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Chronic lymphocytic leukemia: assessing pathogenesis and prognosis by modern molecular cytogenetic studies and microRNAs expression

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is a B-cell clonal lymphoprolipherative disorder characterized by the accumulation of small lymphocytes in the peripheral blood, bone marrow and lymph nodes deriving from the transformation of CD5+ B-cell. Despite a homogeneous immunophenotype consisting of CD19+, CD20+, CD5+ and CD23+, CLL is clinically heterogeneous. Several adverse prognostic features have been identified including stage, CD38 positivity, the unmutated configuration of the variable region of the immunoglobulin heavy chain gene (IGHV), ZAP70 positivity, chromosome aberrations and molecular abnormalities.

Detailed immunophenotypic and genetic analysis allowed for the identification of a number of markers of activation and genetic instability, some of which are gaining relevance in clinical practice to predict outcome. Cell surface CD38 is one of these markers since it is an indicator of cell activation and proliferation that may prelude clonal evolution and worse clinical outcome.

We therefore studied the biological and clinical significance of the presence of genetic heterogeneity in the minor CD38+ leukemic population, in a cohort of untreated low-risk CD38-negative CLL patients, defined by the presence of <7% CD38+ cells, and by the absence of unfavourable genetic lesions. Our data showed that a significant proportion of CD38- CLL patients with low risk FISH findings presented genetic aberrations within CD38+ cells. Most of these abnormalities were high risk lesions (11q deletion and 17p deletion) and, in most of the cases, these lesions were found in different cells indicating that multiple cytogenetically unrelated minor clones may be present in the CD38+ cell fraction. Interestingly, the presence of these additional FISH lesions in the small CD38+ cell fraction was associated with shorter time to first treatment (TTT). To identify biomarkers associated with this phenomenon, we performed miRNA expression analysis. We were thus able to show a deregulated miRNA expression profile in CLL cases with additional FISH lesions in CD38+ cells. In particular, miR-125a-5p was found to be down-regulated both in CD38+ and CD38- cells in patients with FISH abnormal clones as compared to patients without FISH abnormal clones. The relevance of miR-125a-5p as a biomarker of inferior outcome and genetic complexity was then validated in a prospective cohort. In this validation cohort, we were able to confirm the predictive role of miR-125a-5p down-regulation in terms

of shorter TTT. In addition we found that CLL patients with lower levels of miR-125a-5p displayed an increased rate of mutations in CLL-related genes by next-generation sequencing.

Several recent studies have shown that CD38 expression is higher in CLL cells in the bone marrow and lymphoid tissues, especially in the proliferation centres (PCs), which are regarded as the histologic hallmark of this disease. Indeed CLL is a disease in which the host's microenvironment promotes leukemic cell growth, leading to sequential acquisition and accumulation of genetic alterations and proliferation centers may play an important role in the biology of CLL, as they represent its proliferative compartment. To better define the significance of proliferation centers, we studied lymph node biopsies taken from a cohort of patients by fluorescence in situ hybridization (FISH) studies using a 5-probe panel on tissue microarrays (TMA). The cases were classified into two categories: "PCs-rich" and "typical". The PCs-rich group was associated with 17p-, 14q32/IGH translocations and +12. The median survival from the time of TMA for PCs-rich and typical groups was 11 and 64 months respectively. The PCs-rich pattern was the only predictive factor of an inferior survival. These findings establish an association between cytogenetic profile and the amount of PCs in CLL, and show that this histopathologic characteristic is of value for risk assessment in patients with clinically significant adenopathy.

CLL turned out to be a disease with multiple facets in its pathogenic mechanisms including genetic aberrations, antigen drive and microenvironmental interactions. In the first part of this work, we focused our attention on the correlation between CD38-positivity, proliferation centres and development of genetic aberrations.
To translate this knowledge in clinical practice we planned further studies focusing i) on the correlation between chromosomal aberrations and clonal evolution and ii) on how to stratify patients into different risk-groups at diagnosis according to cytogenetic abnormalities and gene mutations.
The presence of cytogenetic abnormalities is a hallmark of CLL. It was reported that a fraction of CLL patients developed new cytogenetic abnormalities at chromosome sites of known prognostic importance during the course of their disease (clonal evolution, CE). To better define the incidence and signature of CE, a cohort of patients were analysed sequentially by FISH. Recurring aberrations at

clonal evolution were 14q32/IGH translocation, 17p-, 11q-, 13q- and 14q32 deletion. The development of CE occurred only in previously treated patients. Our data show that the 14q32/IGH translocation may represent one of the most frequent aberrations acquired during the natural history of CLL. CE occurs in pre-treated patients with short TTT and survival, after the development of CE with and without 14q32 translocation, is relatively short.

Having assessed the incidence of chromosome aberration in CLL with evolution and/or adenopathy we next moved to CLL with an apparently “favourable” profile of cytogenetic lesions, to establish if improved cytogenetic techniques could help refine prognostication. We therefore designed a study to assess whether karyotypic aberrations in patients without FISH anomalies correlate with established clinical and prognostic parameters. The clinical and prognostic significance of karyotypic aberrations in normal FISH CLL was first evaluated in a retrospective single centre series of patients and then validated prospectively in a multicentre series of cases diagnosed and analysed for karyotype with DPS30/IL2 stimulation. Conventional karyotyping using DSP30/IL2 stimulation is an effective method for the detection of chromosome aberrations in approximately one third of CLL with normal FISH. The abnormal karyotype correlated with shorter time to first treatment and shorter survival. This set of data also showed that, in CLL patients with normal FISH, conventional cytogenetic analysis identifies a subset of cases with adverse clinical and prognostic features to be considered for the design of risk-adapted treatment strategies.

In the last part of our experimental work we moved from the consideration that the cytogenetic lesions do not entirely explain the molecular pathogenesis and the clinical heterogeneity of CLL. Indeed, the advent of next-generation sequencing (NGS) technologies has enabled exploration of the CLL genome, uncovering genetic lesions that recurrently target the leukemic cells. NGS studies have further elucidated the genomic complexity of CLL. In order to improve understanding of genetic basis of CLL and to apply NGS to CLL, we sequenced DNA samples from untreated patients affected by chronic lymphocytic leukemia with a panel of 20 genes and we correlated mutational status with clinicobiological parameters.Mutations were identified in the following genes: TP53, SF3B1, POT1 , ATM , MYD88 , FBXW7 , MAPK1 , DDX3X , KLHL6 , KRAS. The presence of mutations correlated with high risk FISH (11q- and/or 17p-) and unfavourable

cytogenetic (11q-, 17p- or complex karyotype) findings. Patients carrying CLLs with gene mutations showed a significant shorter median time to first treatment in comparison to those without mutations (20 months vs not reached at 76 months). The frequency of mutations in the 20 investigated genes is in line with published data in the literature using whole exome sequencing. This study shows that the simultaneous sequencing of a panel of genes implicated in CLL is feasible.

In conclusion, in this work we tried to improve our knowledge on some fundamental pathogenetic aspects of CLL, including:

i) the development of genetic lesions in CD38+ activated cells, obtained from the PB in patients in an initial and indolent phase of the disease;
ii) the pattern of cytogenetic lesions in lymph node microenvironment (proliferation centres) in patients in a more advanced phase of the disease;
iii) to translate this knowledge in clinical practice, we assessed prognosis with modern techniques and we identified cytogenetic lesions associated with disease progression and shorter time to treatment;
iv) we identified recurrent genetic lesions potentially useful for further refinement of our ability to predict prognosis and response to treatment.

ABSTRACT (ITALIAN)

La leucemia linfocitica cronica (LLC) è un disordine linfoproliferativo a carico del linfocita B, caratterizzato dall'accumulo di piccoli linfociti nel sangue periferico, nel midollo osseo e nei linfonodi. Nonostante un immunofenotipo omogeneo costituito dalla presenza degli antigeni CD19, CD20, CD5 e CD23, la LLC è clinicamente eterogenea. Sono stati identificati diversi fattori di prognosi. Tra questi ritroviamo lo stadio di malattia alla diagnosi, la positività per il CD38, la configurazione non mutata della regione variabile della catena pesante dei geni immunoglobulinici (IGHV), la positività per ZAP70, le aberrazioni cromosomiche e le anomalie molecolari.

Analisi immunofenotipiche e genetiche hanno portato all'identificazione di diversi marcatori molecolari di attivazione, alcuni dei quali sono riconosciuti nella pratica clinica al fine di predire la prognosi. L'antigene di superficie CD38 è uno di questi. Esso, in particolare, è un indicatore di prognosi sfavorevole, di attivazione e proliferazione cellulare ed è in grado di predire un'evoluzione clonale ed una prognosi sfavorevole.

Abbiamo quindi studiato, in un gruppo di pazienti a basso rischio (cellule CD38+ <7% e anomalie FISH a basso rischio prognostico), il significato biologico e clinico della presenza di lesioni genetiche nella frazione cellulare CD38 positiva. I nostri dati hanno mostrato che una percentuale significativa di pazienti presentano aberrazioni genetiche all'interno delle cellule CD38+. La maggior parte di queste anomalie sono lesioni ad alto rischio (delezione 11q e 17p) e nella maggior parte dei casi queste lesioni sono stati trovate in cellule diverse indicando che più cloni minoritari possono essere presenti, in maniera indipendente, nella popolazione cellulare complessiva. La presenza di queste lesioni FISH nella piccola frazione CD38+ è associata ad un minore tempo al primo trattamento (TTT). Per identificare marcatori associati a questo fenomeno, abbiamo effettuato un'analisi di espressione dei microRNAs. Abbiamo quindi identificato un profilo di espressione dei miRNAs alterato nei casi di LLC con lesioni FISH supplementari a carico delle cellule CD38+. In particolare, il miR-125a-5p è risultato essere down-regolato sia nelle cellule CD38+ che nelle cellule CD38- nei pazienti con cloni aventi anomalie FISH, rispetto ai pazienti senza cloni anomali. L'importanza del miR-125a-5p come marcatore di prognosi inferiore e complessità genetica è stata

poi validata in una coorte di pazienti indipendente. In questa serie di validazione, abbiamo confermato il ruolo predittivo del miR-125a-5p in termini di TTT più breve. Inoltre abbiamo visto, attraverso tecniche di sequenziamento di ultima generazione, che i pazienti affetti da LLC con bassi livelli di miR-125a-5p presentano un aumento del tasso di mutazione nei geni implicati nella LLC.

