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Distinct transcriptional control in major immunogenetic subsets of chronic lymphocytic leukemia exhibiting subset-biased global DNA methylation profiles

Meena Kanduri,[1,†] Millaray Marincevic,[2,†] Anna M. Halldórsdóttir,[2,†] Larry Mansouri,[2] Katarina Junevik,[1] Stavroula Ntoufa,[3] Hanna Göransson Kultima,[4] Anders Isaksson,[4] Gunnar Juliusson,[5] Per-Ola Andersson,[6] Hans Ehrencrona,[7] Kostas Stamatopoulos[3,8,‡] and Richard Rosenquist[2,‡,*]

[1]Institute of Biomedicine, Department of Clinical Chemistry and Transfusion Medicine; Sahlgrenska University Hospital; Gothenburg, Sweden; [2]Department of Immunology, Genetics and Pathology; Science for Life Laboratory; Uppsala University; Uppsala, Sweden; [3]Hematology Department and HCT Unit; G. Papanicolaou Hospital; Thessaloniki, Greece; [4]Department of Medical Sciences; Science for Life Laboratory; Uppsala University; Uppsala, Sweden; [5]Lund Strategic Research Center for Stem Cell Biology and Cell Therapy; Hematology and Transplantation; Lund University; Lund, Sweden; [6]Section of Hematology and Coagulation; Department of Internal Medicine; Sahlgrenska University Hospital; Gothenburg, Sweden; [7]Department of Clinical Genetics; Lund University Hospital; Lund, Sweden; [8]Institute of Applied Biosciences; Center for Research and Technology; Thessaloniki, Greece

[†]These first authors contributed equally to this work.

[‡]These senior authors contributed equally to this work.

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Chronic lymphocytic leukemia (CLL) can be divided into prognostic subgroups based on the *IGHV* gene mutational status, and is further characterized by multiple subsets of cases with quasi-identical or stereotyped B cell receptors that also share clinical and biological features. We recently reported differential DNA methylation profiles in *IGHV*-mutated and *IGHV*-unmutated CLL subgroups. For the first time, we here explore the global methylation profiles of stereotyped subsets with different prognosis, by applying high-resolution methylation arrays on CLL samples from three major stereotyped subsets: the poor-prognostic subsets #1 (n = 15) and #2 (n = 9) and the favorable-prognostic subset #4 (n = 15). Overall, the three subsets exhibited significantly different methylation profiles, which only partially overlapped with those observed in our previous study according to *IGHV* gene mutational status. Specifically, gene ontology analysis of the differentially methylated genes revealed a clear enrichment of genes involved in immune response, such as B cell activation (e.g., *CD80, CD86* and *IL10*), with higher methylation levels in subset #1 than subsets #2 and #4. Accordingly, higher expression of the co-stimulatory molecules CD80 and CD86 was demonstrated in subset #4 vs. subset #1, pointing to a key role for these molecules in the crosstalk of CLL subset #4 cells with the microenvironment. In summary, investigation of three prototypic, stereotyped CLL subsets revealed distinct DNA methylation profiles for each subset, which suggests subset-biased patterns of transcriptional control and highlights a key role for epigenetics during leukemogenesis.

Introduction

Determination of the immunoglobulin heavy variable (*IGHV*) gene mutational status has proved to be one of the most important prognostic markers in chronic lymphocytic leukemia (CLL), where patients with unmutated *IGHV* genes demonstrate an inferior outcome compared with those displaying mutated *IGHV* genes.[1,2] Furthermore, approximately one-third of CLL cases can be assigned to distinct subsets that express specific IG heavy/light chain genes and carry closely homologous, stereotyped B cell receptors (BcRs).[3-9] These findings have strongly implied a role for antigen selection in CLL development.

Recent studies have also revealed an important association between stereotyped BcRs and clinical outcome for certain subsets.[6,10,11] For instance, subset #1 cases typically express heavy chains encoded by one of the *IGHV1/5/7* clan genes with a highly homologous heavy complementarity determining region 3 (VH CDR3), in combination with kappa light chains encoded by *IGKV1-39/1D-39*. These patients carry unmutated *IGHV* genes and have poorer prognosis compared with non-stereotyped,

*Correspondence to: Richard Rosenquist; Email: richard.rosenquist@igp.uu.se
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unmutated cases using the same *IGHV* genes.[6,11] Furthermore, approximately half of all *IGHV3-21* patients express a stereotyped BcR (denoted as subset #2) with a short and highly similar VH CDR3 in combination with *IGLV3-21* gene usage.[3,10,12] These subset #2 cases have a shorter time to progression, independent of *IGHV* gene mutational status, compared with non-subset #2 patients.[6] In contrast, subset #4 cases express stereotyped *IGHV4-34/IGKV2-30* BcRs that are highly mutated and IgG switched; such cases have a low median age at diagnosis and display an indolent disease course compared with non-stereotyped, mutated *IGHV4-34* patients.[6] Hence, the molecular classification of CLL according to BcR stereotypy seems to refine survival prediction compared with the broader sub-grouping according to *IGHV* gene mutational status alone, likely because subsets are considerably more homogeneous than the broad mutational categories (i.e., unmutated vs. mutated CLL).

