Genomic abnormalities in chronic lymphocytic leukemia influence gene expression by a gene dosage effect

JOHN D. DICKINSON[1], AVADHUT JOSHI[1], JAVEED IQBAL[2], WARREN SANGER[3], PHILIP J. BIERMAN[4] and SHANTARAM S. JOSHI[1]

[1]Department of Genetics, Cell Biology, and Anatomy, [2]Department of Pathology and Microbiology, [3]Center for Human Genetics, [4]Oncology/Hematology Section, Department of Internal Medicine, Center for Research in Leukemia and Lymphoma, University of Nebraska Medical Center, Omaha, NE, USA

Abstract. This work describes the identification and impact of somatic genomic abnormalities in human chronic lymphocytic leukemia (CLL). Using molecular cytogenetics (FISH) and G-banding cytogenetic analysis, chromosome abnormalities were detected in 37 of 46 (80.4%) CLL patients. 13q14 deletion was the most common finding followed by trisomy 12 and 11q22.3 deletion. 17p13 deletion was also detected as were several less frequent chromosome abnormalities. The presence of these abnormalities significantly influenced the period of treatment-free survival as well as other clinical characteristics. In particular, CLL samples with trisomy 12 and 11q22.3 deletion were associated with shorter treatment-free survival. In order to identify the under-lying molecular differences among CLL subgroups with different chromosome abnormalities, gene expression profiling was performed on a custom DNA microarray consisting of 10,000 human gene-specific oligonucleotides. A gene dosage effect was observed where the expression of genes at the genetic loci of the sites of the somatic genomic abnormality was altered in a fashion according to the type of genomic change. This phenomenon was particularly evident in CLL samples with trisomy 12 and 17p13 deletion. Thus, this study demonstrates that genomic abnormalities influence gene expression in CLL by a dosage effect.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in North America and Europe and yet it remains largely incurable (1,2). CLL is derived from malignant B

Correspondence to: Professor Shantaram S. Joshi, Department of Genetics, Cell Biology, and Anatomy, University of Nebraska Medical Center, 986395 Nebraska Medical Center, Omaha, NE 68198-6395, USA
E-mail: ssjoshi@unmc.edu

lymphocytes and is identified by co-expression of CD5, 19, and 23 cell surface markers (3,4). CLL has been characterized by broad clinical and biological heterogeneity and not all patients require immediate therapy (3,5,6). Much of the research in the area of CLL has focused on identifying prognostic factors that can predict which patients will ultimately undergo tumor progression and require therapy. In addition, elucidating the mechanisms of tumor progression in order to develop new molecular therapies and adjuvant therapies to induce long-term remissions is an important goal of CLL research.

Genomic abnormalities have been detected in CLL for decades but no single abnormality has been identified which is common to all CLL cases as in other similar hematological malignancies (7-10). The more precise definition of CLL on the basis of its cell surface phenotype and the increased resolution of detection due to the use of fluorescent *in situ* hybridization (FISH) led to reassessment of the role of genomic abnormalities in CLL. Deletions of 13q14, 11q22.3, and 17p13, and trisomy 12 are all recognized to be common genomic aberrations in CLL (8,11). These findings have led to increased interest in the role of these genomic abnormalities in the pathogenesis and progression of this malignancy. We have previously demonstrated, in this subset of CLL samples, that the presence of distinct genomic abnormalities is correlated with the immunoglobulin heavy chain variable region (IgVH) mutation status of the malignant B cell (12). CLL samples with trisomy 12 or 11q22.3 deletion were found almost entirely in the unmutated IgVH subset. Thus, the presence of certain chromosome abnormalities may be indicative of particular tumor development pathways with differing clinical outcomes.

The purpose of this study was to characterize the gene expression patterns among CLL samples in different genomic abnormality subgroups using a custom DNA microarray spotted with 10,000 human oligonucleotides. Here, we found that the presence of different genomic abnormalities in CLL was associated with treatment-free survival and that they had a specific impact on gene expression by a gene dosage effect.

Materials and methods

Isolation of mononuclear tumor cells from peripheral blood. Peripheral blood samples from 46 CLL patients were collected

by venipuncture under Institutional Review Board (IRB) approval with informed consent from the Nebraska Medical Center, Alegent Health System, and Methodist Hospital in Omaha, NE. Mononuclear cells were isolated from whole blood by density centrifugation with Accu-Prep™ (Accurate Chemical & Scientific Corp., Westbury, NY) lymphocyte separation medium. In order to assess the quantity and purity of the CLL tumor cells from peripheral blood, the cell surface phenotype was assessed by detection of fluorescent labeled antibodies using flow cytometry as previously described (12).

