Gene expression profile and genomic changes in disease progression of early-stage chronic lymphocytic leukemia

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ABSTRACT

The biologic mechanisms involved in the clinical progression from early stages of patients with chronic lymphocytic leukemia (CLL) are not well known. We investigated sequential samples from 16 untreated CLL patients obtained at diagnosis in early stage and after progression before treatment. One patient had a $p16^{INK4a}$ homozygous deletion at diagnosis and progression, and 3 patients acquired a p53 mutation, gains of 5q21-q23 and 11pter-p14, and a gain of chromosome 12 respectively, during the progression of the disease. Gene expression profile analysis showed a significant modulation of 58 genes with a particular downregulation of genes that are inhibitors of cell adhesion and motility.

Key words: chronic lymphocytic leukemia, progression, microarrays, comparative genomic hybridization

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Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults in Western countries. It is characterized by a clonal proliferation and accumulation of mature neoplastic $CD5^+$ B lymphocytes. The individual clinical course of these patients is extremely variable.[1] Patients with CLL in early stage may not require treatment for several years but half of them will progress to a more advanced stage and will need treatment. The median time to disease progression and therapeutic requirement is shorter in patients with unmutated immunoglobulin genes (U-CLL), increased ZAP-70 expression, or adverse cytogenetic alterations.[1] Tumor progression into advanced stages and transformation into large B-cell lymphoma has been associated with diverse factors, such as karyotypic evolution,[2] oncogenic alterations of cell cycle regulatory genes 3 and inactivation of tumor suppressor genes.[4,5] However, the mechanisms that may be involved in the progression of the disease in early stages before the patient requires treatment have not been examined.

To determine possible genetic and molecular alterations related to early clinical progression in CLL, we investigated the genomic and gene expression profile alterations in a series of sequential samples obtained at diagnosis in early stage and at the time of clinical progression before treatment.

Design and Methods

Patients' selection

Sixteen patients diagnosed with CLL were selected on the basis of the availability of two sequential peripheral-blood cell samples in which the first was obtained at diagnosis in an early clinical stage (Binet stage A) and the second when the patient had an active disease but before the onset of treatment (Binet stage B=10 cases, C=6 cases). Active disease was defined according to standard criteria as follows: evidence of progressive marrow failure, massive or progressive splenomegaly or lymphadenopathy, progressive lymphocytosis or lymphocyte doubling time of less than 6 months, autoimmune phenomena poorly responsive to corticosteroid therapy, or presence of disease related symptoms.[6,7] For comparison, sequential samples from three patients with stable CLL disease were also included in the study. All patients gave their informed consent according to the Institutional Ethic Committee. Patients' clinical details are provided in Table 1.

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Table 1. Clinical and biologic characteristics of the CLL patients included in the study.