Recently, we described distinct gene expression profiles in CLL cases that are assigned to two different subsets utilizing the *IGHV4-34* gene, e.g., subset #4 and subset #16 (*IGHV4-34/IGKV3-20*).[13] These differences are especially noteworthy given that both subsets have similar mutational status; furthermore, they support the notion that the molecular classification of CLL based on BcR stereotypy reflects true biological differences stemming from distinct signaling pathways to CLL development. Along these lines, it is relevant to mention that the differentially expressed genes between subsets #4 and #16 are involved in important cell regulatory pathways, including immune response, proliferation and cell cycle control. In a separate study, global expression profiling of *IGHV3-21* CLL patients revealed that genes involved in DNA replication/cell cycle control, transcription and protein kinase activity were preferentially expressed in comparison to non-*IGHV3-21* patients, possibly contributing to the poor outcome associated with *IGHV3–21* gene usage.[14]

Previous reports in CLL have shown a strong correlation between promoter methylation and transcriptional silencing for individual genes (e.g., *ZAP70, TWIST2, DAPK1* and *HOXA4*).[15-18] Additionally, the methylation of several of these genes has been reported to correlate with the *IGHV* mutational status.[15,16] More recently, we reported the first global DNA methylation profiling study in CLL using methylation arrays on patients belonging to the *IGHV*-mutated and *IGHV*-unmutated/*IGHV3-21* groups.[19] Significant differences in methylation patterns were observed when comparing these subgroups; for instance, several tumor suppressor genes were methylated in *IGHV*-unmutated CLL, while a number of genes involved in cell proliferation and tumor progression were methylated in *IGHV*-mutated CLL.

Prompted by these findings, and also taking into account the distinct gene expression profiles of individual CLL subsets with stereotyped BcRs, we here extend our previous study by investigating DNA methylation in relation to BcR stereotypy. Our specific aim was to identify the regulatory processes that may underlie the functional and eventual clinical differences for CLL patients that belong to different subsets. To address this issue, we analyzed a unique collection of 39 CLL cases assigned to three major and paradigmatic stereotyped subsets that represent "extremes" in survival: the poor-prognostic subsets #1 and #2 and the favorable-prognostic subset #4.

Results

Distinct methylation profiles were observed in stereotyped CLL subsets. Genome-wide 27K methylation arrays were applied to analyze the DNA methylation patterns of 39 CLL samples belonging to stereotyped subsets #1, #2 and #4. In particular, each CLL subset was characterized by a unique methylation pattern, as visualized by unsupervised hierarchical and PCA analysis, where CLL samples were separated into three distinct clusters corresponding to their subset assignments (**Fig. 1A and B**). Using stringent criteria based on p values and difference in mean MI (see Materials and Methods) many genes were identified as significantly differentially methylated between each subsets: 106 genes (118 CpG sites) differed between subsets #1 and #4, 60 genes (65 CpG sites) differed between subsets #1 vs. #2, while 56 genes (60 CpG sites) differed between subsets #2 and #4. Supervised hierarchical clustering of cases according to the methylation status of the differentially methylated genes is represented in **Figure 2A–C**.

Importantly, the sets of differentially methylated genes from the current comparisons showed only partial overlap (**Fig. S1**) with the corresponding set from the comparisons performed in our previous study on methylation in *IGHV*-mutated/unmutated and *IGHV3–21* CLL (using the same criteria). This result could be viewed as further evidence that comparing more homogeneous CLL subgroups defined by BcR stereotypy may give more precise information than simply comparing the heterogeneous, broader *IGHV* mutational categories.[19]

Differential methylation of genes involved in immune response. The gene list from each subset comparison was entered into the GOTM database tool in order to investigate the biological functions of the differentially methylated genes. Interestingly, the annotation of genes identified when comparing subset #1 to #2 or subset #1 to #4 revealed a significant overrepresentation of genes involved in various aspects of the immune response including the adaptive immune response, cytokine production, regulation of T helper cell differentiation and T cell proliferation (**Tables S3 and S4**). Among the most frequently listed genes were *CD80*, *CD86, SPN* (*CD43)* and *IL10,* but other immune response genes, such as *IKBKE*, *TOLLIP*, *IL1F9* and *FCRL4,* were also identified (**Table S3A**). Of particular interest was the observation that the majority of these genes were methylated in subset #1 but not in subsets #2 and #4 (**Table S4**).

