Leukemia Research 38 (2014) 1097–1101

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Genetic and cytokine changes associated with symptomatic stages of CLL

Amit Agarwal[a,*], Lawrence Cooke[b], Christopher Riley[a], Wenqing Qi[b], David Mount[a], Daruka Mahadevan[c]

[a] University of Arizona, United States
[b] University of Tennessee, United States
[c] University of Tennessee, West Clinic, United States

ARTICLE INFO

Article history:
Received 30 March 2014
Received in revised form 21 May 2014
Accepted 25 May 2014
Available online 18 June 2014

Keywords:
Chronic lymphocytic leukemia
Stage progression
Gene expression profile
Cytokine profile
Transcription factors
SETD8
CSNK1E
SDF-1/CXCL12

ABSTRACT

The pathogenesis and drug resistance of symptomatic CLL patients involves genetic changes associated with the CLL clone as well as changes within the microenvironment. To further understand these processes, we compared early stage CLL to symptomatic late stage using gene expression and serum cytokine profiling to gain insight of the genetic and microenvironment changes associated with the most severe form of the disease. Patients were classified into low stage (Rai stage 0/I/II) and high stage (Rai stage III/IV). Gene expression profiles were obtained on pretreatment samples using the HG-U133A 2.0 Affymetrix platform. A comparison of low versus high stage CLL revealed a set of 21 genes differentially expressed genes. 15 genes were up regulated in the high stage compared to low stage while 6 genes were down regulated. Analysis of GO molecular function revealed 9 of 21 genes were involved in transcription factor activity. Serum cytokine profiles showed six cytokines to be significantly different in high stage patients. Two chemokines, SDF-1/CXCL12 and uPAR known to be involved in stem cell mobilization and homing were increased in serum of high stage patients. This study has identified therapeutic targets for symptomatic CLL patients.

1. Introduction

Chronic lymphocytic leukemia (CLL) is the most common B-cell malignancy in the Western hemisphere with more than 15,000 cases diagnosed annually [1]. CLL is characterized by the clonal proliferation of CD5 positive B-cells with early leukemogenic events likely involving acquired resistance to apoptosis [2,3]. Recently, studies of novel somatic mutations and clonal evolution have improved our understanding of the pathogenesis of CLL [4,5]. CLL is clinically heterogeneous and the disease can remain dormant without causing any symptoms for several years. Stage progression to symptomatic disease involves genetic changes in the B-cell clone or sub-clone as well as micro-environmental changes in the bone marrow milieu [6]. We sought to study these changes by comparing gene expression and cytokines profiles between asymptomatic and symptomatic stages of untreated CLL patients to better define therapeutic opportunities.

2. Methods

2.1. Sample procurement and processing

This research was conducted using an Institutional Review Board (IRB) approved protocol (project number: HSC #A04-18). After consent we obtained pre-treatment blood samples from CLL patients at the time of diagnosis. Patients were classified into asymptomatic stage (Rai stage 0/I/II) and symptomatic stage (Rai stage III/IV). Mononuclear cells were isolated using Ficoll–Hypaque gradient. Patient characteristics including age, sex, stage, LDH, beta2 microglobulin, cytogenetics and FISH analysis were recorded for analyses. Serum from patients was stored separately for cytokine analysis. Mononuclear cells were flash frozen for RNA isolation.

2.2. RNA isolation and Affymetrix microarrays

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, CA) from flash frozen mononuclear cell preparations. The amount of total RNA isolated from the cells was quantified using spectrophotometric OD260 measurements with yields ≥25 μg/sample. 5 μg of mRNA was used to generate first-strand cDNA by using a T7-linked oligo (dT) primer. After second-strand synthesis, in vitro transcription (Ambion) was performed with biotinylated UTP and CTP (Enzo Diagnostics), resulting in 40–80-fold linear amplification of RNA. 40 μg of biotinylated RNA was fragmented to 50–150-nt size before overnight hybridization at 45 °C to HG-U133A 2.0 Affymetrix array comprising ~18,400 transcripts and 22,000 probe sets (Santa Clara, CA). After washing, arrays were stained with streptavidin—phycoerythrin

* Corresponding author. Tel.: +1 520 445 6858.
E-mail address: aagarwal@uacc.arizona.edu (A. Agarwal).

http://dx.doi.org/10.1016/j.leukres.2014.05.017
0145-2126/

(Molecular Probes) and scanned on a Hewlett–Packard scanner. Intensity for each feature of the array was captured by using GENECHIP SOFTWARE (Affymetrix, CA), and a single raw expression level for each gene was derived from the 10 to 20 probe pairs representing each gene by using a trimmed mean algorithm. Intensity values were scaled such that overall intensity for each chip of the same type was equivalent.