Detection of genomic abnormalities. Conventional cytogenetic analysis with G-banding was performed on CLL samples using either unstimulated cultured bone marrow cells or phytohemmagluttin (PHA) stimulated peripheral blood cells. Cells were cultured for 24-48 h at 37˚C in CHANG BMC media (Irvine Scientific, Irvine, CA) as described previously (13). Briefly, the cultured cells were harvested after 20-min incubation at 37˚C in a hypotonic solution of 0.4% KCL, plus trypsin and 0.08 mg/ml colcemid, which arrests the cells in metaphase. Up to 20 metaphases were used for analysis after the cells had been fixed and prepared for G-banding. Fluorescent *in situ* hybridization (FISH) was performed on all samples. For each CLL cell sample, four hybridization areas were prepared on a Thermatron drying chamber at 25˚C and 47% humidity. FISH probes were mixed in a proportion of 7 μl lysis buffer with 2 μl of probe and 1 μl of sterile water. The cellular DNA and FISH probes were denatured at 75˚C for 1 min and then allowed to hybridize for 18 h at 37˚C in a HyBrite hybridization oven (Vysis Inc., Downers Grove, IL). The fluorescein-labeled anti-digoxigenin detection agent was then added to the hybridization chamber and allowed to incubate for 10 min at 37˚C. The samples were then examined with an Olympus BX-60 fluorescent microscope. Two hundred cells were observed for genomic abnormalities and the average percentage of a given abnormality was taken from two observations. The FISH panel included CEP 12-trisomy 12 DNA probe, which spans the centromere from 12q11 to 12p11, LSI IGH dual color breakpoint probe for 14q32, D13S25 13q14.3 DNA probe, LSI 17p-p53 DNA probe at 17p13.1, and LSI 11q22.3-ATM DNA probe which spans 4 genes at 11q22.3 (Cul5, ACAT, NPAT, and ATM) (Vysis Inc.).

10,000 human oligonucleotide arrays. This research describes the analysis of gene expression data from CLL samples on a 10,000 (10K) gene microarray. The gene clones on the chip consisted of unique 50-mer oligonucleotide sense strand sequences synthesized by MWG Biotech (Ebersberg, Germany). The oligo set from MWG consists of approximately one third of the genes expressed in the human genome (9,850 genes) with 30 replica spots and 104 Arabidopsis negative controls. Each gene is annotated with a specific NCBI accession number (release 126). The oligos were suspended in 3X SSC in 384 V bottom plates and were spotted to the polylysine coated glass slides by a set of 16 robotically controlled printing tips.

Separately, 20 μg of CLL total RNA and 20 μg human reference total RNA (Stratagene, La Jolla, CA) were converted to cDNA by first strand synthesis using oligo dT primers

(Invitrogen Carlsbad, CA). Each cDNA was then incubated with the appropriate nucleotide linked fluorescent marker in the dark for 30 min. An indirect labeling method was used to couple the Cy5 dye to the CLL cDNA and the Cy3 dye to the human reference cDNA after reverse transcription. The labeled cDNA was then washed and purified by standard protocols. The two cDNAs were mixed together and denatured at 95˚C for 3 min followed by a quick chill on ice. The probe mixture was added to the microarray glass slide under a cover slip in the hybridization chamber. After 16 h of hybridization (over-night) at 42˚C, the slide was then washed three times in solutions with successively lower amounts of SSC and SDS. GenePix Pro 5.1 microarray image analysis software (Axon Instruments, Union City, CA) was then used to quantify the image intensity and provide gene identifiers to each spot on the array image. Low intensity spots were filtered out if the signal intensity in both channels was <100 and the signal to noise ratio (SNR) was <2.0. Spots with non-specific binding or contamination were labeled as outliers, flagged, and removed. The median of ratio expression value (Rm) was then $\log_2$ transformed.

Normalization of the raw intensities from different arrays was performed by the median normalization method using BRB Array tools, version 3.2. The dataset was filtered so that genes missing >50% of the median of ratio values were removed from subsequent analyses. Clustering of the gene expression data for CLL samples was performed using hierarchical cluster with Pearson's correlation and average linkage. One-way analysis of variance (ANOVA) was used to compare variance in gene expression across the five CLL subgroups with different chromosome abnormalities relative to variation in gene expression within groups (14,15). In order to identify differentially expressed genes in pairwise comparisons of genomic abnormality groups, non-parametric SAM (significance analysis of microarrays) was used in a manner previously described (16). In order to reduce the number of genes selected and increase the robustness of the comparison, SAM analysis was used on genes previously selected by t-test in a manner as described by Shen *et al* (17).

Gene expression analysis by PCR. Total RNA was isolated from CLL cells by TRIzol™ reagent (Invitrogen). RNA pellets were dissolved in HPLC grade H_2O (Sigma, St. Louis, MO) and stored at -80˚C. The quality and quantity of RNA were determined by the measured 260/280 nm spectroscopic ratio as well as running the total RNA on a 1% TAE (Tris-actetate-EDTA) agarose gel. Total RNA was reverse transcribed using random hexamer primers and SuperScript RT (Invitrogen). The Taqman™ assay (Applied Biosystems, Foster City, CA), which utilizes Taq polymerase with 5' nuclease activity and fluorescent complementary binding cDNA probes to detect *ATM* sequence amplification, was used with the ABI PRISM™ 7700 sequence detection system (SDS) as previously described (12). Briefly, Primer Express™ software (Applied Biosystems) was used to select gene specific forward and reverse primers as well as probes: *ATM* probe, 5'-TGTGCAGCGAACAATC CCAGCCTAA-3'; *ATM* sense primer, 5'-GAGCAGAGTC TTGCCCTGAGTATT-3'; and *ATM* anti-sense primer, 5'-TTGCCACAAACCCTCAGACA-3'. Template cDNA was generated from total RNA of CLL samples as described

Table I. Genomic abnormalities and cell surface phenotype of peripheral blood tumor cells collected from 47 CLL patients.