To investigate this further, GOTM analysis was performed separately for genes methylated in subset #1 but not in subset #2 (n = 65) or #4 (n = 124) after lowering the geometrical distance cutoff from 0.35 to 0.30 to increase the number of genes analyzed. In concordance with the above findings, enriched functions included various immune response categories, and the *CD80*, *CD86* and *IL10* genes were most prominent (**Table S3B**). Additionally, KEGG pathway analysis was performed for genes methylated in subset #1. Enriched pathways

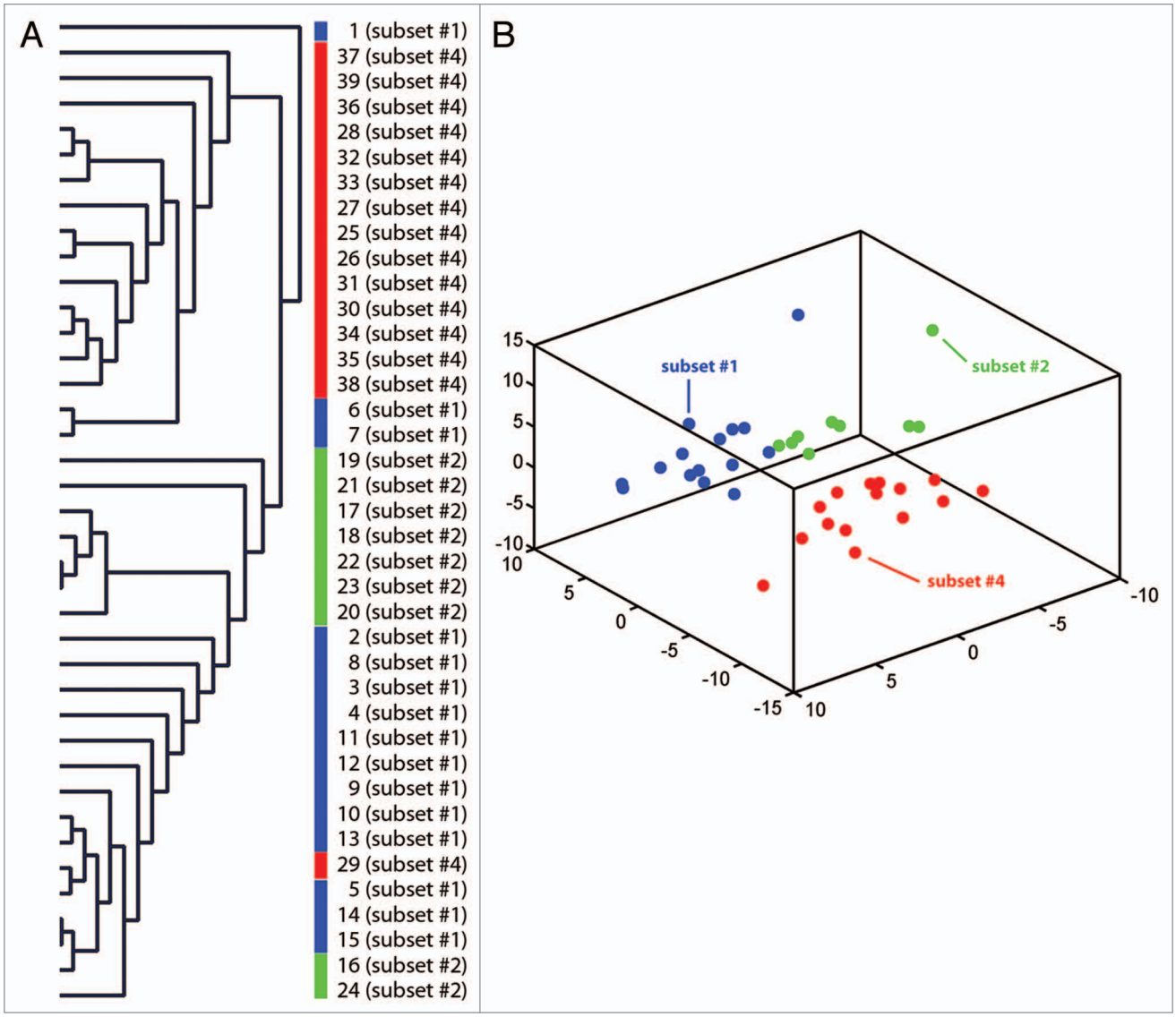

Figure 1. Unsupervised hierarchical clustering (**A**) and three-dimensional principal component analysis (PCA) of DNA methylation data (**B**) comparing samples belonging to subset #1, subset #2 and subset #4. The S refers to the subset number while the following number refers to the specific case as detailed in **Table S1**.

