higher HEM1 expression in high CD38− expressing (low C_t value) compared with low CD38-expressing (higher C_t value) patients. *C,* Kaplan-Meier curve assessing the relationship of HEM1 expression and time to treatment in CLL patients: Higher expression level of HEM1 was associated with shorter time to treatment. *D,* HEM1 antisense treatment. RT-PCR analysis showing down-regulation of HEM1 expression in CLL cells after treatment with HEM1 antisense. *E,* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay demonstrating a significantly increased sensitivity of CLL cells to fludarabine-mediated killing when treated with HEM1 antisense (*AS*) and fludarabine together compared with fludarabine alone (*$P = 0.007$), cells treated with irrelevant antisense and fludarabine (#$P = 0.0001$) or antisense oligonucleotide (*ODN*) alone or an irrelevant oligonucleotide. *F,* mean CTLA4 expression for each high CD38− and low CD38− expressing group. *G,* confirmation of microarray data using real-time PCR demonstrating significantly lower CTLA4 expression in high CD38− expressing (high C_t value) compared with low CD38− expressing (low C_t value) patients. *H,* Kaplan-Meier curve assessing the relationship of CTLA4 expression and time to treatment in CLL patients: Higher expression level of CTLA4 was associated with longer time to treatment. *I,* mean MNDA expression for each high CD38− and low CD38− expressing group. *J,* confirmation of microarray data using real-time PCR demonstrating significantly lower MNDA expression in high CD38− expressing (high C_t value) compared with low CD38− expressing (low C_t value) patients. *K,* Kaplan-Meier curve assessing the relationship of MNDA expression and time to treatment in CLL patients: Higher expression level of MNDA was associated with longer time to treatment.

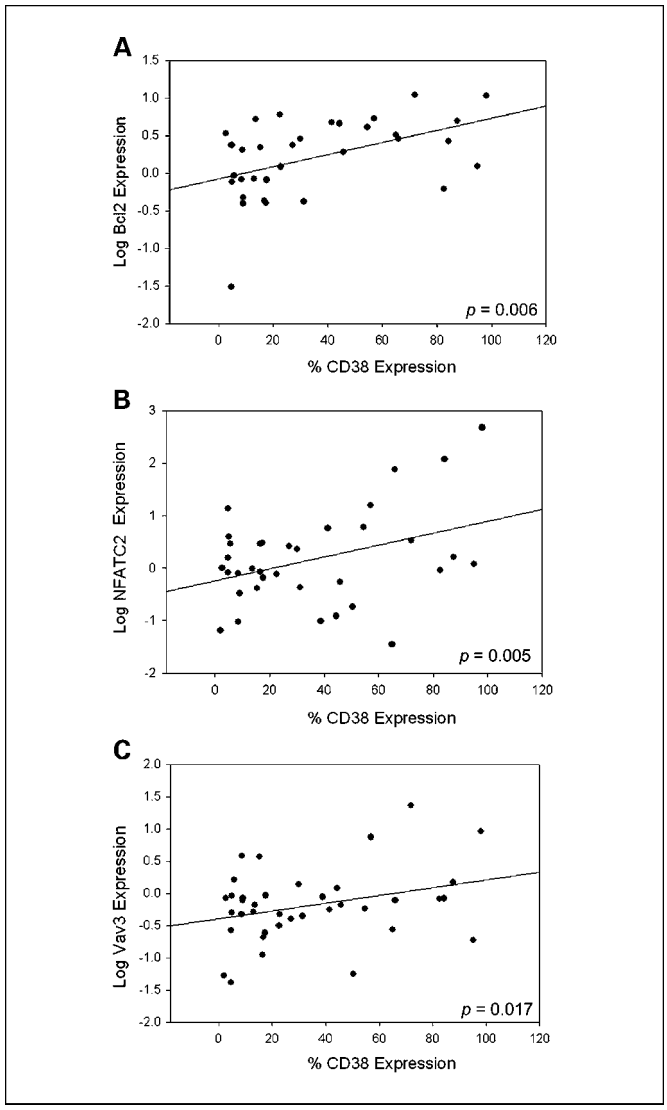

Fig. 3. Correlation of expression of genes involved in BCR signaling with CD38 expression. *BCL2* (*A*), *NFATC2* (*B*), and *VAV3* (*C*) significantly correlated with CD38 expression. A scatter plot is drawn to show the correlation between these genes and CD38 expression.

progression; lower expression of CTLA4 was associated with significantly shorter time to treatment and poor clinical outcome compared with high CTLA4 expression associated with good clinical outcome (Fig. 2H).