#	FISH	Abnormal nuclei (%)	G-banding results	Age	Sex	#	FISH	Abnormal nuclei (%)	G-banding results	Age	Sex
3	Trisomy 12	66	No metaphases	58	F	52	13q14 null; 13q14-	53; 23	NA	61	M
5	Trisomy 12	65	No metaphases	64	F	53	17p13-; 13q14 null	85; 90	No metaphases	82	M
8	Normal		NA	60	M	56	13q14-	79	46,XX,t(1;6)(p34;p23)[2]/ 46,XX[2]	72	F
9	13q14 -	NA	46,XX	64	F	58	Trisomy 12; 13q14-	54.5; 58	46,XY	65	M
10	Normal		46,XX	46	F	60	13q14 null	98	NA	64	M
11	Trisomy 12; 14q32	47; 26	NA	59	M	61	Normal		NA	64	F
16	Trisomy 12; 13q14-	75; 87	a	51	M	63	11q22.3-; 13q14-	21; 36	NA	62	F
19	13q14-	80	NA	56	M	65	13q14-	50	NA	69	M
28	Trisomy 12	81	47,XX, +12(6)/ 46,XX(5)	71	F	66	11q22.3-; 13q14-; 17p trisomy	75; 89; 47	NA	61	F
29	13q14- ; (13q14 null) 11q22.3-; 14q32	96 (61); 91; 5	46,XX	55	F	67	11q22.3-; 13q14-	66; 86	46,XY	42	M
30	Trisomy 12	71	46,XX	55	F	69	Normal		NA	76	M
31	13q14-	58	46,XY	59	M	70	Trisomy 12; 13q14-; 13q34-	58; 92; 18	a	57	M
34	11q22.3-	73	NA	73	M	71	13q14-	82	46, XY	46	M
36	11q22.3-; 13q14-	54; 46	46,XY	57	M	72	13q14-	32	46,XY	59	M
37	Normal		NA	52	M	73	13q14-	23	NA	46	F
38	13q14-; 14q32	94; 94	NA	62	M	74	13q14-	88	46,XY	55	M
40	13q14- (13q14 null)	69; (14)	46,XY	49	M	75	Normal		NA	34	M
41	13q14-	92	46,XY	73	M	76	Normal		46,XX	70	F
44	17p13-;	89	NA	81	M	77	13q14 null; 13q14-	65; 15	No metaphases	48	M
45	13q14 null	86	NA	84	F	79	11q22.3-; 13q14- (13q14 null)	78; 53 (34)	46,XY	62	M
46	17p13-	81	NA	74	F	80	17p13-	61	46,XY	58	M
47	13q14-	82	NA	66	F	82	Trisomy 12	32	46,XY, trisomy 12, t(1;6)(p35;p25)	80	M
49	Normal		NA	63	F	83	11q22.3-; 14q32-	94; 65	NA	28	M
51	Normal		NA	76	F	40b	17p13-; 13q14-	61; 65	46,XY	52	M

aCLL 40b represents a second specimen collection after acquiring 17p13 deletion. G-band analysis for CLL 16: 47,XY,+12[3]/46,XY, +12,add(17)(p13),-18[2]/47,XY,+12,del(17)(p11.2p13). G-band analysis for CLL 70: 46,XY,+X,-2,-7,-8,-13,-15, del(6)(p22.1p24), +10,+12,dup(3) (p21.2p24), der(5)t(5;15)(q13;q11.2),der(8)t(2;8) (q21;q24.3), +10, +12, add (16) (q13]/46,XY[2].

above. *GAPDH* expression values were used to normalize *ATM* expression as previously described (12,18) using the following probe and primer set: *GAPDH* probe, 5'-CAAGC TTCCCGTTCTCAGCC-3'; *GAPDH* sense primer, 5'-GAAG GTGAAGGTCGGAGTC-3'; and *GAPDH* anti-sense primer, 5'-GAAGATGGTGATGGGATTTC-3'.

First-strand cDNA template was generated from 5 μg of total RNA from CLL samples as described above. The following sequences of HEM1 primers were selected for RT-PCR: forward, 5'-CTGGAAAAGTCCATGGAACC-3'; reverse, 5'-CCAGGTAACTCCGAGTGAAA-3'. cDNA template (2.5 μl) for each CLL sample was amplified using 3 μl of 2.5 mM MgCl$_2$, 5 μl of 10X PCR buffer, 1 μl of 10 μM dNTP, 1 ml each of 10 μM forward and reverse primers, 36.75 μl water, and 0.4 μl of AmpliTaq Gold Polymerase (Applied Biosystems). PCR was performed under the following conditions: 95°C for a 3-min initial denaturing incubation, then 32 cycles of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 1 min, followed by a final 7-min 72°C extension incubation. PCR products were visualized on a 1% agarose gel stained with ethidium bromide. Expression of GAPDH was used to normalize HEM1 expression. GAPDH primers were selected for RT-PCR: forward, 5'-GAAGGTGAAGGTCGGAGTC-3'; and reverse, 5'-GAAGATGGTGATGGGATTTC-3'. GAPDH was amplified as described for HEM1 with the following differences: 4 μl of 2.5 mM MgCl$_2$, a 63.5°C annealing temperature, and 30 PCR cycles. The optical density of the HEM1 and GAPDH bands was measured by Image Quant imaging software (Bio-Rad, Hercules, CA) in order to determine relative HEM1 expression as previously described (19).

Statistical analysis. Treatment-free survival, the period from diagnosis to initiation of therapy, was recorded and used as a clinical endpoint to compare among CLL cases with different genomic abnormities. The Kaplan-Meier method was used to look at treatment-free survival measured as months from diagnosis to start of therapy or last contact, if therapy had not yet started. Patients were treated as censored if they had not yet begun therapy. The time to therapy distributions for the various abnormality groups were compared with the log-rank test.