Diversi studi recenti hanno dimostrato che l'espressione del CD38 è maggiore nelle cellule leucemiche all'interno del midollo osseo e del tessuto linfoide, in particolare all'interno dei centri proliferativi (PCs), che sono considerati la peculiarità istologica della LLC. Infatti la LLC è una malattia in cui microambiente promuove la crescita delle cellule leucemiche, conduce all'acquisizione ed al conseguente accumulo di alterazioni genetiche. In questo contesto, i centri proliferativi svolgono un ruolo fondamentale.
Per definire meglio il significato dei PCs, abbiamo studiato le biopsie linfonodali di una coorte di pazienti con metodica FISH su microarray tissutale (TMA). I casi sono stati classificati in due categorie: "PC-rich" e "tipici". Il gruppo "PC-rich" è stato associato alla presenza di delezioni 17p-, traslocazioni 14q32/IGH e trisomia del cromosoma 12. La sopravvivenza media, dal momento del TMA, per i gruppi "PC-rich" e "tipici" è risultato essere, rispettivamente, di 11 e 64 mesi. Questi risultati stabiliscono, quindi, un'associazione tra il profilo citogenetico e la quantità di PCs nella LLC, e dimostrano che questa caratteristica istopatologica è di grande valore per la valutazione del rischio nei pazienti con adenopatie clinicamente significative.

La LLC si è rivelata essere una patologia con molteplici sfaccettature nei suoi meccanismi patogenetici, tra cui la presenza di aberrazioni genetiche, la stimolazione da parte dell'antigene e le interazioni con il microambiente. Nella prima parte di questo lavoro, abbiamo focalizzato la nostra attenzione sulla correlazione tra positività per il CD38, centri proliferativi e sviluppo di aberrazioni genetiche. Per tradurre queste conoscenze nella pratica clinica abbiamo programmato ulteriori studi incentrati i) sulla correlazione tra aberrazioni cromosomiche e l'evoluzione clonale e ii) su come stratificare i pazienti in diversi gruppi di rischio al momento della diagnosi in base ad anomalie citogenetiche e mutazioni genetiche.

La presenza di anomalie citogenetiche è un segno distintivo della LLC. In letteratura è riportato che una frazione di pazienti affetti da LLC sviluppa nuove anomalie citogenetiche durante il corso della malattia (evoluzione clonale, CE). Per definire meglio l'incidenza e lo stato dell'evoluzione clonale, abbiamo analizzato una coorte di pazienti mediante FISH a diversi stadi di malattia. Aberrazioni ricorrenti nell'evoluzione clonale sono risultate essere: traslocazioni 14q32/IGH, delezioni 17p-, 11q-, 13q-e 14q32. L'evoluzione clonale si è verificata solo in pazienti precedentemente trattati. I nostri dati mostrano che la traslocazione 14q32/IGH rappresenta una delle aberrazioni acquisite più frequenti durante la storia naturale della LLC. Il fenomeno dell'evoluzione clonale si verifica in pazienti pretrattati con breve TTT, e la sopravvivenza dopo lo sviluppo di evoluzione clonale è relativamente breve.

Dopo aver valutato l'incidenza di aberrazioni cromosomiche in pazienti LLC con evoluzione clonale e/o adenopatia, ci siamo soffermati sul gruppo di pazienti LLC con lesioni citogenetiche "favorevoli". Abbiamo, quindi, effettuato uno studio per valutare se le aberrazioni cromosomiche in pazienti FISH negativi correlino con i parametri clinici e prognostici. Abbiamo dapprima studiato una coorte retrospettiva di pazienti aventi le suddette caratteristiche. In seguito, abbiamo validato prospetticamente in una serie multicentrica di casi analizzati mediante cariotipo convenzionale con stimolazione metafasica attraverso DPS30/IL2. Abbiamo visto che questa tecnica permette l'individuazione di aberrazioni cromosomiche in circa un terzo dei casi LLC con FISH normale. Il cariotipo anomalo correla con un minore tempo al primo trattamento ed una ridotta sopravvivenza. Questo insieme di dati ha quindi mostrato che, nei pazienti affetti da LLC con FISH normale, l'analisi citogenetica convenzionale identifica un sottoinsieme di casi con caratteristiche cliniche e prognostiche negative da prendere in considerazione al fine di adeguare le strategie di trattamento.

Nell'ultima parte del nostro lavoro sperimentale, abbiamo considerato che le lesioni citogenetiche non sono in grado di spiegare la patogenesi molecolare e l'eterogeneità clinica della LLC. L'avvento del sequenziamento di ultima generazione (NGS) ha permesso l'acquisizione di importanti conoscenze sulla caratterizzazione della LLC, chiarendone ulteriormente la complessità genomica. Al fine di migliorare la comprensione delle basi genetiche delle LLC e di applicare

le tecniche di NGS, abbiamo sequenziato una serie di pazienti non trattati con un pannello di 20 geni, ritrovati frequentemente mutati nella LLC, e abbiamo correlato lo stato mutazionale con i parametri clinicobiologici. Abbiamo ritrovato mutazioni somatiche nei seguenti geni: TP53, SF3B1, POT1, ATM, MYD88, FBXW7, MAPK1, DDX3X, KLHL6, KRAS. La presenza di mutazioni correla con la presenza di anomalie FISH ad alto rischio (11q- e/o 17p-), e cariotipo sfavorevole (11q-, 17p o cariotipo complesso). Pazienti recanti mutazioni genetiche hanno mostrato un tempo al trattamento significativamente più corto rispetto a quelli senza mutazioni. La frequenza di mutazioni nei 20 geni studiati è in linea con i dati pubblicati in letteratura.

In conclusione, in questo lavoro abbiamo cercato di migliorare le nostre conoscenze su alcuni aspetti patogenetici fondamentali della LLC, tra cui:

i) lo sviluppo di lesioni genetiche in cellule attivate CD38+, ottenute da sangue periferico in pazienti in una fase iniziale e indolente di malattia;
ii) il tipo di lesioni citogenetiche nel microambiente linfonodale (centri proliferativi) in pazienti in una fase più avanzata di malattia;
iii) traducendo queste conoscenze nella pratica clinica, abbiamo valutato la prognosi con tecniche moderne ed identificato lesioni citogenetiche associate a progressione di malattia e minor tempo libero da trattamento;
iv) abbiamo identificato lesioni genetiche ricorrenti potenzialmente utili per un ulteriore affinamento della nostra capacità di predizione della prognosi e della risposta al trattamento.

CHAPTER 1

CHRONIC LYMPHOCYTIC LEUKEMIA

1.1 INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in Western countries and is characterized by clonal proliferation and accumulation of mature B lymphocytes. CLL affects individuals with median age at diagnosis ranging between 67 and 72 years. Moreover, the proportion of younger patients with early stage CLL and minimal symptoms seems to increase due to more frequent blood testing. More male than female patients (1.7:1) are affected [1].

CLL is characterized by the clonal proliferation and accumulation of mature, typically CD5-positive B-cells within the blood, bone marrow, lymph nodes and spleen [2]. Typical immunophenotype of the CLL is the presence of the B-cell surface antigens CD19, CD22 and CD 23 with coexpression of T-cell lineage antigen CD5 and expression of CD20 and CD7b lower than that observed in normal B cells [3, 4].

1.2 DIAGNOSIS AND STAGING

The diagnostic criteria as laid out by the International Workshop on Chronic Lymphocytic Leukemia (IWCLL-2008) [4] requires the presence of at least 5.000 B-lymphocytes/ul in the peripheral blood for the duration of minimum 3 months. In most cases the diagnosis of CLL is established by blood counts, differential counts, a blood smear and immunophenotyping. The World Health Organizaton (WHO) classification of hematopoietic neoplasias describes CLL as leukemic, lymphocytic lymphoma, being only distinguishable from SLL (small lymphocytic lymphoma) by its leukemic appearance [5].

CLL is always a disease of neoplastic B-cells and their clonality needs to be confirmed by flow cytometry [4]. The leukemia cells found in the blood smear are characteristically small, mature lymphocytes with a narrow border of cytoplasm and a dense nucleus lacking discernible nucleoli and having partially aggregated chromatin [6] (Fig. 1.1).

Two widely accepted staging methods co-exist, the Rai [7] and the Binet [8] system. Both describe three major prognostic groups with discrete clinical outcomes.