Myeloid cell nuclear differentiation antigen (*MNDA*) was another significantly underexpressed gene in the CD38 high CLL subgroup compared with low CD38 group identified in the microarray analysis. *MNDA* belongs to the IFN-inducible gene family and its expression is limited to the cells of hematopoietic lineage (33). Comparison of expression of genes specifically involved in immunologic functions across different prognostic markers, such as CD38 expression, IgVh mutation status, and chromosome abnormality in CLL patients, revealed that MNDA was consistently overexpressed in CLL patients with good prognosis (Supplementary Table S3). Therefore, underexpression of MNDA in CLL cells expressing high CD38 group compared with low CD38 group was confirmed by semiquantitative RT-PCR and real-time PCR (Fig. 2I and J). Further, to analyze whether expression levels of MNDA correlate with disease progression or not, the log-rank test was applied across the relative expression levels of MNDA obtained from quantitative PCR and disease progression. MNDA expression inversely correlated with the disease progression; higher expression of MNDA was associated with significantly longer time to treatment compared with low MNDA expression that associated with shorter time to treatment (Fig. 2K).

Relationship of BCR signaling molecules BCL2, NFATC2, and VAV3 with the CD38 expression. Because CD38 signaling uses the BCR signaling machinery to transmit the proliferation signal, we specifically looked at the expression levels of genes involved in BCR signaling. A correlative approach was used in this case and Pearson correlation was used to test the correlation of CD38 receptor expression with its downstream effector genes. BCL2, NFATC2, and VAV3 significantly correlated with CD38 expression in CLL patients, implying that the higher the CD38 expression, the higher the BCL2, NFATC2, and VAV3 expression (Fig. 3A-C). BCL2, NFATC2, and VAV3 are known to play a central role in the B-cell development and maturation and proliferation, and its correlation with CD38 expression might explain the possible reason for the observed clinical disparity in CLL patients with high and low CD38 expression patterns.

Overall, these results suggest that higher CD38 expression is associated with higher expression of genes involved in BCR signaling. HEM1, CTLA4, and MNDA may be the key players that are responsible in determining the clinical outcome of CLL patients with high and low CD38 expression. Also, HEM1 can be targeted using antisense oligonucleotides to increase susceptibility of CLL cells to chemotherapy.

Differential expression of ATM in CLL cells with high and low CD38 expression. We have also done a pairwise analysis comparing gene expression profiles in normal B cells with CD38 high CLL cells and CD38 low CLL cells. Pathway analysis was used to identify the functional differences between CD38 high CLL cells and normal B cells, and CD38 low CLL cells and B cells. Pathway comparison in BRB is based on gene ontology comparison and provides a list of Gene Ontology categories that have more genes differentially expressed among the phenotype classes than expected by chance. The genes are then grouped by BioCarta pathways rather than by Gene Ontology categories. BioCarta is a trademark of BioCarta, Incorporated, and the pathways included in BRB array tools provide displays of gene interactions within pathways for human and mouse cellular processes.

Pathway analysis revealed that CLL cells with high and low CD38 shared a common pathway 4-1BB (CD137, a member of tumor necrosis factor receptor family)–dependent immune response. In addition, pathway analysis also showed that apoptotic signaling pathway in response to DNA damage was active in CLL cells with low CD38 expression but not in CLL cells with high CD38 expression (Supplementary Table S4A and S4B). ATM was one of the genes involved in apoptotic signaling pathway (Supplementary Table S5) and was found to be overexpressed in CLL cells with low CD38 expression compared with normal B cells. These observations made us hypothesize that CLL cells with low CD38 expression may have higher ATM expression compared with CLL cells with high CD38 expression (34, 35). To prove this hypothesis, ATM expression in 25 different CLL samples was analyzed using