Results

Detection of genomic abnormalities in CLL. Peripheral blood samples from 46 CLL patients were collected from August of 2001 to March of 2005. CLL #40b represents a second sample collected from CLL #40 after the patient had acquired the 17p13 deletion in addition to the 13q14 deletion previously identified. Therefore, where statistically appropriate, data analysis from this work considered CLL samples in a total of 47 patients.

Genomic abnormalities were detected in 37 CLL samples (80.4%) using fluorescent *in situ* hybridization (FISH) (Table I). 13q14 deletion was the most common abnormality detected (58.7%) and was the sole abnormality in 17 cases (36.9%). Trisomy 12 was the next most common abnormality identified (19.6%) followed by 11q22.3 deletion (17.4%). Five cases (10.6%) of 17p13 deletion were identified when the case of karyotype evolution for CLL 40b was considered

Table II. Classification of genomic abnormality groups for data analysis.

Genomic abnormality group	Total no. of genomic abnormalities detected[a]	Classification
Normal	9	No detectable chromosome abnormalities by FISH or G-banding
13q14 deletion 1x (2x)	27 (7)	13q14 deletion but not trisomy 12, or 11q22.3 or 17p13 deletions
11q22.3 deletion	8	11q22.3 deletion but not trisomy 12 or 17p13 deletion
Trisomy 12	9	Trisomy 12 but not deletions of 11q22.3 or 17p13
17p13 deletion	4 (5)[b]	17p13 deletion but not trisomy 12 or 11q22.3 deletion

[a]Some individuals had more than one abnormality. [b]Five cases of 17p13 deletion were identified when the case of karyotype evolution for CLL 40b was considered which had been previously analyzed with only 13q14 deletion.

as an independent sample. In addition, FISH identified abnormal 14q32 signals that could signify either a deletion or an unidentified translocation in 4 CLL patients (CLL 11, 29, 38, and 83). Trisomy 17p was detected in CLL 66 and a translocation, t(1;6) (p35;p25), was detected by G-banding studies in CLL 56 and 82. Finally, G-banding studies identified multiple genomic abnormalities in CLL 16 and 70 (Table I). In this study, CLL patients were classified into five groups on the basis of the detection of different chromosome abnormalities by FISH in a fashion similar to that of Dohner *et al* (11) (Table II).

Treatment-free survival. To determine the association between major chromosomal abnormalities and clinical outcome, the measurement of treatment survival was used. The treatment-free survival for the various genomic abnormality groups was compared using the log-rank test and two analyses were performed. Firstly, CLL samples were classified into three groups: no abnormalities (normal karyotype), one abnormality (simple karyotype), and two or more abnormalities (complex karyotype). Secondly, treatment-free survival was compared among the five different genomic abnormality groups described above (Table II). The Kaplan-Meier curve demonstrates a significant association between the time from diagnosis to the time of first treatment for both the number and type of genomic abnormality group, p=0.0014 and p=0.0025 respectively (Fig. 1). In particular, CLL samples with 11q deletion and trisomy 12 were associated with shorter time to therapy independent of the number of abnormalities.

Microarray analysis of CLL. Thirty-five CLL samples were available for analysis using the 10K human oligonucleotide
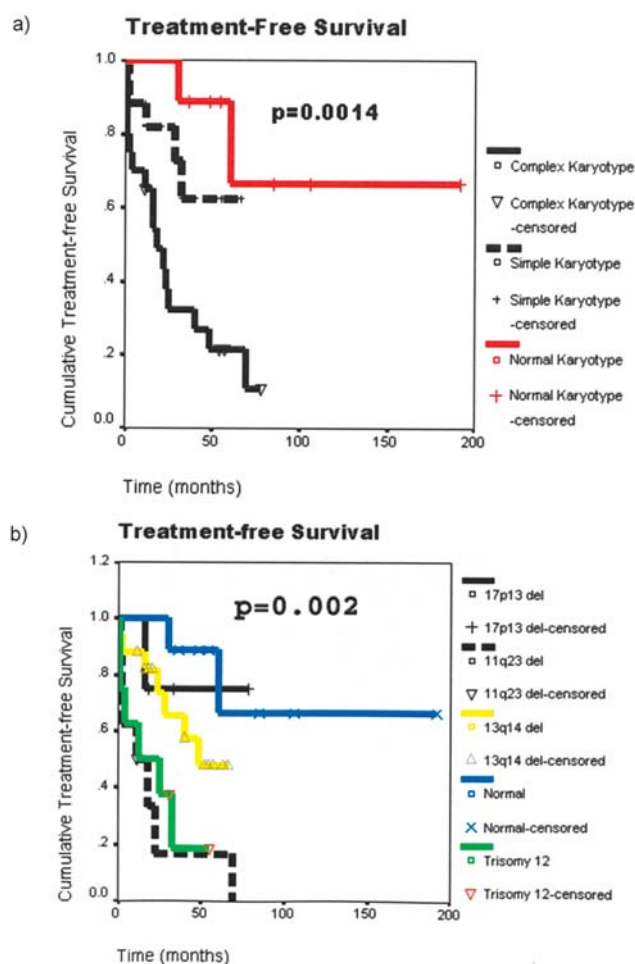
Figure 1. Treatment-free survival was measured as the period from diagnosis to initiation of first therapy. CLL patients that never required therapy were censored at the date of the last encounter. Treatment-free survival was compared among CLL patients according to number of genomic abnormalities (a) or type of genomic abnormality (b).

DNA microarray. This group included CLL 40b which was the second specimen collected from the same patient after the patient underwent karyotype evolution and developed the 17p13 deletion in addition to the previously detected 13q14 deletion.