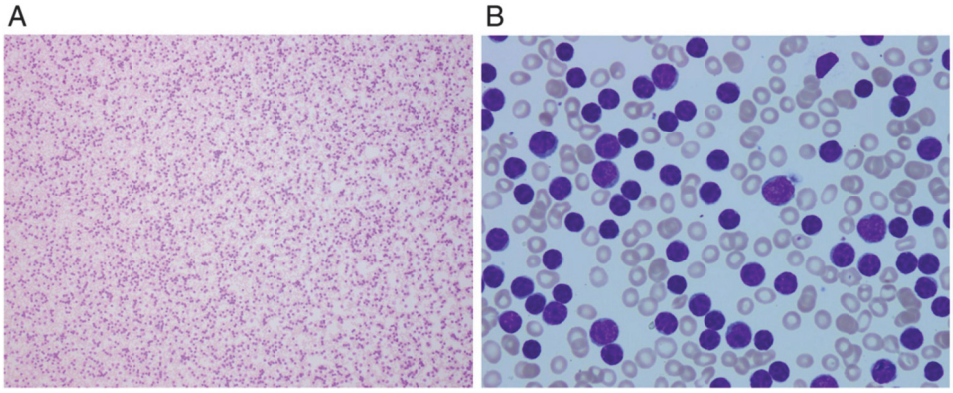


FIGURE 1.1. (A) Peripheral blood smear in a case of CLL with abnormally high lymphocyte counts (4x); (B) Characteristics appearance of small CLL cells with frequent prolymophocytes (40x).

The Rai staging system defines low-risk disease as patients who have lymphocytosis with leukemia cells in blood and/or bone marrow (lymphoid >30%) (Rai stage 0). Patients with lymphocytosis, enlarged nodes in any site and splenomegaly and/or hepatomegaly are defined as having intermediate risk disease (Rai stage I or II). High risk disease includes patients with disease-related anemia (stage III) or thrombocytopenia (stage IV).

The Binet staging system is based on the number of involved areas, as defined by the presence of enlarged lymph nodes of greater than 1 cm or organomegaly, and on whether there is anemia or thrombocytopenia. Binet stage A is defined as having Hb ≥10 g/dL, platelets ≥100 x 10^9/L and up to two areas of involvement. Patients with Hb ≥10 g/dL, platelets ≥100 x 10^9/L and organomegaly greater than that defined for stage A are defined as having stage B. All patients who have Hb of less than 10 g/dL and/or platelet count of less than 100 x 10^9/L, irrespectively of organomegaly are included in stage C.

1.3 Biology and Pathogenesis of CLL

Our understanding of CLL has changed over the last decades. It was once thought to be a homogenous disease, in which mature B cells accumulated largely due to a lack of normal cell death. It is currently known that CLL cells show an antiapoptotic profile, with strong expression of Bcl-2 protein, which suggests that

inhibition of apoptosis is responsible for CLL development. However, several reports have shown that the high lymphocyte count in CLL patients is caused not only by the prolonged survival, but also by proliferating cells. The disease probably develops is a result of accumulation of transformed B cells. In CLL cells, a great imbalance between cell birth and death rate is observed [3, 9].

The gene expression profile suggests that CLL cells originate from transformed, antigen-stimulated B cells [10]. In CLL cells, several mutated IGHV genes are expressed more frequently than in normal B lymphocytes. All those cells express restricted sets of B-cell receptors (BCR). Unique stereotypy of BCR suggests that antigens play a critical role in CLL pathogenesis.

Moreover, CLL has recently been established as a disease of remarkable diversity and differences in cell morphology, immunophenotype, cytogenetics and molecular characteristics. This heterogeneity translates into clinical course and the response to treatment [11]. Approximately one third of patients survive for 20 years or longer and never require treatment [12] and, alternatively, some patients may progress rapidly from the time of diagnosis.

1.4 PROGNOSTIC FACTORS IN CLL

A number of clinical and biological markers of prognostic relevance have been identified. Due to great variability of CLL patients clinical course, there is a need to establish solid prognostic factors for this disease.

CLL patients are currently categorized into risk groups based on the clinical staging systems Rai [7] and Binet [8]. These classifications are helpful for dividing patients in regard to the expected overall survival (OS). However, both systems fail to indicate the higher risk of progression among patients in early stage of disease.

Among prognostic factors in CLL, there are lymphocyte doubling time (LDT), serum markers, IGHV mutational status, ZAP-70 and CD38 expression, cytogenetic abnormalities and somatic mutations [13-16].

LDT longer than 12 months correlates with increased progression-free survival (PFS) and OS. An increase in the lymphocyte count of more than 50% in two months or LDT during less than 6 months is a recommended criterion of active disease and indication for treatment [17].

Serum prognostic factors, such as β2-microglobulin, soluble CD23 or serum thymidine kinase were indicated as an important prognostic factor for CLL patients. In fact they are relevant markers of proliferative activity and a risk disease progression, correlating with other biological prognostic factors [18].
These ones can be immunoglobulin heavy chain variable region (IGHV) mutation status, somatic mutations and some cytogenetic abnormalities, cell membrane expression of CD38 and intracellular expression of ZAP-70 [4, 18, 19]. CLL patients may have either somatically mutated or unmutated IGHV gene, which correlates with a favourable or unfavourable prognosis. Patients with unmutated IGHV gene have significantly shorter OS (approximately 8 years) than those with mutated IGHV gene (approximately 25 years) (Fig. 1.2).

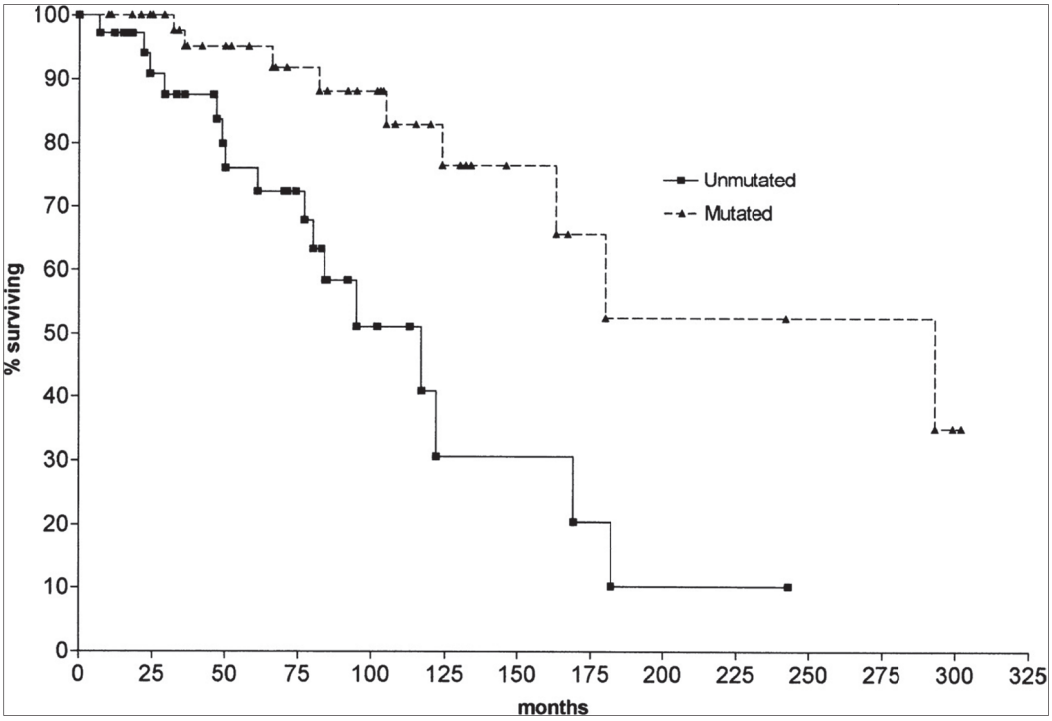


FIGURE 1.2. Kaplan-Meier survival curve comparing CLL patients with mutated and unmutated V_H genes. Median survival for unmutated CLL: 117 months; median survival for mutated CLL: 293 months [14].

The expression of ZAP-70 remains constant over the course of the disease. The cut-off to classify patients as ZAP-70 positive (negative prognostic factor correlating with unmutated IGHV status) or ZAP-70 negative, as measured by flow cytometry, is widely proposed at 20% threshold. However, standardization of ZAP-70 estimation still remains a challenge.

Similar correlation with outcome has been shown for CD38 expression. CD38-positive patients (the threshold proposed is >30% CD38+ CLL cells) were reported to have significantly worse prognosis regarding PFS and OS than those who were CD38 negative. It was observed that CD38 expression on CLL cells correlates with the absence of IGHV mutations [3, 4, 13, 18, 19].

A crucial prognostic importance is assigned to chromosomal aberrations. In approximately 80% of patients, there is a mutation detected by fluorescence in situ hybridization (FISH) [15]. The most common recurrent chromosomal abnormalities include deletion 13q, trisomy 12 and deletions 11q, 17p and 6q. A subdivision based on these aberrations is important, as they are predictor of disease outcome. Five prognostic categories have been identified in a hierarchical model, showing poor survival in patients with 17p deletion or 11q deletion (median survival 32 and 79 months, respectively) but better survival for patients with trisomy 12, normal karyotype, and deletion 13q as the sole abnormality (114, 111 and 133 months, respectively) [15]. Deletions of 11q22-q23 and 17p13, resulting in abnormalities of ATM and TP53 genes, respectively, are independent prognostic factors identifying patients with a rapid disease progression and a short OS in a multivariate analysis. Patients with del(11q) have more frequently B symptoms, advanced clinical stage and extensive peripheral, abdominal and mediastinal lymphoadenopathy. Deletions 17p and 11q are more often detected in advanced stages of the disease, among patients with unmutated IGHV gene. In contrast, deletion of chromosome band 13q14 is associated with a favourable CLL outcome. Moreover, patients with trisomy 12 have a shorter OS than those with 13q deletion [3, 4, 9, 13, 15, 18].

TP53 mutations have been described in 4-12% of patients with untreated CLL. Approximately 80-90% of cases with a deletion of one copy of TP53 locus will have a TP53 mutation on the remaining copy [16, 20-23]. TP53 mutations are more prevalent in progressive and refractory CLL [22] (Fig 1.3). A TP53 mutation is an independent predictor of poor prognosis and confers even shorter OS than del(17p) in the absence of TP53 mutation [20]. Mutations of the ATM gene may also have prognostic implications independent of those associated with the deletion of chromosome 11q. Patients harbouring ATM mutations have a more aggressive clinical course and are more resistant to traditional chemotherapeutic agents [24, 25].