real-time RT-PCR. The ATM expression was normalized using glyceraldehyde-3-phosphate dehydrogenase as an internal control. Results showed that ATM was significantly underexpressed in CLL cells with high CD38 (Fig. 4A and B). In addition, to further confirm the differential expression of ATM, we also looked at the transcription levels of CHK2, a target for ATM in CLL cells (36, 37) with high and low CD38 expression (Fig. 4C). There was a significant increase in the expression of CHK2 in the CD38 low group compared with the CD38 high CLL subgroup. These results with CHK2 expression are in line with ATM expression in CD38 high and low CLL subgroups. Thus, overexpression of ATM in CLL cells with low CD38 expression may be responsible for making these cells more susceptible to apoptosis in response to chemotherapy-induced DNA damage.

Discussion

Clinical heterogeneity in CLL warrants the immediate necessity of new avenues to predict the prognostic indicator and, thereby, treatment. In our gene expression analyses, significance analysis of microarray identified 76 differentially expressed genes in high and low CD38–expressing CLL patients, revealing an underlying gene expression pattern. Similar microarray study has been done in the past comparing the gene expression profiles of CD38-positive and CD38-negative CLL (16). There are several differences between our study and the study by Durig et al. (16), including number of genes on the array chip. Their study concentrated on the CLL subgroup revealed by an unsupervised cluster, whereas in our study the unsupervised cluster did not reveal any such subgroup (data not shown). In addition, they used the Wilcoxon rank sum test for identifying the differentially expressed genes in CD38-positive and CD38-negative CLL subgroups, which may be less robust compared with the significance analysis of microarray, used in our study.

This study reports the differential expression of *HEM1*, *CTLA4*, and *MNDA* genes for the first time in CLL groups with high and low CD38 expression. HEM1, a transmembrane protein with an unknown function, is also found to be significantly overexpressed in CLL patients with trisomy 12 abnormalities (data not shown). Also, all the CLL patients with trisomy 12 expressed high CD38 (Supplementary Table S1). Similarly, Haslinger et al. (38) have described HEM1 overexpression in trisomy 12 subgroups. HEM2, a homologue of HEM1 that is expressed only in the brain, when down-regulated with antisense treatment, induces apoptosis in neurons (39). This observation also prompted us to use an antisense against HEM1. Down-regulation of HEM1 by antisense increased the susceptibility of CLL cells to fludarabine even at a 75% lower dose than the suggested IC_{50} of fludarabine (40), suggesting a role of HEM1 in therapy resistance. However, the precise role of HEM1 in cell proliferation or chemoresistance remains unknown. HEM1 protein has a transmembrane domain, suggesting its role in cell signaling. It can be speculated that HEM1 signaling pathways may activate cell survival or proliferation. These observations warrant further in-depth study of HEM1-mediated signaling in CLL cells.

A study by Kosmaczewska et al. (32) showed that CTLA4 was overexpressed in CLL cells compared with their normal B cells and its expression inversely correlates with Rai stages in CLL patients and suggested a role of CTLA4 in arresting leukemic B cells in G_0-G_1 phase. In our study, good prognosis indicators such as low CD38 expression, 13q deletion, and normal karyotype, and immunoglobulin mutation correlated with higher expression of MNDA, indicating a growth-inhibitory/tumor-suppressor role of MNDA in CLL, which needs to be confirmed with additional studies.

Furthermore, because CD38 signaling uses the BCR signaling pathway for transmitting the intracellular signals, we specifically looked for the expression of genes involved in BCR signaling across high and low CD38–expressing groups. Expression of BCL2 directly correlated with the CD38 positivity (Fig. 3A). BCL2 has been regarded as the main culprit in CLL (41) and this is the first report demonstrating direct correlation between BCL2 levels and CD38 expression levels. Similarly, expression of other genes, such as *NFATC2* and *VAV3*, that are involved in B-cell differentiation and survival, respectively (42, 43),