CLL with clinical progression

Case	Age(yrs)	Gender	Stage at diagnosis	Stage at progression	Sampling interval (months)	IgV_H	Mutation	ZAP70	p53	$p16^{INK4a}$	CGH Initial	CGH progressed
CLL1	46	F	A (I)	B (I)	11	VH4-39	Unmutated	High	wt	wt	gain 12	gain 12
CLL2	71	F	A (I)	B (I)	14	VH3-30	Unmutated	High	wt	wt	not altered	not altered
CLL3	83	M	A (0)	C (IV)	9	VH3-33	Unmutated	High	wt	wt	not altered	gain (5q21-q23), gain (11pter-p14r)
CLL4	54	M	A (I)	B (II)	18	VH1-2	Unmutated	High	wt	wt	del(13q14-q21)	del(13q14-q21)
CLL5	71	M	A (I)	C (IV)	6	VH1-69	Unmutated	High	wt	wt	del10p	del10p
CLL6	71	F	A (0)	B (I)	9	VH3-7	Unmutated	High	wt	wt	not altered	not altered
CLL7	54	F	A (I)	C (IV)	14	VH1-69	Unmutated	High	wt	wt	not altered	not altered
CLL8	84	F	A (0)	B (I)	38	VH1-69	Unmutated	Low	mut	wt	del(9pter-p23), del(17pter-p12)	del(9pter-p23), del(17pter-p12)
CLL9	48	M	A (II)	B (II)	20	VH3-20	Unmutated	High	wt	wt	not altered	gain(12)
CLL10	69	F	A (0)	C (IV)	17	VH3-30	Unmutated	High	wt	del	del (9pter-p23)	del (9pter-p23)
CLL11	59	M	A (II)	B (II)	34	VH3-53	Unmutated	Low	wt	wt	gain(12)	gain(12)
CLL12	86	F	A (0)	C (III)	13	VH1-69	Unmutated	High	wt	wt	gain(12)	gain(12)
CLL13	55	F	A (I)	B (I)	20	VH4-39	Mutated	Low	wt	wt	del(1q41-qter)	del(1q41-qter)
CLL14	56	F	A (0)	B (I)	12	VH6-11	Unmutated	High	wt	wt	not altered	not altered
CLL15	69	M	A (0)	C (IV)	14	VH3-11	Unmutated	High	wt	wt	not altered	not altered
CLL16	51	F	A (I)	B (I)	18	VH1-69	Unmutated	Low	wt	wt	gain(17q22-qter), del (11q14-q24)	gain(17q22-qter), del (11q14-q24)

CLL with stable clinical evolution

Case	Age (yrs)	Gender	Stage at first sample	Stage at second sample	Sampling interval (months)	IgV_H	Mutation	ZAP70	p53	$p16^{INK4a}$	CGH first sample	CGH second sample
CLL control 1	n.a.	F	A (0)	A (0)	34	VH1-69	Unmutated	High	wt	wt	gain (12)	gain (12)
CLL control 2	70	F	A (0)	A (0)	40	VH1-69	Unmutated	High	wt	wt	not altered	not altered
CLL control 3	59	M	A (0)	A (0)	49	VH2-70	Unmutated	High	wt	wt	gain (4q)	gain (4q)

Abbreviations: n.a., not available; F: female, M: male; wt: wild type; mut, mutated; del, deleted

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Isolation of tumor cells, purification and ZAP-70 analysis

Mononuclear cells were isolated from peripheral blood by gradient centrifugation, frozen in DMSO and stored at –180 °C until analysis. Samples were thawed and tumor cells were purified using anti-CD19 magnetic microbeads (Miltenyi Biotech®, Bergisch Gladbach, Germany) prior to nucleic acid extraction. A purity greater than 98% of CLL cells was obtained in all samples. The expression of ZAP-70 was determined by flow cytometry as previously described.[8]

DNA analysis

DNA was isolated according to standard protocols. Comparative genomic hybridization (CGH), the mutational status of the immunoglobulin heavy chain genes (IgV_H), p53 mutations, and $p16^{INK4a}$ deletions were performed in all sequential samples as previously described.[2,8]

RNA extraction and microarray analysis

Total RNA was extracted with the TRIzol reagent (Invitrogen Life Technologies®, Carlsbad, CA, USA). High quality RNA samples were hybridized to HU133plus2.0 GeneChips (Affymetrix®, Santa Clara, CA, USA), and processed according to standard protocols. Data normalization was performed with the GeneChip® Operating Software (GCOS, Affymetrix®) and using the global scaling method (target intensity=500). The data was then filtered with the DNA-Chip analyzer software v1.3 (dChip, Boston, MA, USA). To perform the unsupervized analysis, genes were filtered according to their variation across samples (1<standard deviation for logged data < 10) and the expression level (≥5 in ≥30% samples). For the supervized analysis, only the probe sets with values above 10 for at least 60% of the samples were considered for further analysis. Differential expression of paired samples was assessed with the Linear Model for Microarray Data Analysis (LIMMA) software package.[10] Only those genes which met both conditions of p-value<0.05 (corrected using the False Discovery Rate method) and Bayes statistic (B)>1 were considered significant. Clusters were built with the dChip software. Gene identity and functional annotation was studied with the Ingenuity Pathways Analysis® v.4.0 program (Redwood City, CA, USA).