Gene expression was compared among the five genomic abnormality groups described in Table II by supervised microarray analysis. Hierarchical clustering, using uncentered Pearson's correlation and average linkage, was to visualize the 316 genes that were identified with significantly different expression among the 5 genomic abnormality groups (Fig. 2a). All 4 samples with trisomy 12 had a common expression pattern. In addition, 6 out of 8 CLL samples with 11q22.3 deletion and 8 of 12 CLL samples with 13q14 deletion as the sole abnormality had common expression patterns. A group of overexpressed genes was observed in cluster 3 that primarily involved CLL samples with 11q22.3 deletion as well as those with trisomy 12. A comparison of the Gene Ontology (GO), http://www.geneontology.org/, description among the genes of this cluster revealed that many of the overexpressed genes were involved in cell signaling and cell cycle pathways.

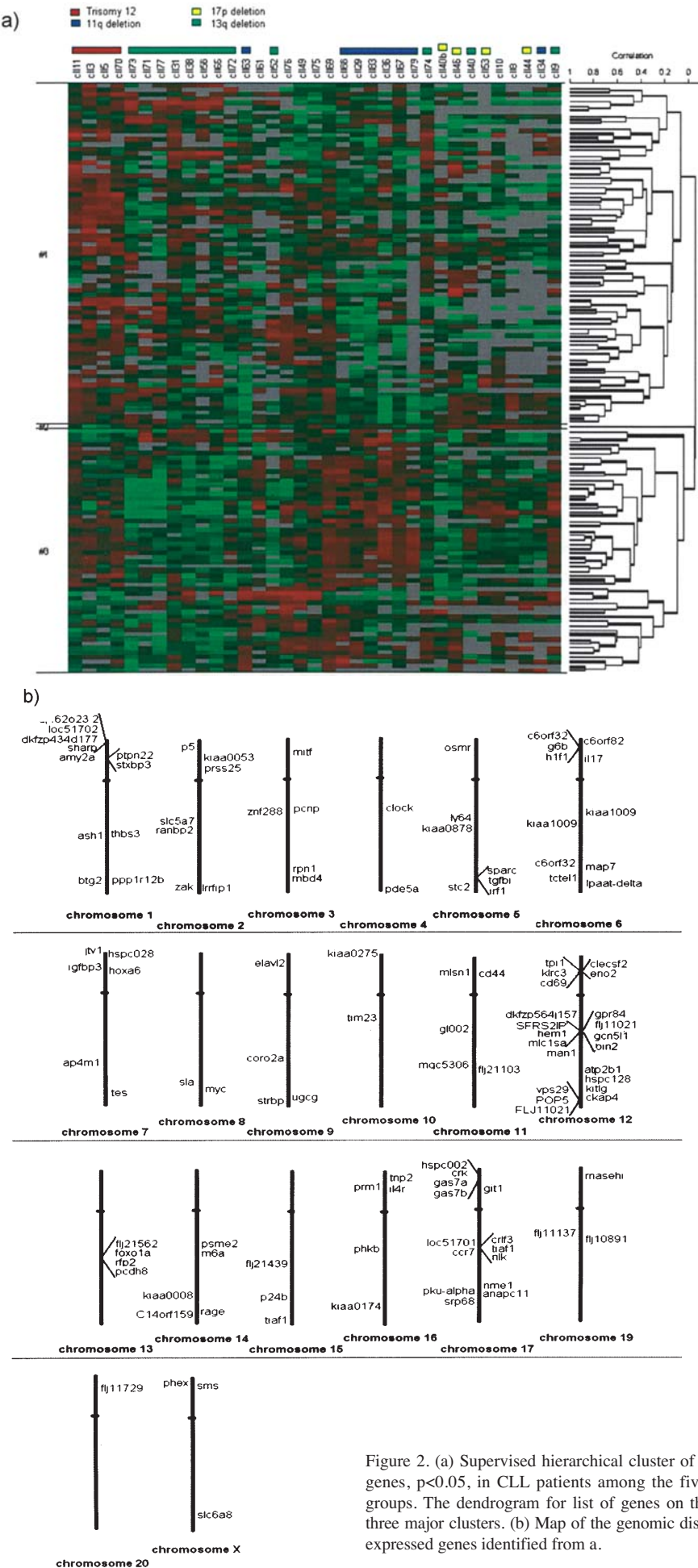The initial identification of GO annotations among the differentially expressed genes (Fig. 2a) revealed that many of them were found at loci of one of the four common genomic abnormalities in CLL. Therefore, the genetic loci of the differentially expressed genes were identified. A map of the loci indicated a non-uniform distribution of the differentially expressed genes from the hierarchical cluster in Fig. 2. Because of the indication of a gene dosage effect, follow-up pairwise comparisons for each of the genomic abnormalities were then performed. Significance analysis of microarrays (SAM) was used to compare the gene expression between CLL samples versus those without each of the four primary genomic abnormalities. The genomic loci of each of the differentially expressed genes from the comparisons between CLL samples with and without a particular genomic abnormality were identified. The genes were then clustered to identify genes at approximately the same locus that were also co-regulated (Fig. 3). As expected, 21 genes from all along chromosome 12 were overexpressed in the CLL samples with trisomy 12 such as CD69, CLECSF2, and HEM1. PCR confirmation of HEM1 revealed higher expression in CLL cases with trisomy 12 than without (Fig. 4). Likewise, 7 genes from locus 17p13 were down-regulated in the 17p deletion comparison. Thus, a specific gene dosage effect was observable for CLL samples. Interestingly, in the 11q deletion comparison, no differentially expressed genes were identified in the minimally deleted gene segment defined by Stilgenbauer *et al* for the 11q22.3 deletion (20). There were, however, 3 genes from 13q14 which were down-regulated in the 11q deletion group. Finally, 1 gene at locus 13q14.3, hypothetical protein FLJ11712, was down-regulated in the 13q deletion CLL group relative to those CLL samples without 13q deletion.