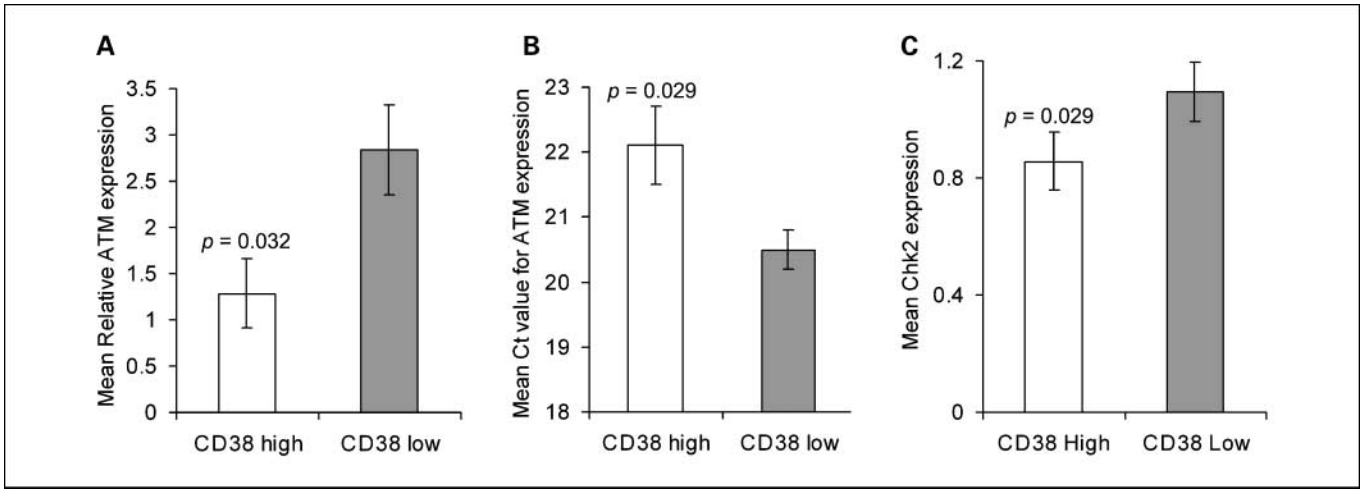

Fig. 4. Differential expression of ATM in CD38 high and low CLL subgroups. The expression of *ATM* gene in CLL cells was determined using real-time PCR analysis. *A,* mean ATM expression for each CD38 high – and CD38 low – expressing group. *B,* demonstration of significantly lower ATM expression in CD38 high using raw C_t value compared with low CD38 – expressing patients. *C,* correlation of CHK2 expression in CD38 high and low CLL subgroups.