Real time quantitative RT-PCR (qRT-PCR) validation

cDNA was synthesized from 1 μg of total RNA by using the High-Capacity cDNA Archive kit (Applied Biosystems®). *ACTL6A*, *OGG1*, *PLCB2* and *RAPGEF1* expression levels were determined with the following pre-developed assays (Applied Biosystems®): *ACTL6A* Hs00188792_m1; *OGG1* Hs00213454_m1; *PLCB2* Hs00190117_m1 and *RAPGEF1* Hs00178409_m1. Expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method using human β-glucoronidase (GUS) as endogenous control. The differences of expression between sequential samples were analyzed using a paired T-test approach (BRB-Array Tools v.3.3.0 software®, National Cancer Institute, USA).

Results and Discussion

IgV$_H$ and ZAP-70 status

All sequential samples were clonally related showing the same immunoglobulin heavy chain genes (IgV$_H$) family switch and mutational status (Table 1). Fifteen out of the 16 cases with progressive disease were unmutated and the sequence was stable during the evolution of the disease. Twelve out of the 15 unmutated cases were ZAP-70 positive. Two of the 3 discordant unmutated CLL had a 17p deletion (*CLL8*) and a 11q loss (*CLL16*) respectively, a common finding in CLL with discordance between the IgV$_H$ mutational status and ZAP-70 expression.[11] The 3 patients with stable disease (*CLL control 1-3*) had CLL with unmutated IgV$_H$ genes and were ZAP-70 positive.

Comparative genomic hybridization

The comparative genomic hybridization (CGH) profiles showed a global low number of chromosomal alterations in all the patients (Table 1, Figure 1). Chromosomal imbalances were detected in 9 out of the 16 (56%) initial samples from CLL with clinical progression. The most common alteration was trisomy 12 (27%). Two patients with a non-altered initial genomic profile acquired new karyotypic aberrations: a gain of 5q21-q23 and 11pter-p14 (*CLL3*) and a gain of chromosome 12 (*CLL9*) respectively. Gains of 5q and 11p are uncommon in CLL but have been observed in cases with complex karyotypes,[12,13] although their association with disease progression had not been previously observed. Interestingly, the patient that acquired trisomy 12 as a single alteration upon disease progression subsequently transformed into a large B-cell lymphoma in 35 months. Clonal evolution in CLL has been documented in occasional studies, usually associated with advanced disease and shorter survival.[2,14] Our observations indicate that a moderate increase of chromosomal imbalances may already occur in early phases of clinical progression in CLL.

P53 and p16 status

In the present series, 2 patients had an inactivation of the two tumor suppressor genes p53 and p16^{INK4a} at tumor progression (Table 1). One case (*CLL8*) with a 17p loss at diagnosis acquired the p53 mutation during the progression of the disease, supporting the view that inactivation of this tumor suppressor gene in CLL may occur early in disease progression.[14] Concerning the p16^{INK4a} locus, 1 case (*CLL10*) had a homozygous deletion in both sequential samples, associated with a small deletion of 9pter-p23 observed by CGH. The case with a p53 mutation also had a loss of 9pter-p23, but in this case p16^{INK4a} was not deleted, confirming previous observations that 9p losses in CLL are not always associated with p16^{INK4a} gene aberrations.[2]