In recent times, the improvements in next generation sequencing technologies have provided a novel opportunity to examine the CLL genome [26], and have

allowed previously unknown genomic alterations to be identified, such as mutations of NOTCH1 (neurogenic locus notch homolog protein 1) [27, 28], SF3B1 (splicing factor 3B unit 1) [29] and BIRC3 (baculoviral IAP repeat-containing protein 3) [30]. Mutations of these genes have been associated with short time to progression and survival. Each of these lesions recognizes a different distribution across different clinical phases and biological subgroups of the disease.

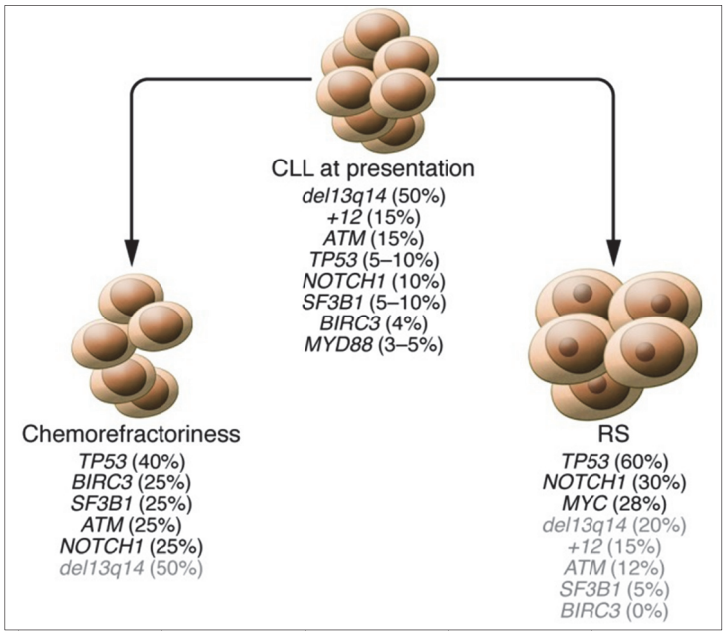


FIGURE 1.3. Genetic lesions of CLL at different phases of the disease. The frequency (in parenthesis) of genetic lesions is shown for CLL at presentation and for two different type of CLL progression: chemorefractoriness without evidence of histologic transformation, and histologic transformation to Richter Syndrome (RS). the two type of CLL progression follow distinct molecular pathways in terms of type and frequency of genetic lesions. Genetic lesions that occur at CLL presentation but are not enriched at CLL progression are indicated in gray.

1.5 TREATMENT OF CLL

Despite the great improvement in CLL treatment during the last decades, the disease still remains difficulty curable. Chemotherapy is not recommended in early or stable disease patients. In this group, a “watch and wait” approach is widely indicated. Treatment should be introduced in patients with advanced, symptomatic or progressive disease. The choice of therapy depends on clinical stage, the disease activity, age and comorbidities.

1.5.1 Cytostatic Agents

For many years, chlorambucil (with or without steroids) was the drug of choice in previously untreated patients with progressive or advanced CLL [31]. Even today, this drug remains an appropriate option for elderly and unfit patients. The advantages of chlorambucil are its moderate toxicity and low cost; the major disadvantages are its low complete remission (CR) rate and some side effects that occur after extended use.

Three purine analogs are currently used in CLL: fludarabine, pentostatin and cladribine. These drugs showed high antileukemic activity. Significantly higher overall response (OR), complete remission and progression free survival (PFS) in patients treated with fludarabine or cladribine were documented in several randomized clinical trials [32, 33]. Next, combination of fludarabine or cladribine with cyclophosphamide appeared to be more effective than purine analogs in monotherapy in regard to OR, CR and PFS [34-36].

1.5.2 Antibodies

Rituximab. CD20 is an activated, glycosylated phosphoprotein expressed on the surface of mature B-cells. The protein has unknown natural ligand and its function is not yet discovered [37]. As CD20 is expressed on most B-cell malignancies, the introduction of the anti-CD20 antibody Rituximab in 1998 improved the treatment of most CD20-positive on-Hodgkin lymphomas including CLL [38]. In CLL, Rituximab is less active as single agent than in follicular lymphoma, unless very high doses are used [39, 40]. In contrast, combinations of Rituximab and chemotherapy have proven to be very efficacious therapies of CLL.

Ofatumumab. It is a fully humanized antibody targeting a unique epitope on the CD20 molecule expressed on human B-cells. Ofatumumab was found to be effective in a phase III randomized study, as monotherapy for heavily pretreated patients with CLL resistant to fludarabine or fludarabine-alemtuzumab regimen [41].

Alemtuzumab. It is another MoAb, recombinant, humanized anti-CD52, highly active in CLL. In previously untreated patients, an OR rate of more than 80% was achieved, being effective in patients with high risk genetic markers such as deletions of chromosome 11 or 17 and TP53 mutations [42, 43], and in those patients with Alemtuzumab can be used as a first-line treatment [44].

Obinutuzumab (GA101). The humanized and glycol-engineered monoclonal antibody Obinutuzumab showed impressive results in vitro with higher rates of apopotosis in B-cells in comparison to Rituximab [45]. Encouraging results were reported in the interim analysis of CLL11 trial on CLL patients with comorbidity. The primary endpoint was PFS [46].

1.5.3 Combination chemotherapy

A major advance in CLL treatment was achieved by the combined use of different treatment modalities. Because purine analogs and alkylating agents have different mechanisms of action and partially nonoverlapping toxicity profiles, they were combined to achieve synergistic effects. The most thoroughly studied combination chemotherapy for CLL is fludarabine plus cyclophosphamide (FC) [47]. In noncompertive trials, the overall response rates did not appear to be better than with fludarabine alone, but the addition of cyclophosphamide appeared to improve the CR rate up to 50% [47]. A phase II study of cladribine in combination with cyclophosphamide has also demonstrated activity in advanced CLL, but results seemed inferior to FC [48].

1.5.4 Chemoimmunotherapy

The results of recent studies showed that Rituximab in combination with purine analogs or purine analogs and cyclophosphamide can increase the OR and CR rates and PFS time [49]. The combination of Rituximab with FC (FCR regimen) demonstrated particularly high rates of OR, CR and duration of PFS in both previously untreated and relapsed/refractory CLL [50]. However FCR is acceptable for younger, physically fit patients. This regimen has limitations in the unfit group, mainly due to the risk of myelosuppression and other side effects. Because CLL often occurs in elderly patients with relevant comorbidities, a dose modified FCR-Lite regimen was designed to maintain the efficacy but decrease the toxicity of the FCR regimen [51]. This regimen reduced the dose of fludarabine and cyclophosphamide and increased the dose of Rituximab. Recent trials proved that combination of Rituximab with pentostatin or cladribine and cyclophosphamide is a highly active treatment modality in CLL. More recently, bendamustine, a bifunctional agent composed of an alkylating nitrogen mustard group and a purine-

like benzimidazole ring, has been included in CLL treatment regimens. In a randomized trial compared activity of bendamustine or chlorambucil in untreated CLL patients [52], OR and CR rates were significantly higher in patients treated with bendamustine. Several other combinations have been investigated, such as, cladribine with rituximab, methylprednisolone plus rituximab followed by alemtuzumab, or rituximab plus alemtuzumab.

1.5.5 New drugs targeting pathogenic pathways of CLL cells

There are an increasing number of interesting new compunds in clinical development. The common denominator of these compounds is that thei mechanism of action targets a relatively specific signalling abnormality or redirects the immune system against CLL cells.

Agents targeting BCR signalling. Idelalisib (CAL-101) is an inhibitor of phosphatidylinositol 3-kinase (PI3K) signalling. CAL-101 reduces survival signals derived from the BCR and inhibits BCR and chemokine-receptor-induced AKT and MAP kinase (ERK) activation [53]. In preclinical studies, it was found to induce apoptosis of CLL cells. Protein kinase C and PI3K pathways have an influence on the survival of B cells in CLL. In a phase I clinical trial, Idelalisib showed acceptable toxicity, positive pharmacodynamics effects and favourable clinical activity [54]. Results on Idelalisib in combination with Rituximab, Ofatumumab or bendamustine/rituximab were presented in preliminary form and showed encouraging results .

Ibrutinib is an orally active small molecule inhibiting BTK that plays a role in the signal transduction of the BCR. Inhibition of BTK might induce apoptosis in B-cell lymphomas and CLL cells [55]. Ibrutinib showed significant activity in patients with relapsed or refractory B-cell malignancies, including CLL [56].

Bcl-2 inhibitors. Proteins in the B-cell CLL/Lymphoma 2 (Bcl-2) family are key regulators of the apoptotic process [57]. The Bcl-2 family comprises proapoptotic and prosurvival proteins. Shifting the balance toward the latter is an established mechanism whereby cancer cells evade apoptosis. ABT-263 (Novitoclax) is a small molecule Bcl-2 family inhibitor that binds with high affinity to multiple antiapoptotic Bcl-2 family proteins. Initial studies showed very promising results for this drug as single agent [58]. However its therapeutic use seemed somewhat limited by severe thrombocytopenia being a prominent side effect.

Therefore the compound was reengineered to create a highly potent and cl-2 selective inhibitor, ABT-199 [59]. This compound inhibits the growth of Bcl-2-dependent tumors in vivo and spares human platelets. Recent data from clinical trials indicate that selective pharmacological inhibition of Bcl-2 holds great promise for the treatment of CLL.