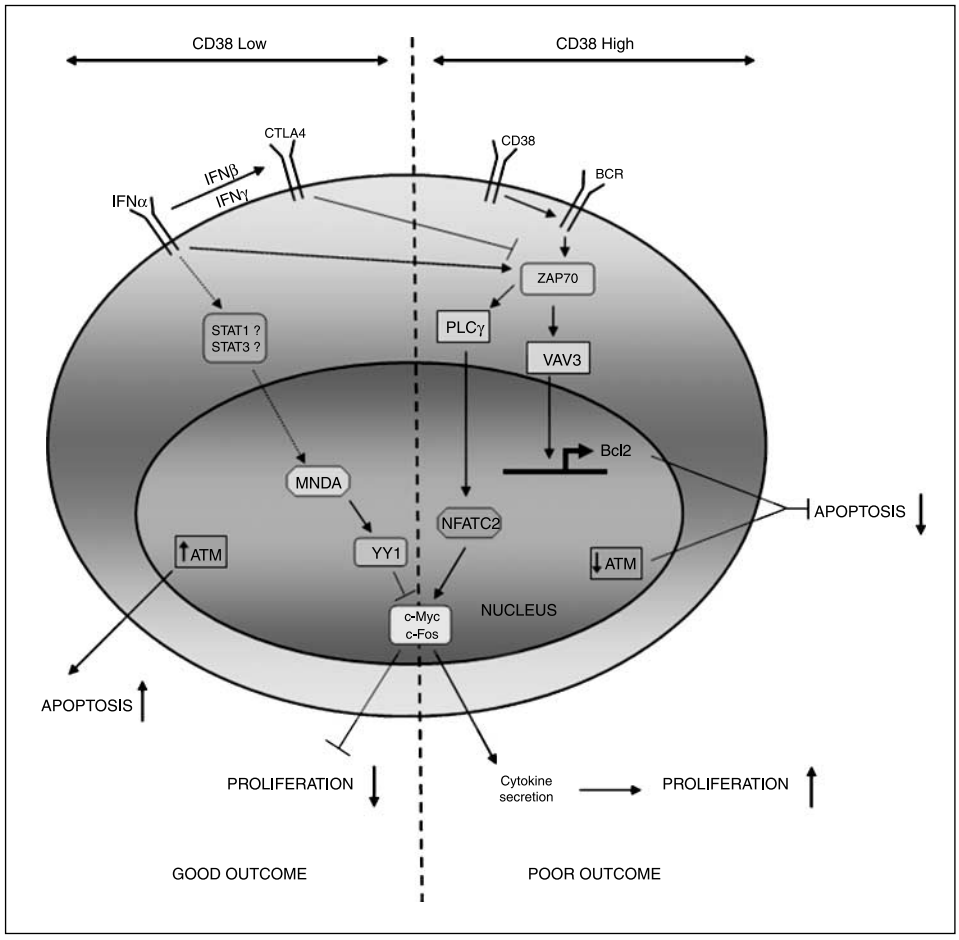

Fig. 5. Hypothetical model describing interplay of various genes that can define the fate of CLL cells. Left, the signals that reduce the proliferation of CLL cells; right, the various genes responsible for inhibition of apoptosis of CLL cells and thus increasing their proliferation. The final fate of the CLL cells will be determined by the balance between the right and the left sides of the panel. CLL patients may have good outcome if the balance is in the favor of the left side, whereas shift of balance toward the right side will result in poor outcome. The left side represents tumor-suppression properties in CLL via molecules such as CTLA4, IFN, MNDA, YY1, c-Myc, and c-Fos, resulting in decreased proliferation. Roles of intermediate molecules in the pathway such as signal transducers and activators of transcription 1 (*STAT1*) or 3 (*STAT3*) have been speculated. The right side represents tumor-progressing effects involving CD38 molecules via activation of ZAP-70, NFATC2, the BCR signaling molecules along with cytokine secretion leading to cell proliferation; and alternatively or simultaneously ZAP-70, VAV3 and BCL2, leading to inhibition of apoptosis.

directly correlated with CD38 expression. VAV3 up-regulates BCL2 via nuclear factor-κB and thus shows that higher VAV3 expression in CD38 high expressing CLL cells may lead to higher survival in these cells (44). NFATC2 is a member of the NFAT family of transcription factors that regulates lymphocyte activation and plays a direct role in cell cycle regulation (45, 46). Higher expression of NFATC2 shows activated phenotype of CLL cells with high CD38 expression. It remains unclear how NFATC2 may affect disease aggressiveness of CLL cells expressing high CD38 and warrants further in-depth studies to ascertain its role in CLL biology. Other genes involved in BCR signaling, such as *BTK, SYK*, and *BLNK*, did not show any correlation with CD38 expression and it may be speculated that activity of these genes may be regulated by their phosphorylation status. Together, these observations suggests that CD38 may be responsible for driving the underlying gene expression pattern in addition to its role as a signaling molecule (19, 20) in CLL cells. Whether CD38 directly affects the expression of the identified differentially expressed genes remains unknown and would be a part of future studies in the laboratory.

Previous studies have compared gene expression differences in normal B cells and CLL cells (47) showing a gene expression signature associated with disease progression as well as between CD38 high and low CLL cells (16) demonstrating a role for translation-related gene in the pathogenesis of CLL. On the other hand, our results point at certain genes that might be involved in the behavior of CLL cells leading to either aggressive clinical outcome or an indolent disease. The genes involved in the regulation of CLL cell proliferation/survival or death are *HEM1*, *MNDA*, *CTLA4*, and *ATM* as well as *BCL-2*, *VAV3*, *NFATC2*, and *CHK2*. The precise interaction(s) of these genes in CLL cells that regulate their pathogenesis are currently being investigated.