After hybridization, the quality of the data was tested with several methods. The arrays were first inspected visually for artifacts and B2 Oligo performance per manufacturer's instructions (Affymetrix, Inc. "Gene Chip© Expression Analysis Technical Manual", November 2004). Chip CEL files were then imported into R-BioConductor (v. 1.7) (http://genomebiology.com/2004/5/10/R80). MAS5 present/absent calls were generated with the simpleaffy (http://bioinformatics.picr.man.ac.uk/simpleaffy/) package using a target scaling factor of 100. The scale factors were within 3-fold of each other as recommended in the guidelines provided in the Affymetrix Gene Chip© Expression Analysis: Data Analysis Fundamentals manual. The quality control metrics were generated using the "qc" function provided in the simpleaffy package. All chips performed as follows: the average backgrounds fell between 20 and 100, hybridization controls (bioB, bioC, bioD and cre) were all called present with increasing signal intensities and the Formula ratios for the internal controls (GAPDH and β-actin) were less than 1.25 and 3 respectively. Further quality control was performed using the affy PLM package provided in the BioConductor suite. The microarray data were normalized by robust multi-chip analysis (RMA) using the "fitPLM" function (default values). Chip pseudo-images of the weights and residuals, normalized unscaled standard error (NUSE) and relative log expression (RLE) values were used for chip-level quality assessment. Chip pseudo-images displayed no apparent artifacts that warranted excluding a chip from further analysis. NUSE and RLE plotting revealed the chips used in the experiments to be consistent. Next, chips were normalized by RMA using the simpleaffy package. Log-fold changes were calculated from the normalized log2 expression values.

2.3. Gene expression data analysis and visualization

BRB Array tool was used for gene expression data analysis and visualization [7]. CEL files were imported into the software using the import function. Using a supervised clustering analysis, samples were grouped into 'low' stage and 'high' stage as described above. A list of genes that are differentially expressed between the two groups was generated using the class comparison function of BRB Array tools to generate a list with a false discovery rate (FDR) of 1% with 80% confidence. BRB Array tools uses a multivariate analysis test to generate a list of genes that are differentially expressed between two groups with an ability to control the proportion of false discoveries in the list of genes [8,9]. The list of genes that were differentially expressed was further analyzed using PANTHER software which allows for GO over-representation analysis. The list was also analyzed using Ingenuity Pathway Analysis software to define networks of closely linked genes. Data set containing specific gene identifiers and their relative fold change were uploaded into both the applications. PANTHER performs an overrepresentation analysis of GO molecular function and GO biological processes based on the number of expected versus number of observed genes for each group. After using a correction for multiple tests, a list of GO molecular functions that are statistically significantly overrepresented is generated. IPA generates 'network' of genes from the differentially expressed genes based on their connectivity with other genes. The fold change information is overlaid on these gene networks and significant networks are generated using an IPA algorithm.

2.4. Serum cytokine profiling

Stored (−80 °C) patient serum samples were utilized. Array slides were thawed at room temperature as serum was diluted with 1× blocking buffer (Cytokine Array Protocol, RayBiotech, Inc. #AAH-CYT-G1000). Slides were blocked with blocking buffer and 50 μL of each sample was hybridized with the slide for 2 h; 1 μL internal controls were also added. After washing, provided biotin-conjugated anti-cytokine antibodies were diluted with 300 μL of blocking buffer, added to the slide, and incubated for 2 h at room temperature. Washing steps were repeated, followed by addition of a 1:1500 dilution of Alexa Flour 555-conjugated streptavidin and incubated overnight at 4 °C. Slides were washed three times and read on the Axon GenePix using the cy3 channel. Quality control was ensured with negative and graded positive controls present on the chip. Analysis was conducted using the RayBiotech analysis tool to obtain the expression value for each cytokine after background subtraction.