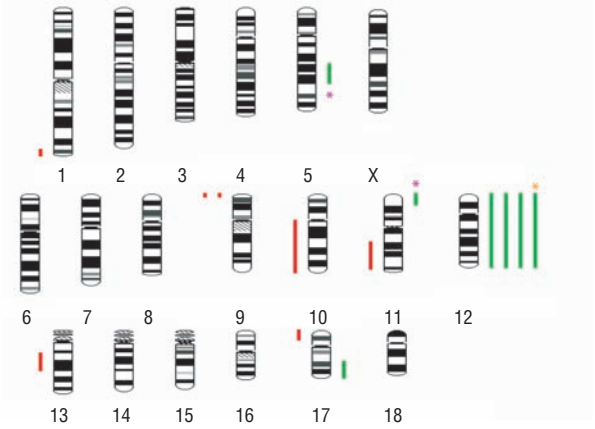

Figure 1. Summary of the CGH data for the 16 B-CLL cases with clinical progression. Red lines on the left side of the profile indicate loss of the chromosomal material. Green lines on the right side indicate gain of chromosomal material. Alterations acquired by unaltered cases at diagnosis CLL3B (violet) and CLL9B (orange) are indicated by an asterisk.

Microarray data analysis

The unsupervized analysis of the 26 paired samples from 13 patients (*CLL1-CLL13*) failed to identify the two groups of samples at diagnosis and progression, indicating that the individual variations in the expression profile were higher than the differences related to the behavior of the disease. These observations are in line with the results obtained in a previous gene expression profile study that compared unrelated tumor samples from progressed and stable CLL and did not find single genes significantly associated with this phenomenon.[15] However, the supervized analysis comparing the initial and progressed sample of the same patients allowed us to identify a group of 58 genes differentially expressed in these two situations (Figure 2A). Of these, 37 genes were overexpressed (fold-change ratio between 0.3 and 1.7) whereas 21 were downregulated (fold-change ratio between -0.3 and –2). No significant differences were observed in the expression of these genes in the sequential samples of the 3 CLL cases with stable clinical evolution (Figure 2B). The functional analysis of the upregulated genes showed that they are involved in different pathways, including cell cycle and cell growth (*MCM4, RAPGEF2, OGG1, ESCO1, ESR1, ACTL6A, CENPJ, ATG5*) and calcium and ion regulation (*MYLC2PL, ADRB1, TRPV5, TMCO3*). Interestingly, 6 out of the 21 (29%) downregulated genes are considered negative regulators of integrin mediated cell adhesion and motility (*PRAM1, CDC42EP4, COL4A2, PLCB2, RAPGEF1, FLNA*). These findings agree with recent experimental studies suggesting that CLL progression is associated with increased ability to respond to migratory signals and that ZAP-70 positive tumors are more sensitive to these signals.[16,17] Four genes were chosen to further validate the microarray results by qRT-PCR, accord-