ATM down-regulation in CLL patients with high CD38 expression may be responsible for a progressive disease and confirms similar observations by others (48, 49) showing that ATM-deficient CLL patients are refractory to DNA-damaging chemotherapeutic drugs. Thus, these observations suggest that patients with high CD38 expression with ATM deficiency may not respond to fludarabine and chlorambucil, which are the frontline chemotherapeutic drugs in CLL that act by inducing DNA damage in CLL cells. Therefore, novel therapeutic strategies that bypass the ATM pathway need to be devised to treat this subgroup of CLL patients with high CD38 expression.

Although there is a report suggesting changes in CD38 levels, the extent of shift of CD38 remains controversial. In our data, the CD38 expression levels were measured at multiple times for the same patient and we did not see a significant change. Role of CD38 as a surrogate marker for IgVh mutation status has always been controversial. In our data presented here, the CLL patients with unmutated IgVh had an overall significantly higher CD38 expression compared with patients with mutated IgVh. Although these observations suggests that there might be

a correlation between IgVh mutation status and CD38 expression, use of CD38 expression as a surrogate marker for IgVh mutation remains inconclusive. Moreover, a change in CD38 expression in response to treatment has been reported (50). However, in our laboratory, CD38 expression levels were measured at multiple times for the same patient and we did not see a significant change. However, untreated patients or patients not treated for the prior 6 months were enrolled in the study, assuming that 6 months would be a long enough time interval to overcome the treatment-induced alterations in gene expression pattern. Also, previous gene expression studies from our laboratory revealed similar gene expression profile in untreated and 6 months posttreatment scenario of the same CLL patient, suggesting a minimal chance of selection of subclones either with regard to CD38 status or with regard to gene expression.

To summarize the findings of this study, we put forth a possible CLL model (Fig. 5). CD38 signaling may play a role in increased proliferation and decreased apoptosis of CLL cells by activating BCR signaling genes, such as *BCL2*, *VAV3*, and *NFATC2*. CD38 is known to directly activate/phosphorylate ZAP-70 in T cells and natural killer cells (51, 52), and, therefore, it can be assumed that ZAP-70 may play a role in transmitting the intracellular signals from CD38 to VAV3 and VAV3 may directly up-regulate nuclear factor-κB, thereby activating BCL2, which results in decreased apoptosis. NFATC2 may be activated via PLCγ pathway, which may result in proliferation of CLL cells. On the other hand, higher expression of antiproliferative genes, such as *CTLA4* and *MNDA*, may decrease proliferation of CLL cells. In this case, a shift of the balance in one direction or the other will determine the cell fate (i.e., either the cell will proliferate or its proliferation will be arrested). Among all these genes, *HEM1* remains the least studied player and needs to be further studied to determine its role in CLL biology.

Acknowledgments

We thank Dr. Richard Simon and Amy Peng Lam for developing the BRB Array Tools used in microarray analyses.