Given the impressive choice of options, the right choice of treatment becomes a task that requires experience, a good clinical judgement and an appropriate use of diagnostic tools. The following parameters should be considered before recommending a treatment for CLL: the clinical stage of disease; the fitness of the patient; the genetic risk of leukemia; the treatment situation (first versus second line, response versus non-response of the last treatment).

CHAPTER 2

PATHOGENESIS OF CLL: CD38 AND PROLIFERATIONS CENTERS

2.1 INTRODUCTION

In 1995, chronic lymphocytic leukemia (CLL) was defined as a homogeneous disease of immature, immune-incompetent, minimally self-renewing B cells [2], which accumulate relentlessly because of a faulty apoptotic mechanism. Since ten years, these views have been transformed by a wealth of new information about the leukemic cells. CLL is currently considered a clinically heterogeneous disease originating form B lymphocytes that may differ in activation, maturation state or cellular subgroup. Leukemic cell accumulation occurs because of survival signals delivered to a subgroup of leukemic cells from the external environment through a variety of receptors and their cell-bound and soluble ligands [60].

CLL is seen as a disease characterized by a dynamic balance between cells circulating in the blood and cells located in permissive niches in lymphoid organs [61]. The former are primarily mature-looking small lymphocytes resistant to apoptosis, whereas the latter are composed of lymphocytes that undergo either proliferation or apoptosis according to environment [60].

B lymphocytes mature in the bone marrow and in the process rearrange immunoglobulin variable (V) gene segments to create the code for an immunoglobulin molecule that serves as the B-cell receptor for antigen. When an antigen of adequate affinity engages the receptor, the cell enters a germinal center in lymphoid follicles, where, as a centroblast, it rapidly divides and its V genes undergo somatic hypermutation. This process introduces mutations in the rearranged V_HDJ_H and V_LJ_L gene segments that code for the binding site of the receptor. Through these mutations, the receptors of the descendent B cells, called centrocytes, acquire new properties. Centrocytes with receptors that no longer bind the antigen or do bind autoantigens are normally eliminated [62].

This stimulation and selection pathway usually requires the help of T lymphocytes and occurs in germinal centers [62], the structure of which ensures the selection of B cells capable to recognize antigens. However, the process can proceed without T cells [63] and outside germinal centers, in the marginal zones around lymphoid follicles [64]. Both process lead to the development of plasma cells or memory (antigen-experienced) B cells. Concomitant with B-cell activation, the proteins on the surface of the B cell change. These modifications help activated B cells to interact with other cells and soluble mediators. One surface molecule that supports B cell interactions and differentiation is CD38 [65].

The heterogeneous clinical outcome of CLL patients is dictate, at least in part, by the nature of these microenvironmental signals and interactions that can promote or impair accumulation of genomic alterations [66]. Detailed immunophenotypic and genetic analysis of different neoplastic clones have led to the identification of a number of molecular markers of activation, some of which are gaining relevance in clinical practice to predict outcome [14, 15, 17, 67]. Cell surface CD38 is one of these markers.

2.2 CD38 IS ASSOCIATED WITH GENETIC HETEROGENEITY AND CLONAL EVOLUTION

2.2.1 Biology of human CD38

CD38 is expressed at high levels by B lineage progenitors in bone marrow and by B lymphocytes in germinal center, in activated tonsils, and by terminally differentiated plasma cells [68]. On the other side, mature virgin and memory B cells express low levels of this molecule. CD38 is located in the cytosol and/or in the nucleus and not on the cell membrane [68].

CD38 is a 45 kDa type II transmembrane glycoprotein. The functional CD38 molecule is a dimer, with the central part hosting the catalytic site [69]. The transition from monomer to dimer modulates the function of the molecule. CD38 has the tendency to associate with other proteins, forming large supramolecular complexes. Molecules that associate with CD38 include the CD19/CD81 complex, the chemokine receptor CXCR4, ad adhesion molecules, such as CD49d [70, 71]. CD38 is also found in exosomes [72], membrane vescicles secreted by B cells, and is probably part of an intracellular communication network.

The functional properties of CD38 on human B cells appear to be strictly linked to the stage of maturation. The presence of blocking mAbs in cultures of CD19+ B-cell precursors on stromal layers markedly suppress B-cell lymphopoiesis by inducing apoptosis [73]. In contrast, in mature circulating B lymphocytes and tonsillar germinal center B cells, CD38 ligation is followed by activation, apoptosis inhibition, proliferation and cytokine secretion [74, 75]. In both cases, the mechanisms are attributed to the activation of an intracellular signalling pathway ruled by CD38 and requiring an association with CD19.

2.2.2 CD38 as a marker in CLL

CD38 identifies two subgroups of CLL patients with different clinical outcomes [67]; this distinction is based on the percentage of CD38+ leukemic cells within a CLL clone. The two patient subgroups differ clinically in several ways, including overall survival [67, 76], time to first treatment [77, 78], number of leukemic cells with atypical morphology [79], extent and level of adenopathy [76] and absolute lymphocyte counts [80]. These subgroups also differ in responsiveness to various therapies [80].

The past decade has highlighted several molecular differences between CD38+ and CD38- members of the same clone, including expression of specific activation markers [81], which reflect quantitative and temporal differences in response to stimulation. CD38+ CLL cells express high levels of CD69 and HLA-DR [82], both indicative of recent activation. CD38 also marks a cellular subset enriched in Ki-67+ and ZAP-70+ cells. In addition, CD38+ CLL clones display enhanced ability to migrate in response to chemokine and to transduce BCR-mediated signals.

These findings suggest that cellular proliferation might be at the root of the association between higher levels of CD38, aggressively growing CLL clones, and poor patient outcome [60]. This suggestion is consistent with in vivo labelling experiments using $^{2}H_2O$ (“heavy water”) that demonstrated larger than anticipated rates of CLL birth, especially in patients with poor clinical course and outcome [83] and a several-fold higher percentage of newly born cells in CD38+ fractions of CLL clones than in CD38- counterparts of the same clones [84]. Furthermore, preliminary results from a large clinical study suggest that the percentage of CD38+ CLL cells strongly correlates with the level of leukemic cell turnover observed in vivo [85].

2.2.3 CD38 and genetic subclonal complexity

Current models of cancer progression are based on the concept that tumors are subject to the Darwinian process of evolution and selection. Recent studies in acute lymphpoblastic leukemia have provided pivotal insights into the complex sequence of events during leukemogenesis, showing that the initiating mutation is followed by copy number alterations (CNAs) that drive the emergence of the disease [86, 87]. These data imply that at least some CNAs/copy neutral loss of heterozigosity regions (cnLOHs) are likely to be involved in driving leukemia progression and therefore might contribute to relapse. Knight and colleagues [88] hypothesized that subclones containing driver CNAs/cnLOHs would newly occur or expand in relapse samples compared with samples taken before treatment. They tested their hypothesis by systemically tracking the presence and subclonal distribution of CNAs/cnLOHs in CLL patients before treatment and at subsequent relapse. The results reveal complex changes in the subclonal architecture of paired samples at relapse compared with pre-treatment, suggested that genomic complexity correlate with a poor clinical outcome. Clinically, the existence of multiple, genetically distinct, subpopulations that escape therapeutic intervention might present formidable challenges for the development of effective treatment for patients with relapsed refractory CLL.

In order to better understand the ongoing evolution of genetic lesions in patients with CLL, Grubor and colleagues [89] compared the leukemic genome with the patient's normal DNA by using a high-resolution CGH technique called representational oligonucleotide microarray analysis (ROMA) [90]. This technique have an incredible resolution and it is so sensitive to permits the examination of the clonal heterogeneity within the same CLL patient form mixed population. To find clearer evidence of intraclonal heterogeneity within patients, they searched for genomic differences between CD38+ and CD38- populations in the same patient. So they profiled the genomes of CLL cells separated by the surface marker CD38 and found evidence of distinct subclones of CLL within the same patient. In fact, they observed copy number differences between CD38+ and CD38- fractions in 3 of 4 cases. In one case, this involved a loss of p53 locus in the CD38+ fraction, a marker that was not observed in the parallel CD38- fraction.

Another molecular basis for the association between CD38 expression and inferior clinical outcome emerged in the study of Pepper and colleagues [91]. They analysed sorted CD38+ and CD38- CLL cells derived from the same patient by

gene expression profiling. Their conclusion is that CD8+ fraction possess a distinct gene expression profile when compared with CD38- subclones. Subclonal populations, having identical IgHV rearrangements, derive from a single malignant transforming event and differences in gene expression between them cannot be explained by heterogeneous genetic background. Importantly, CD38+ CLL cells relatively over expressed vascular endothelial growth factor (VEGF). Elevated VEGF expression was associated with increased expression of the anti-apoptotic protein Mcl-1. Moreover, the expression of the CD38 antigen defines a sub-population of CLL cells with a distinct transcriptional profile that may be the cause or effect of an increase in cellular activation and reduced apoptosis.
More generally, within an overall apparently constant leukemic burden, the outgrowth of a subclone with additional genomic lesions and distinct gene expression profile might signal the start of a new phase of the disease.

In order to better understand the biological and molecular features predicting disease progression in CLL patient, we choose a subset of patients with favourable prognostic features and designed a two-phase study having the following aims:

- Phase I, a) to assess whether genetic lesions may be present in the minority of CD38+ cells in a series of untreated low risk patients as defined by CD38 negativity (CD38+ cells <7%) and favourable genetic findings
  b) to identify biologic factors associated to genetic lesions in the small CD38+ fraction of CD38- CLL patients and predicting for disease progression;
- Phase II, to validate our findings in an independent cohort of consecutive untreated CD38- CLL patients with favourable FISH findings.

Details are shown in Appendix I (paper Rigolin GM et al., Oncotarget 2013).