2.5. Cytokine profile analysis

Post normalization values were compared between the 'low' and 'high' stages of CLL using STATA 11. A *t*-test was performed using a *p*-value of 0.005 as cut-off to account for multiple cytokines being compared. The fold change was calculated separately by generating the ratio of the average expression value of 'low' stage versus 'high' stage.

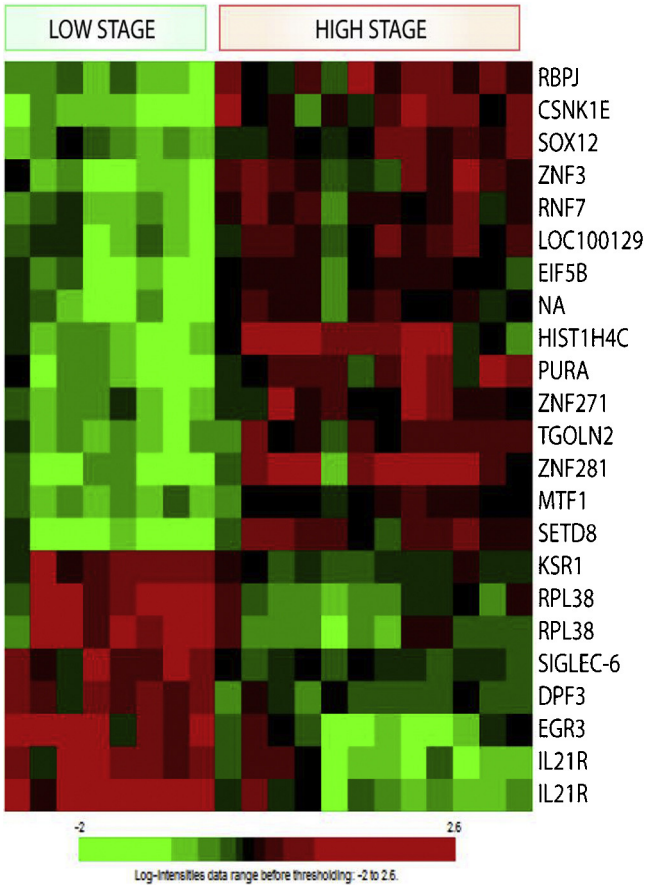

Fig. 1. Heat map of genes/probe sets that are differentially expressed between asymptomatic and symptomatic stages of CLL.

3. Results

3.1. CLL patient characteristics

Data was collected from 29 patients (14 low and 15 high stage). The patients were ages 48–91 years (median - 61 years). The male to female ratio was 3:1. Among asymptomatic stage patients, 8 were Rai stage 0, 3 were Rai stages I and 3 were Rai stage II. The hemoglobin value for these patients ranged from 12.4 to 15.8 g/dL and platelet count ranged from 131,000 to 305,000. For symptomatic patients, 2 were Rai stages III and 13 were Rai stage IV. The hemoglobin for these patients ranged from 5.3 to 11.7 g/dL and platelet count ranged from 24,000 to 214,000. A majority of patients in both groups had normal cytogenetics and no adverse risk cytogenetic change was over-represented in either group.

3.2. Gene expression profile associated with stage progression

We compared the gene expression analysis of 8 'low' and 12 'high' stage patients. Using BRB Array tools, we generated a list of genes that were differentially expressed between these two groups. A multivariate analysis with a cut-off of 1% false discovery rate (FDR) and 80% confidence provided a list of 23 probe sets. Of these, 2 probe sets were duplicated (IL21R and RPL38) resulting in 21 unique probe sets that were differentially expressed. 15 probe sets were up-regulated in high stages as compared to low stages and 6 were down-regulated (Fig. 1). GO molecular function analyses of differentially expressed genes were performed to define underlying mechanisms involved in progression from asymptomatic to symptomatic stages of CLL. PANTHER, a web based analysis software that