References

1. Morton LM, Wang SS, Devesa SS, Hartge P, Weisenburger DD, Linet MS. Lymphoma incidence patterns by WHO subtype in the united states, 1992-2001. Blood 2006;107:265–76.
2. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med 2005;352:804–15.
3. Binet JL, Auquier A, Dighiero G, et al. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. Cancer 1981;48:198–206.
4. Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. Blood 1975;46:219–34.
5. 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;94:1840–7.
6. Dohner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med 2000;343:1910–6.
7. Rosenwald A, Alizadeh AA, Widhopf G, et al. Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. J Exp Med 2001;194:1639–47.
8. Wiestner A, Rosenwald A, Barry TS, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood 2003;101:4944–51.
9. Reinherz EL, Kung PC, Goldstein G, Levey RH, Schlossman SF. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. Proc Natl Acad Sci U S A 1980;77:1588–92.
10. Campana D, Suzuki T, Todisco E, Kitanaka A. CD38 in hematopoiesis. Chem Immunol 2000;75:169–88.
11. Malavasi F, Funaro A, Alessio M, et al. CD38: a multi-lineage cell activation molecule with a split personality. Int J Clin Lab Res 1992;22:73–80.
12. Lund FE, Yu N, Kim KM, Reth M, Howard MC. Signaling through CD38 augments B cell antigen receptor (BCR) responses and is dependent on BCR expression. J Immunol 1996;157:1455–67.
13. Yasue T, Nishizumi H, Aizawa S, et al. A critical role of lyn and fyn for B cell responses to CD38 ligation and interleukin 5. Proc Natl Acad Sci US A 1997;94:10307–12.
14. Matrai Z. CD38 as a prognostic marker in CLL. Hematology 2005;10:39–46.
15. Durig J, Naschar M, Schmucker U, et al. CD38 expression is an important prognostic marker in chronic lymphocytic leukaemia. Leukemia 2002;16:30–5.
16. Durig J, Nuckel H, Huttmann A, et al. Expression of ribosomal and translation-associated genes is correlated with a favorable clinical course in chronic lymphocytic leukemia. Blood 2003;101:2748–55.
17. Hamblin TJ, Orchard JA, Ibbotson RE, et al. CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease. Blood 2002;99:1023–9.
18. Del Giudice I, Morilla A, Osuji N, et al. Zeta-chain associated protein 70 and CD38 combined predict the time to first treatment in patients with chronic lymphocytic leukemia. Cancer 2005;104:2124–32.
19. Deaglio S, Capobianco A, Bergui L, et al. CD38 is a signaling molecule in B-cell chronic lymphocytic leukemia cells. Blood 2003;102:2146–55.
20. Deaglio S, Vaisitti T, Bergui L, et al. CD38 and CD100 lead a network of surface receptors relaying positive signals for CLL growth and survival. Blood 2005;105:3042–50.
21. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A 2001;98:5116–21.
22. Hromas R, Collins S, Raskind W, Deaven L, Kaushansky K. Hem-1, a potential membrane protein, with expression restricted to blood cells. Biochim Biophys Acta 1991;1090:241–4.
23. Brunner MC, Chambers CA, Chan FK, Hanke J, Winoto A, Allison JP. CTLA-4-mediated inhibition of early events of T cell proliferation. J Immunol 1999;162:5813–20.
24. Greenwald RJ, Oosterwegel MA, van der Woude D, et al. CTLA-4 regulates cell cycle progression during a primary immune response. Eur J Immunol 2002;32:366–73.
25. Geng Y, Choubey D. Differential induction of the 200-family proteins in Daudi Burkitt's lymphoma cells by interferon-α. J Biol Regul Homeost Agents 2000;14:263–8.
26. Wang P, Munger CM, Joshi AD, Pirruccello SJ, Joshi SS. Cytotoxicity of cord blood derived Her2/neu-specific cytotoxic T lymphocytes against human breast cancer *in vitro* and *in vivo*. Breast Cancer Res Treat 2004;83:15–23.
27. Dickinson JD, Smith LM, Sanger WG, et al. Unique gene expression and clinical characteristics are associated with the 11q23 deletion in chronic lymphocytic leukaemia. Br J Haematol 2005;128:460–71.
28. Jaimes MC, Rojas OL, Kunkel EJ, et al. Maturation and trafficking markers on rotavirus-specific B cells during acute infection and convalescence in children. J Virol 2004;78:10967–76.
29. Dickinson JD, Joshi A, Iqbal J, Sanger W, Bierman PJ, Joshi SS. Genomic abnormalities in chronic lymphocytic leukemia influence gene expression by a gene dosage effect. Int J Mol Med 2006;17:769–78.
30. Dickinson JD, Gilmore J, Iqbal J, et al. 11q22.3 deletion in B-chronic lymphocytic leukemia is specifically associated with bulky lymphadenopathy and ZAP-70 expression but not reduced expression of adhesion/cell surface receptor molecules. Leuk Lymphoma 2006;47:231–44.
31. Wang P, Pavletic ZS, Joshi SS. Increased apoptosis in B-chronic lymphocytic leukemia cells as a result of cyclin D3 down regulation. Leuk Lymphoma 2002;43:1827–35.
32. Kosmaczewska A, Ciszak L, Suwalska K, Wolowiec D, Frydecka I. CTLA-4 overexpression in CD19+/CD5+ cells correlates with the level of cell cycle regulators and disease progression in CLL patients. Leukemia 2005;19:301–4.
33. Miranda RN, Briggs RC, Shults K, Kinney MC, Jensen RA, Cousar JB. Immunocytochemical analysis of MNDA in tissue sections and sorted normal bone marrow cells documents expression only in maturing normal and neoplastic myelomonocytic cells and a subset of normal and neoplastic B lymphocytes. Hum Pathol 1999;30:1040–9.
34. Boultwood J. Ataxia telangiectasia gene mutations in leukaemia and lymphoma. J Clin Pathol 2001;54:512–6.
35. Shiloh Y. ATM: Ready, set, go. Cell Cycle 2003;2:116–7.
36. Choudhury A, Cuddihy A, Bristow RG. Radiation and new molecular agents part I: Targeting ATM-ATR checkpoints, DNA repair, and the proteasome. Semin Radiat Oncol 2006;16:51–8.
37. Niida H, Nakanishi M. DNA damage checkpoints in mammals. Mutagenesis 2006;21:3–9.
38. Haslinger C, Schweifer N, Stilgenbauer S, et al. Microarray gene expression profiling of B-cell chronic lymphocytic leukemia subgroups defined by genomic