2.2.4 Methods and results

2.2.4.1 Patients

In this study 2 cohorts of patients have been considered. Cohort one (C1) included 28 untreated CLL patients seen between 2005 and 2006. Cohort two (C2) consisted of 71 consecutive untreated CLL patients diagnosed between 2007 and 2011. The principal characteristics of C1 and C2 are reported in Table 2.1.

TABLE 2.1. Principal clinical and biologic characteristics of the patients of the cohort 1 and cohort 2

	Cohort 1	Cohort 2
N of patients	28	71
M/F	16/12	49/22
Age mean yrs (range)	65 (50-91)	64 (38-86)
Stage (Binet) A/B/C	28/0/0	63/8/0
FISH neg/13q deletion	14/4	40/31
ZAP70 (>30%) neg/pos	22/5	61/10
IGHV mut/unmut	20/2	60/11
TP53 mut/unmut	0/18	0/69

2.2.4.2 FISH analysis on immunomagnetically sorted cells in patient C1

In the 28 patients in C1, CD38+ and CD38- CLL cells were isolated by immunomagnetic sorting. The purity of sorted fractions was >98% as determined by flow cytometric analysis. For CLL risk assessment, FISH was performed in all patient on peripheral blood samples obtained at diagnosis using probes for 13q14, 12q13, 11q22/ATM, 17p13/TP53. In C1 patients, FISH analysis was performed on both CD38+ and CD38- sorted cells, and the following region was investigated: 13q14, 12q13, 11q22/ATM, 17p13/TP53 and 14q32. Co-hybridization experiments were performed in order to evaluate the coexistence on the same cells of more genetic lesions.

TABLE 2.2. FISH results in CLL patients with detectable genetic lesions in CD38+ cells

	FISH results on CD38- cells (%)	FISH results on CD38+ cells (% of positive cells)						
case		del(13q)	del(11q)	Trisomy 12	14q32 rearr	del(17p)	Number of additional lesions in CD38+ cells	Cohybridizations
56	13q del (20%)	25	20	Neg	32	24	3	Different cells
58	13q del (30%)	28	30	Neg	28	18	3	Different cells
41	Neg	23	24	Neg	Neg	40	3	Different cells
50	13q del (60%)	37	21	15	Neg	33	3	ND
46	13q del (18%)	42	22	Neg	Neg	3820	2	ND
49	13q del (60%)	37	23	Neg	21	23	3	Different cells
61	Neg	33	26	Neg	22	Neg	3	Different cells
43	Neg	34	Neg	Neg	27	Neg	2	Same cells
56	Neg	19	Neg	Neg	49	21	2	Different cells
63	Neg	20 (69*)	Neg	Neg	Neg	Neg	2	ND
45	13q del (69%)	58	33	17	Neg	Neg	2	Different cells
54	Neg	62*	Neg	Neg	18	Neg	2	Same cells
60	Neg	18	Neg	Neg	Neg	Neg	1	NA
48	13q del (38%)	45	20	Neg	Neg	Neg	1	Different cells
57	13q del (30%)	37	Neg	19	Neg	Neg	1	ND
64	Neg	34	Neg	33	Neg	Neg	1	Same cells

*biallelic 13q deletion.

Result of FISH analysis in CD38- cells and CD38+ cells in C1 are reported in Table 2.2. In 16/28 patients, genetic aberrations were detected in CD38+ cells and

not in CD38- cells. In the remaining 12 patients no additional genetic lesions were found in the CD38+ population as compared to the CD38- cells.

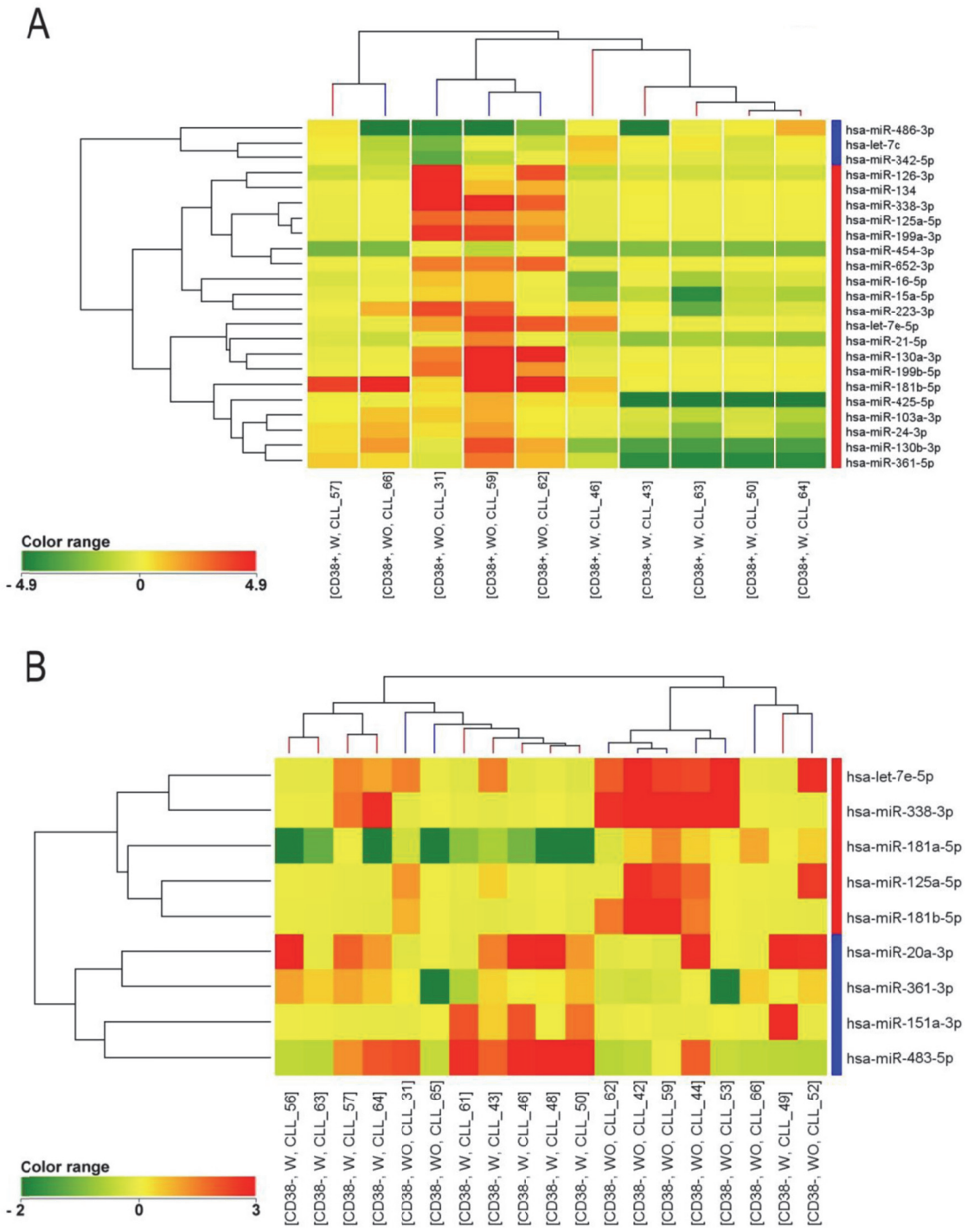


FIGURE 2.1. Cluster analysis of patients with (W) and without (WO) lesions in the CD38+ fraction. miRNA profiling of CD38+ (A) and CD38- (B) cells from CD38- CLL patients with (red) and without (Blue) FISH lesions in the CD38+ fraction. A distinctive miRNA profile characterized patients with and withput FISH lesions both in the CD38+ (23 microRNAs) and CD38- (9 microRNAs). The coors of the genes represented on the heatmap correspond to the expression values normalized on miRNA mean expression across all samples: green indicates down-regulated; red indicates up-regulated.

To assess whether the genetic lesions were on different clones, co-hybridization experiments using appropriate probes were performed in 11 cases with >1 aberration in CD38+ cells. In these experiments it was shown that genetic lesions involved different CD38+ cells in 8 cases and involved the same cells in remaining 3 cases.

2.2.4.3 miRNA profiling on immunomagnetically sorted cells in C1

MiRNA expression was investigated using Agilent Technologies platform. Samples were grouped according to the presence or not of genetic lesions by FISH analysis in the CD38 positive cells. So we evaluated the global miRNA expression profile of 19 patients by considering CD38+ and CD38- cell populations separately.

We found that at diagnosis most of the patients with genetic lesions in CD38+ (W) had a distinctive miRNA profile when compared to those without genetic lesions (WO), both in CD38+ (Fig. 2.1 A) and CD38- fraction (Fig. 2.1 B).

Twenty-three miRNAs were found to be differentially expressed in CD38+ population (corrected $p<0.05$) and nine miRNAs were found to be differentially expressed in CD38- population (corrected $p<0.05$). Four miRNAs were found to be down-regulated in patients with in contrast to those without genetic aberrations in CD38+ cells, both in the CD38+ and CD38- subpopulations: let-7e-5p, miR-125a-5p, miR-181b-5p and miR-338-3p. Interestingly, miR-125a-5p showed the higher degree of significance both in CD38+ and CD38- cell fractions and was therefore chosen for further clinical correlations. The down-regulation of miR-125a-5p was confirmed by RT-qPCR analysis, using Taqman MicroRNA assay specific for miR-125a-5p and normalized on 18S ribosomal RNA.

2.2.4.4 Clinical outcome

In C1, the presence of additional FISH abnormalities in the CD38+ cells correlated with a more aggressive course of the disease that was characterized by a shorter time to treatment (TTT) (HR 8.052, range 1.332-16.760, $p=0.0162$) (Fig. 2.2 A). Having shown that miR-125a-5p down regulation was strongly associated with additional FISH lesions on CD38+ cells and with shorter TTT, we investigated in an independent cohort of 71 consecutive untreated low-risk CLL the clinical relevance of miR-125a-5p expression.