included immune signaling such as the Toll-like receptor signaling pathway (4 genes, *CD80*, *CD86, IKBKE* and *TOLLIP,* p = 0.004 vs. subset #2, p = 0.04 vs. subset #4). A separate GOTM analysis was performed for genes methylated in subsets #4 (n = 66) and #2 (n = 23) but not in subset #1 using the 0.30 cutoff. Among genes methylated in subset #4 enrichment for genes involved in biological/cell adhesion was observed (11 genes, p = 0.01) such as *PECAM1*, *ITGAM* and *LAMA1* (**Table S3C**). No significant enrichment was seen among genes methylated in subset #2 vs. #1, probably due to the low number of genes.

Pyrosequencing confirmed differential methylation of *CD80* and *CD86*. Prompted by (1) the herein reported significant differences in methylation status of the *CD80* and *CD86* immune response genes and; (2) our recent immune profiling studies of subsets #1 and #4 demonstrating significantly lower CD86 expression in subset #1, we sought to evaluate further the DNA methylation levels for *CD80* and *CD86* by a quantitative method using pyrosequencing technology in subset #1 and #4 CLL patient samples. The methylation percentage as measured by pyrosequencing was significantly higher in subset #1 than subset #4 samples for both the *CD80* gene (median methylation 64% vs. 13%, p = 0.001) and the *CD86* gene (median methylation 41% vs. 18%, p = 0.001) (**Fig. 3A**). Furthermore, there was a good correlation between the methylation levels as detected by the Illumina array and pyrosequencing for individual patient samples for the *CD80* gene (r = 0.88). Hence, we were able to validate our observations from the array data using an independent method.

Correlation of *CD80* and *CD86* methylation status with gene expression. The mRNA expression levels of *CD80* and *CD86* were compared in subset #1 and #4 samples using RQ-PCR. For both *CD80* and *CD86,* methylated in subset #1 but unmethylated