aberrations and VH mutation status. J Clin Oncol 2004;22:3937–49.
39. Suzuki T, Nishiyama K, Yamamoto A, et al. Molecular cloning of a novel apoptosis-related gene, human Nap1 (NCKAP1), and its possible relation to Alzheimer disease. Genomics 2000;63:246–54.
40. Di Raimondo F, Palumbo GA, Romeo MA, et al. *In vitro* sensitivity of B-CLL cells to fludarabine and interferons. Leuk Lymphoma 1995;17:449–53.
41. Keating MJ. Chronic lymphocytic leukemia. Semin Oncol 1999;26:107–14.
42. Peng SL, Gerth AJ, Ranger AM, Glimcher LH. NFATc1 and NFATc2 together control both T and B cell activation and differentiation. Immunity 2001;14:13–20.
43. Vigorito E, Gambardella L, Colucci F, McAdam S, Turner M. Vav proteins regulate peripheral B-cell survival. Blood 2005;106:2391–8.
44. Katzav S. Vav1: An oncogene that regulates specific transcriptional activation of T cells. Blood 2004;103:2443–51.
45. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol 1997;15:707–47.
46. Serfling E, Berberich-Siebelt F, Avots A, et al. NFAT and NF-κB factors—the distant relatives. Int J Biochem Cell Biol 2004;36:1166–70.
47. Jelinek DF, Tschumper RC, Stolovitzky GA, et al. Identification of a global gene expression signature of B-chronic lymphocytic leukemia. Mol Cancer Res 2003;1:346–61.
48. Faderl S, Keating MJ, Do KA, et al. Expression profile of 11 proteins and their prognostic significance in patients with chronic lymphocytic leukemia (CLL). Leukemia 2002;16:1045–52.
49. Austen B, Powell JE, Alvi A, et al. Mutations in the ATM gene lead to impaired overall and treatment-free survival that is independent of IGVH mutation status in patients with CLL. Blood 2005;106:3175–82.
50. Gentile M, Mauro FR, Calabrese E, et al. The prognostic value of CD38 expression in chronic lymphocytic leukaemia patients studied prospectively at diagnosis: a single institute experience. Br J Haematol 2005;130:549–57.
51. Zubiaur M, Izquierdo M, Terhorst C, Malavasi F, Sancho J. CD38 ligation results in activation of the Raf-1/mitogen-activated protein kinase and the CD3-ζ/ζ-associated protein-70 signaling pathways in Jurkat T lymphocytes. J Immunol 1997;159:193–205.
52. Mallone R, Funaro A, Zubiaur M, et al. Signaling through CD38 induces NK cell activation. Int Immunol 2001;13:397–409.