Identification of the loci of differentially expressed genes revealed a non-uniform distribution in regions outside of the four known chromosome abnormalities at 13q14, 11q22.3, 17p13, and chromosome 12. In particular, a number of differentially expressed genes were identified at 1p33, and 6p21. However, the expression of the genes at these loci was not regulated in a coordinate fashion on the basis of the presence of trisomy 12, or 11q22.3, 13q14, or 17p13 deletions. It is possible that the genes at these loci are co-regulated on the basis of another genomic aberration that is yet to be identified.

ATM, the gene responsible for the recessive genetic condition, ataxia telangiectasia, is located at 11q22.3-23.1, which is within the minimally deleted genome segment in CLL and other hematological malignancies (20). CLL patients with lower levels of atm protein expression have shorter survivals (21). Therefore, a gene dosage effect may underlie the unique clinical characteristics and biological differences associated with CLL patients with 11q22.3 deletion. TaqMan real-time PCR was used to compare *ATM* gene expression in 27 CLL samples. As was expected, the CLL samples with 11q22.3 deletion had the lowest mean *ATM* expression but several other samples with trisomy 12 or 17q13 deletion also had very low *ATM* expression and the overall difference was not significant, p=0.061 (Fig. 5).

Discussion

Genomic abnormalities were detected in 80.4% of CLL patients by fluorescent *in situ* hybridization (FISH) and significantly
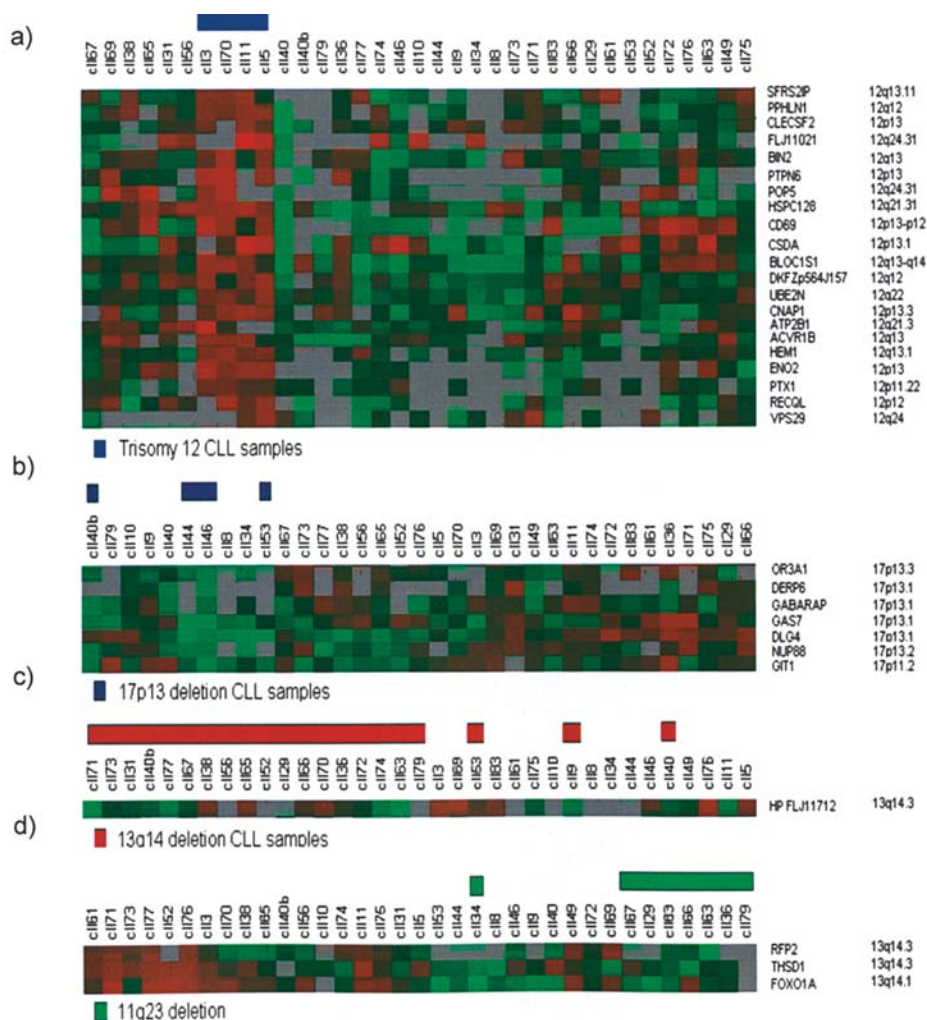
Figure 2. (a) Supervised hierarchical cluster of 316 differentially expressed genes, p<0.05, in CLL patients among the five chromosome abnormality groups. The dendrogram for list of genes on the y axis was clustered into three major clusters. (b) Map of the genomic distribution of the differentially expressed genes identified from a.