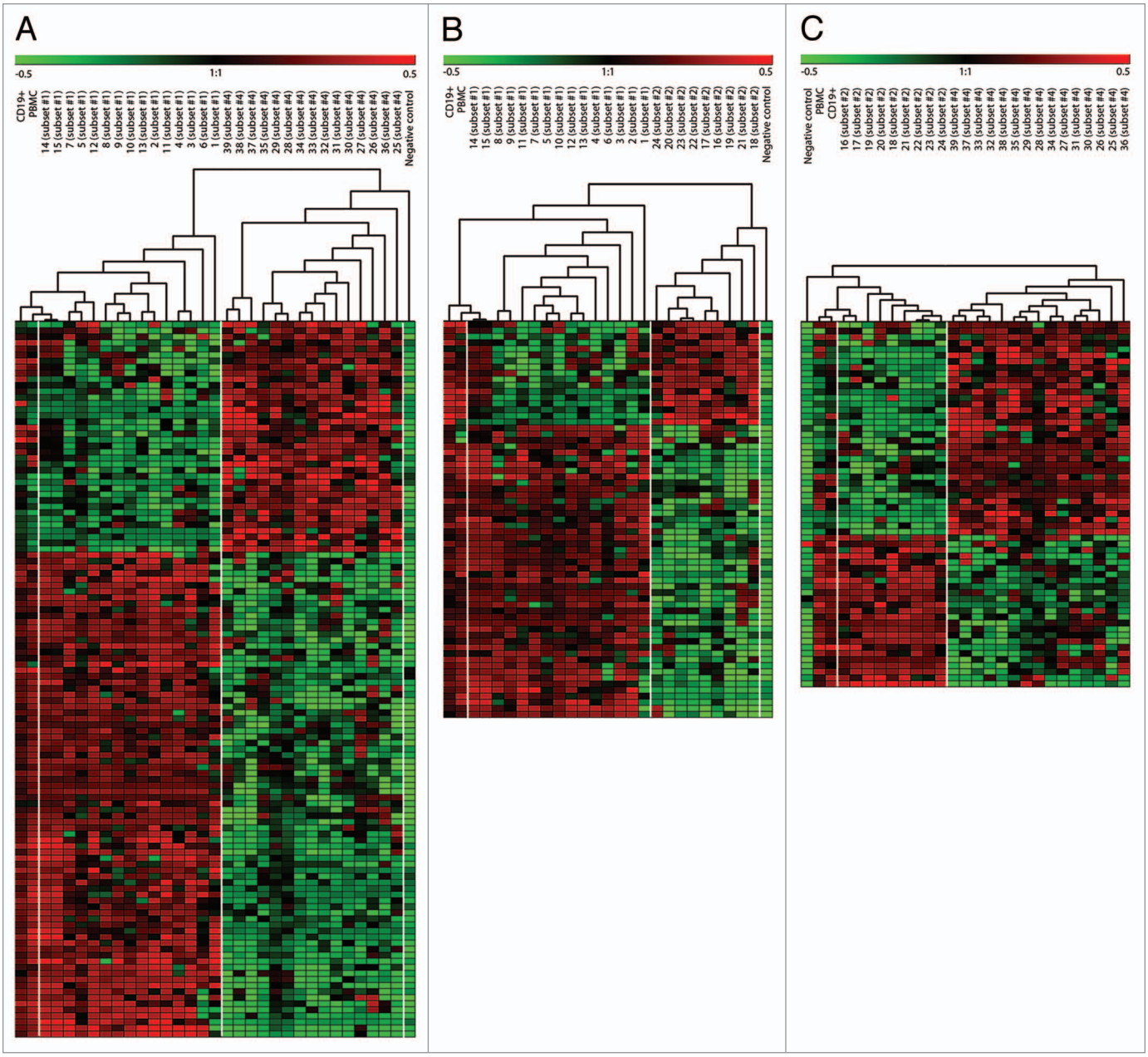

Figure 2. Supervised hierarchical clustering of differentially methylated genes (methylation index geometric mean difference > 0.35, p < 0.05). Comparisons were made between subsets #1 and #4 (**A**), subsets #1 and #2 (**B**) and subsets #2 and #4 (**C**). A gradient color scale represents the methylation index values; green as unmethylated and red as methylated.

in subset #4, the gene expression correlated with the methylation status. Specifically, the expression was significantly lower in subset #1 samples compared with subset #4 samples (*CD80* p = 0.0002, *CD86* p = 0.002, **Fig. 3A**). Along with the mRNA expression levels, the protein expression levels also supported the data showing higher expression of CD80 and CD86 in subset #4 samples (n = 4) compared with the expression levels in both *IGHV*-unmutated/subset #1 samples (n = 3), which were analyzed by FACS analysis using CD80 and CD86 antibodies (**Table S5**). In the case of CD86, it is relevant to note that the results herein reported accurately recapitulate our previous findings in subsets #1 and #4 from an independent series of experiments.[20,21]

Furthermore, the expression of nine additional genes was compared in subset #1 and #4 specimens. For two genes (*ABI3,* p = 0.002; and *NGFR,* p = 0.0004), the gene expression correlated significantly with the DNA methylation status, but for seven genes there was either no difference (*SPN*, *CIAS, GRP55*, *IKBKE*, *NCOR2* and *RIPK3*) in gene expression between the subsets, or an inverse correlation (*CST7*) with DNA methylation status (data not shown).