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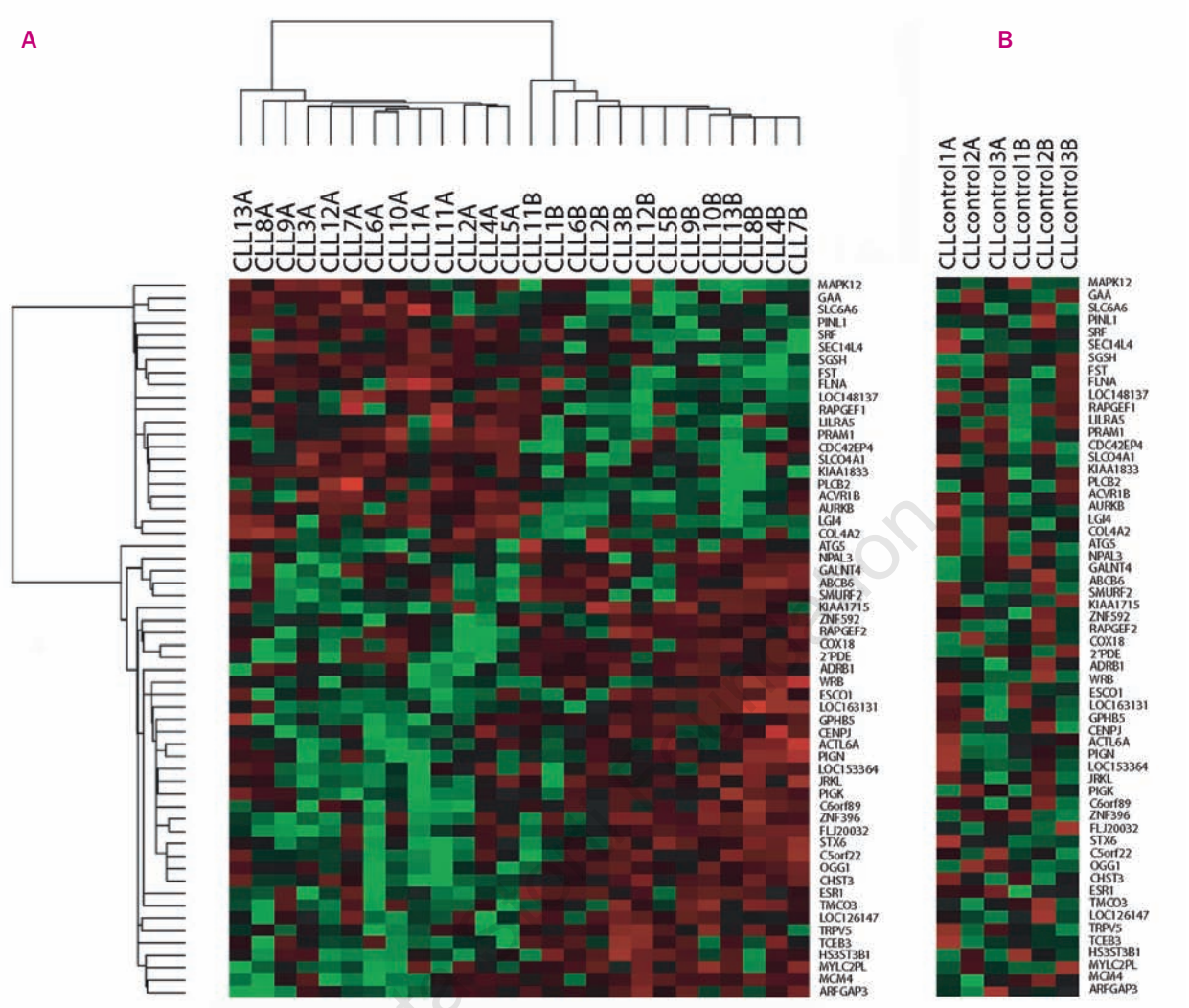

Figure 2. Supervised clustering of the B-CLL sequential samples, performed with the DNA-Chip Analyser (dChip) software and according to the 58 differentially expressed genes found in the microarray data analysis. (A) CLL with clinical progression; CLLA: initial samples, CLLB: progressed samples. (B) CLL with stable clinical evolution; CLLA: first samples, CLLB: second samples.

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ing to their higher variation between samples, B-statistic score and biologic meaning. These four genes were *ACTL6A* (upregulated, cell cycle and cell growth pathway), *OGG1* (upregulated, cell cycle and cell growth pathway), *RAPGEF1* (downregulated, cell adhesion and motility pathway) and *PLCB2* (downregulated, cell adhesion and motility pathway). The validation was performed in 10 paired samples from the microarray study and 3 new paired samples (*CLL14-CLL16*) (Table 1, *Online Supplementary Figure 1*). The microarray results were confirmed in all cases. To summarize, our results indicate that clinical progression of early stage CLL is associated with karyotype evolution, inactivation of tumor suppressor genes and modulation of the expression of a small number of genes, especially a subset that are inhibitors of cell adhesion and motility.

Authorship and Disclosures

VF extracted the RNA and DNA of the samples and performed the molecular characterization of the tumors; VF and PJ performed the microarray analysis; IS and SB analyzed the chromosomal alterations of these tumors by comparative genomic hybridization; EG, DC, NV, FB, EM and EC selected the B-CLL samples and reviewed their clinical and pathological characteristics; MA and NV determined the B-CLL cells purity of the samples and the expression of ZAP-70 by flow cytometry; VF and PJ drafted the manuscript and EC wrote the final version; PJ, FB, EM, EC designed the whole study. All authors critically reviewed the manuscript, and EM and EC approved the final version.

The authors reported no potential conflicts of interest.

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