Figure 3. Genes differentially expressed using class comparisons was found at the same genetic locus, and with similarity of expression: trisomy 12 comparison (a), 17p deletion comparison (b), 13q deletion comparison (c), and 11q deletion comparison (d).

influenced the tumor progression, as measured by treatment-free survival (Fig. 1). In addition, we have previously shown that genomic abnormalities influence gene expression among a subset of genes selected for known roles in cell cycle and cell signaling regulation (12). Yet the mechanism of the effect of the different chromosome abnormalities on tumor progression in CLL remains elusive. It is possible that the direct loss and gain of genetic material due to somatic genomic abnormalities is responsible for the variations in phenotype observed in CLL patients with different chromosome abnormalities.

When compared in terms of the five different genomic abnormalities groups described above (Table II), 316 genes were differentially expressed (Fig. 2a). An initial comparison of gene annotations among the differentially expressed genes revealed that many were located along chromosome 12 (Fig. 2b). Follow-up pairwise comparisons were then performed between CLL samples with and without a particular genomic abnormality. Genes that were found at the same locus and were regulated in the same fashion, i.e. all over- or underexpressed, were then identified (Fig. 3). Twenty-one genes found on both the short and long arms of chromosome 12 were overexpressed in the trisomy 12 group. Most genes were clustered around 12p13 and 12q13. This represents a gene dosage effect where the expression of genes is increased

due to amplification of the genomic segment where the genes are located. There were many other genes located along chromosome 12 that were not up-regulated in a coordinated fashion in CLL samples with trisomy 12. Genomic amplification itself is unlikely to alter transcriptional activity within a cell. The developmental status of the cell and activity of specific transcriptional factors will determine which genes will be expressed regardless of gene dosage. Haslinger *et al* recently reported a similar phenomenon using Affymetrix Human Genome U95A or U95Av2 arrays to compare 100 CLL samples with different chromosome abnormalities (22). Unlike Hasslinger *et al*, who found an up-regulation of genes that were approximately primarily located from 12q13 to 12q24, we identified an up-regulation of genes on both 12p and 12q. Einhorn *et al* demonstrated that trisomy 12 in CLL is caused by the simple duplication of one of the complete chromosomes (23). Our data is consistent with these findings.

The biological consequence of the genomic amplification and subsequent overexpression of genes along chromosome 12 remains uncertain. Two type ii transmembrane proteins, *CLECSF2* and *CD69*, are both located at 12p13 and may be relevant to the biology of CLL. Both contain c-lectin domains and share a high degree of sequence homology. *CD69* has been described as an early marker of lymphocyte activation
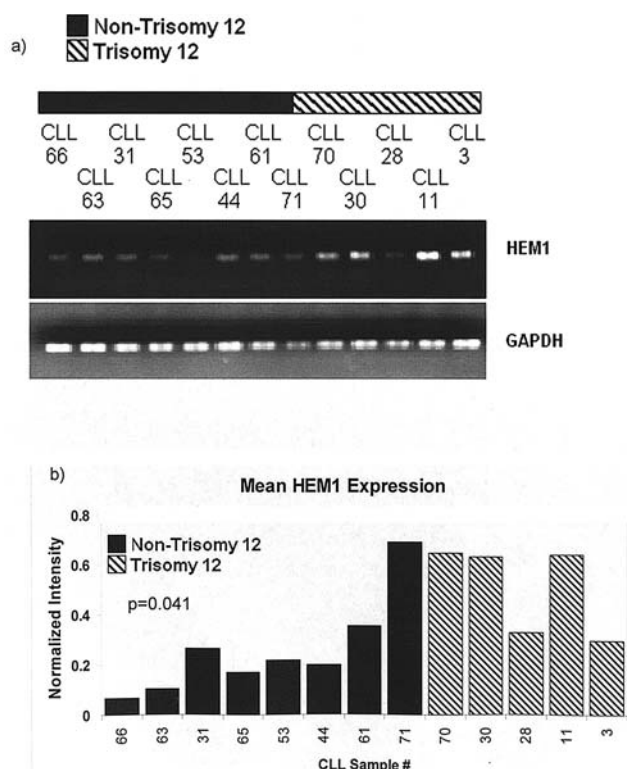
a)

b)

Figure 4. (a) HEM1 expression was measured by PCR and normalized by GADPH expression. (b) Values below represent the average normalized optical density of HEM1 PCR bands from two independent experiments. Mean optical density values of HEM1 were compared between CLL samples with and without trisomy 12 by Student's t-test.
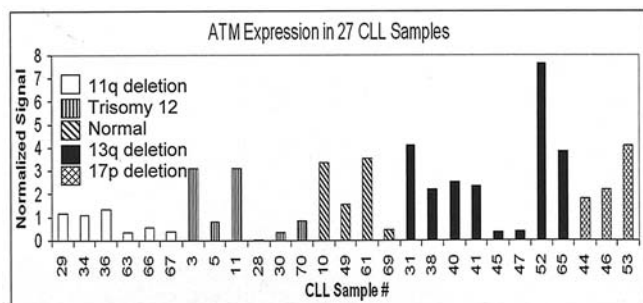

Figure 5. ATM expression by TaqMan real-time PCR in 27 CLL samples. The ATM signal intensity was normalized by endogenous GAPDH expression. Mean ATM expression was compared between those CLL samples with and without 11q22.3 deletion (p=0.061).