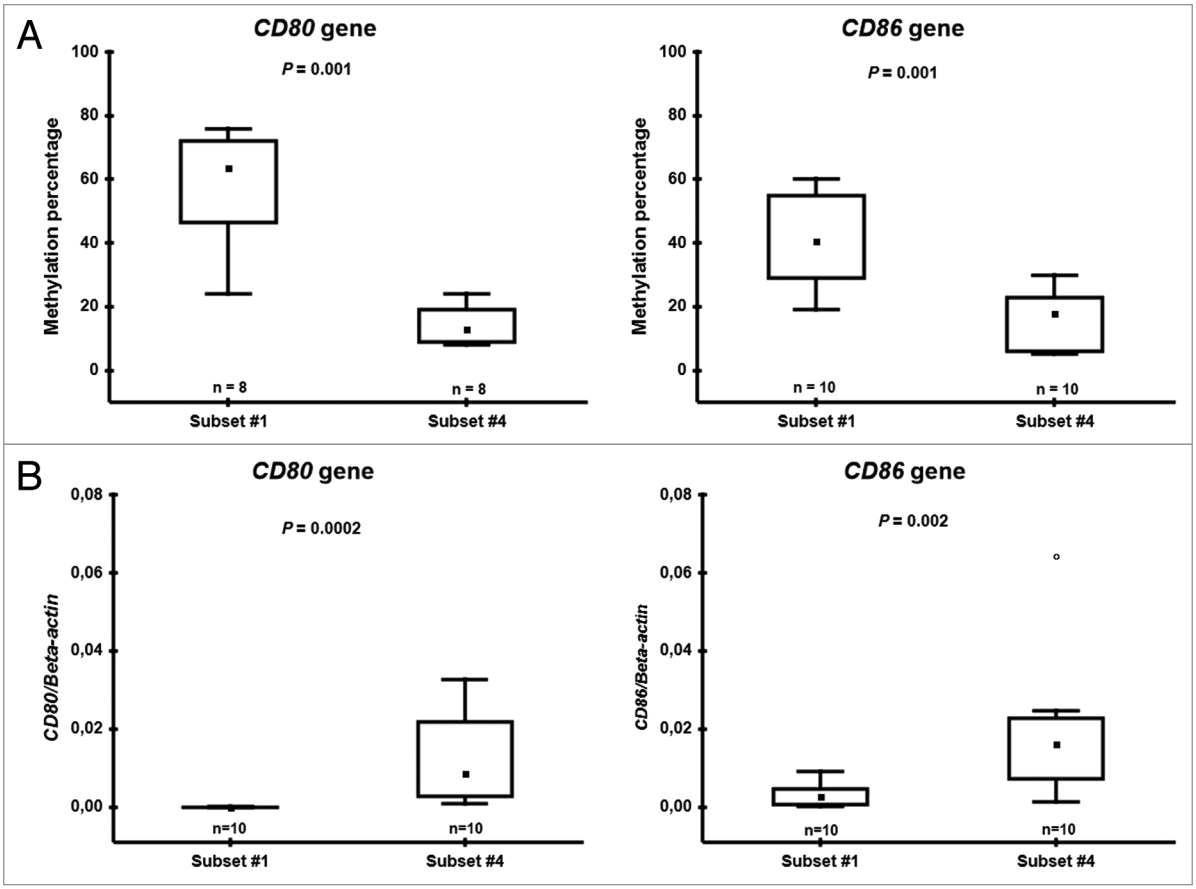

Figure 3. Pyrosequencing (**A**) and RQ-PCR (**B**) data on *CD80* and *CD86* in subset #1 and #4 patient samples. Boxes indicate the interquartile range (25–75%) while the small inner square indicates the median value. The whiskers represent the minimum and maximum values, except for outliers (circles) and extremes (stars).

Discussion

It is now widely recognized that DNA methylation is an important epigenetic modification and that aberrant methylation patterns are associated with tumorigenesis.[22,23] In a previous study using methylation arrays we were able to observe differential methylation profiles between *IGHV*-mutated/-unmutated and *IGHV3-21* CLL subgroups.[19] More recently, we and others could confirm and extend this finding using 450K-arrays.[24,25] In the present study, we further explored global methylation in subsets with BcR stereotypy. This approach is justified by ever-increasing evidence that different subsets are characterized by high intra-subset homogeneity and consistency for clinical presentation and outcome,[6,11] genomic aberrations,[26,27] antigen reactivity[28,29] and immune signaling[20,21] even beyond their *IGHV* gene mutational status.

The present study is based on the analysis of a unique collection of samples belonging to three major stereotyped CLL subsets,[9] i.e., the poor-prognostic subsets #1, #2 and the good-prognostic subset #4. Overall, significant differences in the global methylation profile were observed between the three subsets (**Fig. 2**) that were clearly separated from each other in the PCA (**Fig. 1**), thus further corroborating the notion that the subgrouping of CLL into distinct subsets based on BcR sequence stereotypy reflects actual biological differences and is also functionally and clinically relevant. Perhaps even more importantly, only a partial overlap was observed between differentially methylated genes identified in the present study compared with our methylation profiling according to *IGHV* gene mutational status (**Fig. S1**).[19] This finding further underscores that the *IGHV*-mutated/-unmutated subgroups include a considerably more heterogeneous assortment whereas stereotyped CLL subsets represent more homogeneous groups, enabling to reach more consistent and robust conclusions even when analyzing a more limited number of cases as in the present study.

Gene ontology analysis revealed enrichment of distinct biological processes in different subset comparisons, where one of the most striking findings was a prominent overrepresentation of genes involved in immune response, such as regulation of adaptive immune response, cytokine biosynthesis and T cell proliferation, which were generally more methylated in subset #1 vs. subsets #2 and, especially, #4 (**Tables S3 and S4**). In more detail, targets methylated in subset #1 were enriched for adaptive immune response genes (e.g., *CD80, CD86* and *IL10*) and NF-κB-inducing kinase activity (*IKBKE, PPP4C* and *RIPK3)*, while the only enriched categories among targets

methylated in subset #4 were biological and cell adhesion (e.g., *ITGAM, PECAM1* and *LAMA1*). In contrast, no particular gene category was enriched among genes methylated in subset #2.