For the purpose of this analysis patients were subdivided into 2 groups based on the 50th percentile of miR-125a-5p distribution (range 0.00276-6.57599 ΔΔCq, 50th percentile 0.85082 ΔΔCq). Patients with a lower miR-125a-5p expression were characterized by a more aggressive course of the disease and a shorter TTT both in univariate and in multivariate analysis (Fig. 2.2 B).

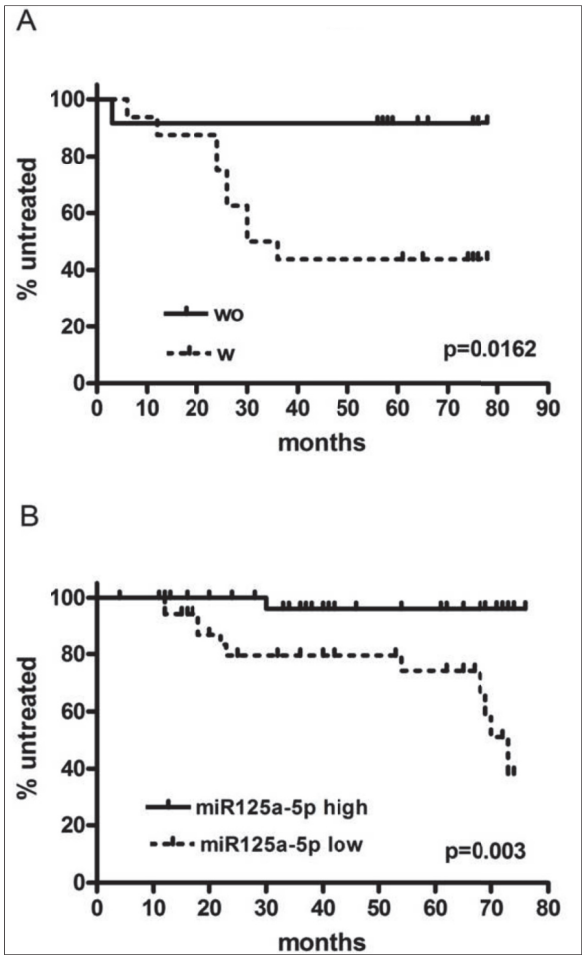


FIGURE 2.2. Time to first treatment in cohort 1 (A) and cohort 2 (B) respectively, according to the presence (W) or not (WO) of FISH lesions in CD38+ cells and to the level of expression of miR-125a-5p (low or high), respectively.

2.2.4.5 Mutational analysis by Next Generation Sequencing (NGS)

Because down-regulation of miR-125a-5p was associated with the presence of genetic lesions in a minor CD38+ fraction of total neoplastic cells in C1, we screened 20 consecutive patients in C2 using Next Generation Sequencing (NGS).

NGS analysis was performed using Ion Torrent PGM. Libraries of spot exonic regions of 20 genes (ATM, BIRC3, BRAF, CDKN2A, CTNNB1, DDX3X, FBXW7, KIT, KLHL6, KRAS, MAPK1, MYD88, NOTCH1, NRAS, PIK3CA, POT1, SF3B1, TP53, XPO1, ZMYM3) were constructed using Agilent Haloplex Target Enrichment kit starting from genomic DNA. Enriched libraries linked to Ion Sphere Particles were loaded in one Ion chip and sequenced using Ion Torrent PGM, according to manufacturer instructions. Seven out of 12 patients with low miR-125a-5p expression displayed mutations in the CLL population (Table 2.3) as compared with no patients out of 8 cases with high miR-125a-5p expression (7/12 vs 0/8, p=0.015).

TABLE 2.3. NGS mutations as assessed by Ion Torrent technology in 7 patients with low miR-125a-5p expression

Patient	Gene	Chr	Genomic position	Location	Ref sequence	Var sequence	Mutation type	Freq. %
1	DDX3X	chrX	41204747	exonic	GAAAGTAGTTTGGGTGGAAGA	GAAAGTAGTT----TGGAAGA	Frameshift deletion	39,1
2	SF3B1	chr2	198267491	exonic	C	G	SNV	52,7
3	MYD88	chr3	38182641	exonic	T	C	SNV	10,8
4	TP53	chr17	7577575	exonic	A	C	Stop codon	6,4
	TP53	chr17	7577580	exonic	T	C	SNV	18,9
5	XPO1	chr2	61719472	exonic	C	T	SNV	26,3
6	ATM	chr11	108138003	exonic	T	C	SNV	64,1
	FBXW7	chr4	153249384	exonic	C	T	SNV	32,9
7	TP53	chr17	7578536	exonic	T	C	SNV	28,6

SNV, non synonymous variation.

2.3 THE PATHOGENETIC ROLE OF "PROLIFERATION CENTERS" IN CLL: SITES WITH CD38+ CELLS AND A HIGH FREQUENCY OF GENETIC INSTABILITY

2.3.1 The role of microenvironment in CLL

Available evidence exists suggesting that the interaction of leukemic cells with stromal and T cells in the microenvironment has a key role in CLL pathogenesis and evolution. As there is increasing evidence that individual cancer samples are heterogeneous and include subclonal populations and that tumors likely evolve through competition and interaction between different sublclones, this tumoral microenvironment appears to be the site where acquisition of additional genetic lesions in the clone occur, which, should greatly influence clinical outcome [92]. In this hypothesis CLL could emerged as a chronic disease in which the host physiologically provides essential elements and conditions leading to the acquisition and accumulation of genetic alterations by leukemic cells. This scheme accommodates the existence of structures that provide replicating and surviving signals to B cells on their way to neoplastic transformation [5, 10]. A key element in this view is that leukemic cells can and do proliferate, with division taking place not in the blood, but primarily in specialized morphologically discrete structures of lymph nodes and bone marrow known as proliferation centers (PCs) [66, 84, 93, 94].

So important advances in the molecular pathogenesis were obtained through the study of the role of the microenvironment.

CLL can be defined as a low-grade CD5+ B-cell tumor, whose tumoral cells have previously encountered the antigen, escaped programmed cell death and undergone cell cycle arrest in the G0/G1 phase. In CLL cells, elevated levels of

the cyclin-negative regulator p27-Kip1 protein are found in a minority of patients [95]. Given the key role of this protein in cell cycle progression, its overexpression in CLL cells may account for the accumulation of B cells in early phases of the cell cycle [96]. In addition, overexpression of the anti-apoptotic BCL-2, BCL-XL, BAG-1 and MCL-1 molecules and the absence of microRNAs miR-15 and miR-16 [97], whereas proapoptotic proteins like BAX and BCL-XS are under expressed [98] could explain the resistance of tumoral cells to apoptosis. Despite the fact that most leukemic cells are arrested in cell cycle G0/G1 stages, Messmer et al. [83] demonstrated that CLL is not only a static disease but also a disease in which a proliferative pool coexist. In consequence, evolution of this leukemia depend on the relative balance between these subpopulations.

In contrast with in vivo results, apoptosis occurs after in vitro culture, suggesting a role of the microenvironment in CLL cell survival [99]. Within leukemic microenvironment, two cellular components appear to be potential players: stromal cells and T-lymphocytes. Direct contact between CLL and mesenchymal stromal cells (MSCs) is required for this inhibition of apoptosis, and tumor cell-stromal cell interactions are also important in controlling migration into and retention of CLL cells within tissue compartments (bone marrow or lymphoid tissue). MSCs are an important component of the bone marrow and support the maintenance of normal hematopoietic stem cells [100]. When co-coltured with CLL cells, MSCs have also been shown to protect the neoplastic B cells from apoptosis induced by fludarabine, bendamustine and steroids [101, 102]. Closely related to MSCs is a population of monocyte-derived “nurse-like” cells (NLC) that are also able to protect CLL cells from apoptosis [103, 104]. Both MSCs and NLCs therefore have anti-apoptotic activity and the combination of these two cell types provides a supportive microenvironment for tumor cells in CLL. In the other side, also T lymphocytes are attractive candidates to the role of elements that amplify microenvironment able to inhibit the malignant B-cell apoptosis and to favour disease progression. The weight of evidence points to a dialogue between CLL cells and CD4+ T cells, based upon bidirectional interactions that are regulated by adhesion molecules and chemokine and translate into the production of several cytokines by both cell types [99]. Many reports suggest that CD40/CD40L interactions are central to the dialogue between CLL cells and T cells. CD40 is a member of the tumor necrosis factor (TNF) receptor superfamily that is expressed by B cells, dendritic cells and monocytes [105]. CD40L is a member of the TNF

family expressed by activated T cells. The stimulation of CD40 rescues CLL cells from apoptosis and induces their proliferation [98, 106].

2.3.2 Proliferation centers

At this time, it is clear that crosstalk with accessory cells in specialized tissue microenvironments favours disease progression by promoting malignant B-cell growth and the emergence of new genetic alterations, which will lead to drug resistance. Therefore, understanding the crosstalk between malignant B-cells and their milieu could give us new keys on the cellular and molecular biology of CLL that can finally lead to novel strategies for disease treatment. In this regard, the isolation and analyses of the tumoral subset that is being triggered in the proliferative compartments of progressive CLL cases is an important aim to understand CLL pathogenesis.