by mitogen or antigen. Alizadeh *et al* described *CD69* over-expression in resting/activated T cells, resting/activated B cells, follicular lymphoma, and in some cases of CLL (24). Damle *et al* noted higher *CD69* expression in CLL samples with unmutated IgVH and high CD38 expression (25). In addition, several studies noted that *CD69* expression increased after *in vitro* activation with CD40 ligand and tetradecanoyl phorbol acetate (TPA) (26,27). The expression of C-type lectin superfamily member 2 (*CLECSF2*) is also increased upon lymphocyte activation and shows a similar expression pattern as *CD69* in different normal and malignant lymphocyte subsets (24). A number of other overexpressed genes located

on chromosome 12 may be significant to CLL biology. Protein tyrosine phosphatase non-receptor 6 (*PTPN6*) is restricted to hematopoietic cells and contains 2 SRC homology domains that are probably involved in cell signaling. The activin binding receptor type 1 B (*ACVR1B*) gene expresses a protein that forms part of a heteromeric receptor complex. Activin, which is related to the TGF-ß family, binds to the *ACVR1B* receptor and induces serine/threonine kinase activity. Hematopoietic protein 1 (*HEM1*) is a newly cloned transmembrane protein restricted to hematopoietic cells (28). Its function remains unknown but follow-up PCR studies confirmed overexpression of HEM1 in CLL samples with trisomy 12 (Fig. 4). HEM1 was also found to be overexpressed in trisomy 12 cases by Haslinger *et al* (22). In addition, early laboratory studies indicated that gene expression knockdown of HEM1 by anti-sense oligonucleotides induces increased cell apoptosis when treated with fluodarabine *in vitro* (unpublished data).

Others have shown that 17p13 deletion was related to poor response to treatment and poor survival (8,29). It is likely that this phenotype is related to loss of the tumor suppressor gene, p53, since the remaining p53 allele is often mutated in CLL (30). We observed that the 17p13 deletion affects the expression of genes at that particular chromosome locus by a gene dosage effect. Hasslinger *et al* identified 14 down-regulated genes located at 17p13, including *TP53* (22). While our data does not provide direct evidence for the down-regulation of *TP53* (not included in the MWG oligo set used to construct the array), we observed the down-regulation of 7 genes located at 17p13, *OR3A1, GAS7, DERP6, GABARAP, DLG4,* and *NUP88*, along with *GIT1* at 17p11.2.

11q22.3 deletion has been associated with lower survival rates in CLL and probably involves loss of the DNA repair gene, *ATM*, yet there remains some controversy regarding the role of *ATM* in CLL (31-33). We have shown using commercially available cDNA arrays that this subgroup of CLL was related to an overexpression of cell cycle and cell signaling genes such as *CDC2* and *ZAP-70* and an under-expression of cell adhesion related genes such as *CD44* and *CD11a* (12,19). In addition, CLL patients with 11q22.3 deletion were associated with shorter treatment-free survival, shorter lymphocyte doubling time, and bulky abdominal/mediastinal lymphadenopathy. Unlike samples with trisomy 12, however, there was no correlation of these genes with location on chromosome 11. It is likely that the loss of the *ATM* tumor suppressor gene is significant to the tumor progression and altered gene expression profile of CLL patients with 11q22.3 deletion. How the expression or activity of *ATM* is related to these clinical and biological features remains uncertain. *ATM* expression was lower but not significantly different between CLL samples with and without 11q22.3 deletion by FISH (Fig. 5). In fact, the pair-wise comparison of the genomic loci of genes differentially expressed between CLL samples with and without 11q22.3 deletion revealed no genes located from 11q22-11q23. It did reveal that 3 genes located at the 13q14 locus (*RFP2, THSD1,* and *FOXO1A*) were all down-regulated. This could be a bystander effect since 6 out of 8 of the CLL samples in the 11q22.3 deletion group also had 13q14 deletion. Yet, these 4 genes were not underexpressed when CLL samples with 13q14 deletion were compared to CLL samples without

13q14 deletion (Fig. 3). Ret finger protein 2 (*RFP2*) is located within the minimally deleted sequence mapped in the 13q14.3 deletion which occurs in hematological malignancies such as CLL (34,35). *RFP2* may be an important regulator of the cell cycle and NF-κB pathways (36,37). *RFP2* is also down-regulated among CLL samples with trisomy 12 (data not shown). Therefore, the down-regulation of *RFP2* in the trisomy 12 and 11q22.3 deletion cytogenetic subgroups may indicate the overall aggressiveness of the tumor rather than a consequence of loss of genomic material at that locus.

The lack of significant down-regulation of the expression of *ATM* and other genes at the11q22.3 locus is in disagreement with the recent work of Haslinger *et al* that showed the down-regulation of 8 genes, including *ATM*, from the 11q22-11q23 locus (22). There are several possibilities regarding this discrepancy. The results from our report come from an oligonucleotide microarray with oligos representing only 10,000 human genes compared to the 30,000 genes on the Affymetric array used by Haslinger *et al* (22). With regard to the expression levels of the *ATM* gene, our results by real-time PCR were close to significant and an increase in the number of samples may demonstrate a greater difference in expression between those with and without 11q22.3 deletion. Finally, *ATM* is a large, complex molecule that is highly regulated (38-42). Therefore, the pathological features of CLL samples with 11q22.3 deletion may not simply be related to measurement of a gene dosage effect of *ATM*.

This work provides further evidence that distinct genomic abnormalities play a significant role in tumor progression in CLL. This concept was supported by the correlation of shorter treatment-free survival in the trisomy 12 and 11q22.3 genomic abnormality subgroups. Further, supervised analysis of gene expression among the 5 genomic abnormality subgroups identified broad differences in gene expression in the CLL subgroups. In the trisomy 12 and 17p13 deletion subgroups, this difference in gene expression was the result of a direct gene dosage effect.

Acknowledgements

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