We decided to focus the validation part on the two prototypic subsets, subsets #1 and #4, where data on distinct immune profiling has started to emerge,[30] as well as on two genes, *CD80* and *CD86*, which repeatedly came up in our analyses as methylated in subset #1 and unmethylated in subset #4. The observation of differential methylation of *CD80* and *CD86* in the array data was first validated by an independent method, i.e., pyrosequencing, and, importantly, the gene and protein expression of both *CD80* and *CD86* were found higher in subset #4 cases than subset #1 cases (**Fig. 3**). CD80 and CD86, which are both expressed on the surface of antigen presenting B cells, have been shown to physically interact with the CD28 receptor on T cells. These co-stimulating signals are absolutely necessary for optimal T cell activation and prevent T cell death during primary immune activation.[31] Both CD80 and CD86 have previously been reported to be upregulated on CLL cells after triggering via CD40 or after exposure to pre-activated T cells in vitro.[32,33] Recent gene expression data also demonstrated high *CD80/CD86* expression in *IGHV*-mutated CLL cases compared with unmutated cases and in particular *CD86* was highly expressed in subset #4 compared with subset #1 CLL.[20] Furthermore, *CD86* expression could be upregulated in subset #4 CLL cells after stimulation via Toll-like receptors but to a much lesser extent in subset #1 CLL cells.[21]

Taken together, these results may imply that subset #4 CLL clones expressing higher levels of CD80 and CD86 engage in and perhaps are actively dependent on crosstalk with their microenvironment, including T cells. We obtained additional support for this hypothesis by performing co-culturing experiments with CLL cell lines expressing CD80/CD86 and allogeneic T cells from healthy donors, which revealed induction of T cell activation (**Figs. S2 and S3**). Admittedly, these findings have to be corroborated also on primary CLL cells, yet, despite their preliminary nature, they can be taken to imply an important role for CD80 and CD86 in the pathophysiology of CLL in general and CLL subset #4 in particular. Questions remain especially as to how this capacity for immune signaling can be reconciled with the particularly indolent clinical behavior of CLL subset #4 that points to diminished responsiveness to microenvironmental stimulation. A propos this latter point, it is relevant to note that subset #4 is also outstanding among all other CLL for exhibiting pronounced intra-clonal diversification due to ongoing somatic hypermutation, very likely in the context of active immune signaling.[34,35]

Certainly, our study can offer only a partial view of the global DNA methylation patterns in the CLL subsets analyzed, as the Illumina 27K array is biased toward promoter CpG islands and covers only a proportion of all CpG sites in the genome. Interestingly, we did observe that the majority (62–78%) of differentially methylated CpG sites in our three subset comparisons were located outside CpG islands compared with only 27% of CpG probes on the array. Recent studies have also shown that CpG sites that are located outside CpG islands, in regions known as shores and shelves, appear more differentially methylated and play an important role in regulation of gene expression.[24,25,36,37] Future approaches using next-generation sequencing may hence reveal the full spectrum of aberrant methylation in different parts of the genome in stereotyped subsets of CLL.

In conclusion, distinct methylation profiles were identified for three major and paradigmatic stereotyped CLL subsets. Importantly, gene ontology analysis revealed enrichment for immune response genes, such as *CD80* and *CD86*, among targets methylated in subset #1 but remaining unmethylated and expressed in subset #4. In line with our previous report of distinct gene expression patterns for stereotyped CLL,[13] these novel findings provide additional evidence for distinct patterns of transcriptional control among CLL subsets and further underscore the validity of the molecular sub-classification of CLL according to BcR stereotypy.

Patients and Methods

Patient samples. Tumor samples from 39 CLL patients (24 males, 15 females) were included in this study; 37 derived from peripheral blood, while 2 samples were from bone marrow. Samples were collected from collaborating institutes in Greece (n = 18) and Sweden (n = 21). Cases were diagnosed and classified according to recently revised criteria[38] and displayed the typical CLL immunophenotype with a tumor percentage of ≥ 70%. Based on IG gene sequence features and following previously established criteria defined according to Stamatopoulos et al. and Murray et al.,[6,7] cases were assigned to three groups: (1) subset #1: 15 cases; (2) subset #2: 9 cases and (3) subset #4: 15 cases. Clinical and molecular characteristics for all patients, including immunogenetic features of the clonotypic BcRs, are summarized in **Table S1**. In total, 7 samples (2 subset #1 and 5 subset #2 cases) overlapped with our recent methylation array study on *IGHV* mutated/unmutated and *IGHV3-21* CLL (**Table S1**).[19] A negative control (whole-genome amplified DNA) was included in the analysis as well as two healthy control samples, one with peripheral blood mononuclear cells (PBMC) and the other with CD19 sorted B cells.[19] Written informed consent was obtained according to the Helsinki declaration and the study was approved by the local Ethics Review Committee from each institution.

Methylation array and data analysis. In this study, Illumina Infinium HumanMethylation27 BeadChip arrays (Illumina, WG-311-2201), which allow analysis of 27,578 CpG dinucleotides covering 14,495 genes, were applied. Bi-sulfite conversion of genomic DNA was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, D5005) as outlined previously.[19] The BeadStudio software (Illumina) was used to analyze fluorescence as previously described.[19] Quality control of array data revealed that all cases had > 98% of CpG probes (> 27,026) with a p value < 0.01, except two cases where > 95% of CpG probes (> 26,199) had a p value < 0.01. The methylation status for each detected CpG site ranged between 0 (completely unmethylated) to 1 (completely methylated). Subsequent bioinformatics analysis

of the methylation data was performed in R (www.r-project.org). In order to identify any differences in methylation between the subsets (i.e. subsets #1, #2 and #4), data was quantile normalized and arcsin transformed, and an empirical Bayes moderated t-test was applied using the "limma" package. P values were adjusted using the method of Benjamini and Hochberg and a level of $p < 0.05$ was used as a cut-off. In order to identify genes displaying the greatest difference in methylation among subsets, a further filter incorporating an average geometric difference of 0.35 was applied, although the cutoff was lowered to 0.30 for selected gene ontology analyses. This measure was added to ensure that only genes with large absolute differences remained. Results were visualized by heatmaps and dendrograms using Genesis TreeView version 1.2.7 (www.genome.tugraz.at). Principal component analysis (PCA) was applied to visualize the data; more specifically, the "princomp" function in Matlab was used. Furthermore, the Gene Ontology Tree Machine (GOTM) online software (http://bioinfo.vanderbilt.edu/gotm) was applied for identification of significant GO and KEGG pathway categories within the data set, using the Illumina 27K array as a reference set and the Benjamini and Hochberg method for multiple test adjustment where $p < 0.05$ was considered significant.

Gene expression analysis using real-time quantitative PCR. Real-time quantitative PCR (RQ-PCR) analysis was performed to measure the expression levels of 11 genes. Total RNA was extracted from sorted CLL cells (Dynal B Cell Negative Isolations Kit, Invitrogen, 113.13D) from subset #1 (n = 10) and subset #4 (n = 10) patients using the RNeasy Mini kit (Qiagen, 74104) according to the manufacturer's protocol. Reverse transcription (RT) reaction was performed using the MMLV-RT kit (Invitrogen, 28025-013) and random hexamers (Fermentas, SO142) according to the manufacturer's protocol. RQ-PCR primers were designed with the Primer3 software (Broad institute) and RQ-PCR analysis was performed using the Maxima™ SYBR Green Master Mix according to manufacturer's protocol (Fermentas, K0251). *Beta-actin* expression was used as an internal reference. Gene expression was analyzed using the ΔCt method and differences between subsets were evaluated using the Mann-Whitney U nonparametric test and the Statistica 9.0 software (Stat Soft). $p < 0.05$ was considered significant.

Pyrosequencing assay. Pyrosequencing was performed for two genes (*CD80* and *CD86*) in order to measure methylation levels in subset #1 and subset #4 patient samples. Genomic DNA was bisulfite converted using the EZ DNA Methylation Kit according to manufacturer's protocol as described previously.[19] The PyroMark™ software was used to design pyrosequencing primers; one forward and one reverse primer for PCR amplification of the desired product (one biotin labeled in the 5' end) and one sequencing primer. The sequenced target region contained the specific CpG site targeted by the Illumina methylation array along with nearby CpG sites. Primer sequences, total number of CpG sites covered in the target region and the size of the amplified product are specified in **Table S2**. In brief, bisulfite converted DNA was subjected to PCR amplification in total of 25 μl. Eighteen to twenty microliters of PCR product were immobilized to 2 μL of Streptavidin Sepharose High Performance (GE Healthcare, 17-5113-01) followed by annealing in 25 μL of annealing buffer containing 0.3 μM of sequencing primer for 2 min at 80°C. The analysis was performed using the PyroMark™ Q24 pyrosequencer instrument (Bioetage Inc., 9001514). CpG site methylation analysis was done with PyroMark Q24 software (9019062), and for each gene CpG methylation percentage was calculated for CLL patient samples. Methylation differences between subsets were evaluated using Mann-Whitney U nonparametric test and the Statistica 9.0 software. $p < 0.05$ was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/22901

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