The CLL proliferating compartment is represented by focal aggregates of proliferating prolymphocytes and para-immunoblasts that give rise to the so-called pseudofollicles or proliferation centers (PCs) [107]. Proliferation centres are present in approximately 90% of lymphocytic lymphomas. They are the histological CLL hallmark in lymph nodes, white pulp of the spleen and bone marrow where they appear as vaguely nodular areas never surrounded by a mantle zone. They consist of loosely arranged larger cells that often contain prominent nucleoli. In contrast to true B-cell follicle, which may be found entrapped within the small lymphocytic infiltrate in sections of B-CLL, proliferations centers are said not to contain follicular dendritic cells, although their presence has been occasionally recorded. Proliferation centers contain numerous T cells, most of which are CD4+. Notably, as compared to the non-proliferation center component of CLL, cells clustered in the proliferation centers have increased expression of the proliferation-associated markers Ki-67 and CD71, co-expression of survivin and BCL-2 and also higher expression of CD20, CD23 and MUM1/IRF-4 [81, 107]. Cells are surrounded by and interspersed with new vessels. It is still unclear whether these newly formed vessels are fully functional and bring nutrients to the proliferating cells or whether they represent an epiphenomenon of the angiogenetic factors that are produced by actively proliferating malignant B cells [108].

Although the size of PCs and the amount of para-immunoblasts in lymph node sections did not show a correlation with clinical course [109, 110], the presence of more extensive PCs in follow-up biopsies has been reported [111]. Interestingly, a recent study suggested that an association may exist between the expanded and highly active PCs and an aggressive variant of CLL [112].

2.3.3 Tissue microarray

Recently, techniques were developed that allow for the application of fluorescence in situ hybridization (FISH) on paraffine-embedded fixed tissues [113]. This technique is based on tissue microarray (TMA) [114]. It was first described by Kononen in 1998 [115], and represents a high-throughput technology for the assessment of histology-based laboratory test, including immunohistochemistry and FISH. Small cylindrical cores are extracted from standard formalin-fixed, paraffin-embedded tissue and arranged in a matrix configuration within a recipient paraffin block. The TMA has proven to be invaluable in the study of tumor biology, the development of diagnostic tests and the investigation of oncological biomarkers. The preparation of tissue microarray for FISH analyses made this technique a potentially ideal method for the screening of large number of cases in a single hybridization experiment [114].

As only few data are available on the cytogenetic profile of lymph nodes in CLL and the presence of chromosome lesions in correlation with the extension of PCs has never been previously tested, we designed a study aiming to:

- analyze the sensitivity and reproducibility of FISH on paraffin-embedded fixed tissues arranged on TMA from 183 consecutive lymph node biopsies corresponding to histologically ascertained CLL;
- estimate the frequency of chromosome lesions on lymph node samples
- analyze the possible correlation of specific chromosome lesions and the extension of PCs, and how these features may impact on clinical outcome. Details are shown in Appendix II (paper Ciccone M et al., Leukemia 2011).

2.3.4 Methods and result

2.3.4.1 Patients

A total of 183 consecutive patients with CLL diagnosed according to NCI criteria [4], submitted to excisional lymph node biopsy between 2002 and 2008 for diagnostic purposes, were included in this study. Lymph node biopsy in these patients was performed in the presence of progressive disease requiring treatment.

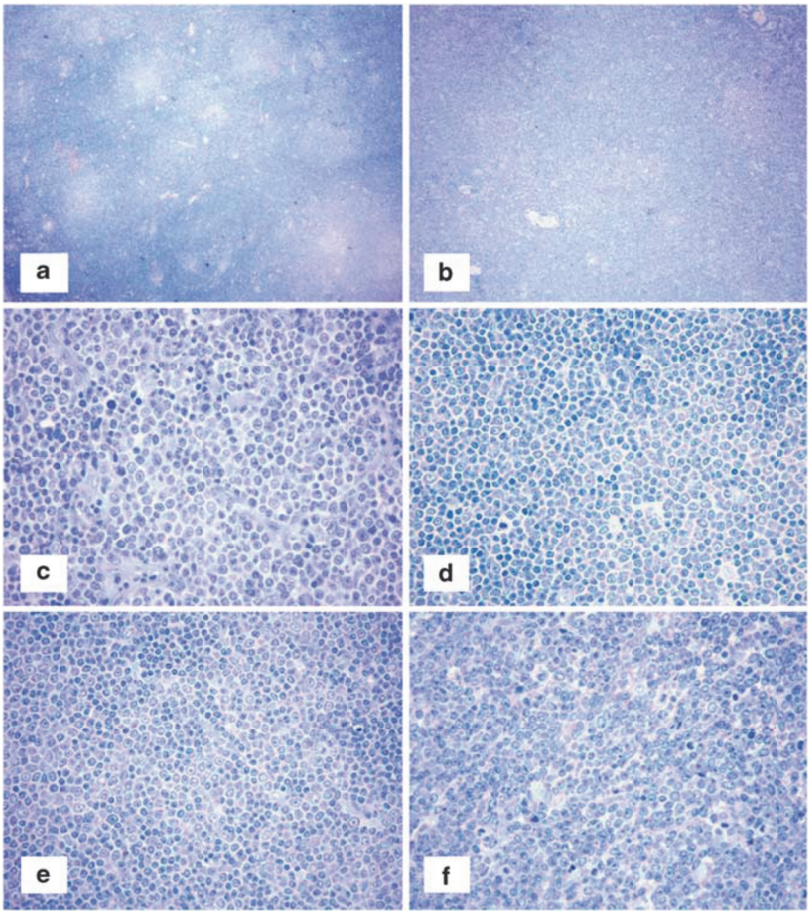


FIGURE 2.3. (a) Typical PC pattern (x40); (b) PC-rich pattern (x40); (c) cellular composition of a typical PC with a mixture of small lymphocytes, prolymphocytes and paraimmunoblasts (x400); (d) an example of PC-rich case with predominant prolymphocytes composition (x400); (e) in a case with PC.rich pattern, there is a mixture of prolymphocytes and paraimmunoblasts (x400); (f) in another case with similar pattern, paraimmunoblasts predominate (x400)

Each biopsy had been fixed in formalin. The cases were classified into two categories: “PCs-rich” and “typical”. The former included those cases with confluent PCs whereas the latter showed scattered, small, ill-defined PCs in a monotonous background of small, relatively round lymphocytes [110] (Fig. 2.3).

2.3.4.2 FISH studies: efficiency of hybridization

A slide was prepared from each paraffin block, and representative tumor regions including scattered or confluent PCs were morphologically identified and marked on every slide. The biopsies were arranged in seven TMAs, each containing material from 32-64 cases. Each TMA was submitted to hybridization using the following 5-probe panel in dual color hybridization tests using a chromosome 10 centromeric probe as internal control in each experiment: 11q22, 13q14.3, 17p13.1, chromosome 12 and 14q32/IGH.

Hybridization with each single probe was successful in 61.7-80.3% of the cases (Fig. 2.4).

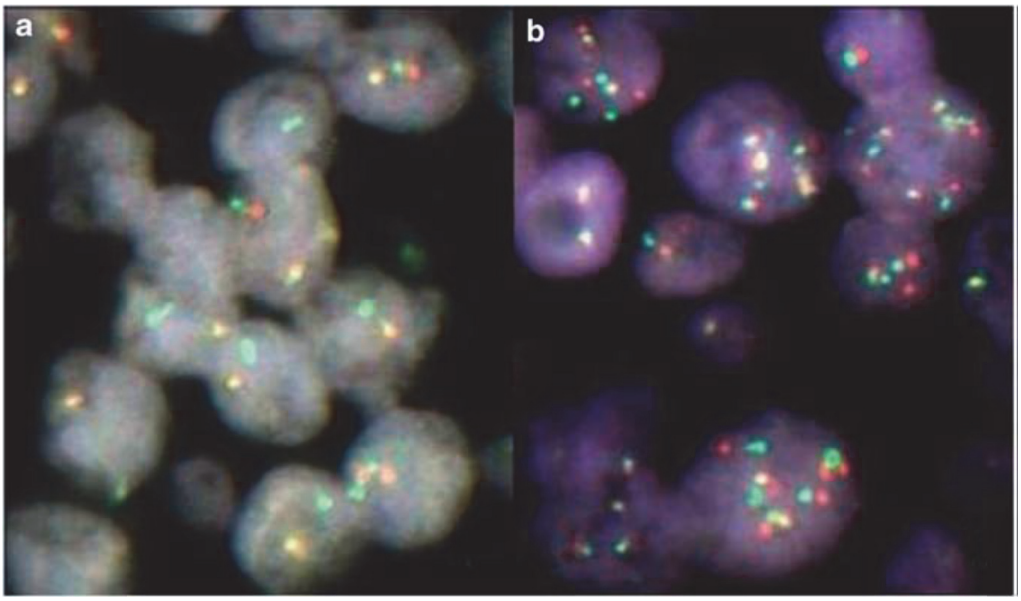


FIGURE 2.4. (a) The presence of one fusion signal (yellow) and of one green and one red signal (split signals), is indicative of 14q32/IgH translocation. (b) 14q32/IgH amplification documented by the presence of multiple nuclei with ≥3 fusion signals; the presence of red and green split signals indicates concomitant 14q32/IgH translocation.

Assessable data for the complete 5-probe panel were obtained in 101/183 cases (55.1%). In 58 cases (31.6%) assessable data were obtained with 1-4 probes, whereas no data were obtained in 24 cases (13.1%). Technical failures were accounted for by the absence of adequate number of cells on TMA corresponding cores in 12.5-26.2% of the cases; inefficient hybridization and overlapping nuclei precluded accurate signal screening in 7.1-13.2% of the cases.

2.3.4.3 Incidence of chromosome lesion in PCs

The incidence of each single aberration was as follows: 13q14 deletion 36.7% (54/147 cases), 14q32 translocations 30.8% (42/136), 11q deletion 24.7%

(28/113), trisomy 12 19.5% (26/133) and 17p deletion 15.6% (23/147). There was no significant difference in the incidence of 13q-, +12, 11q- and 14q32 translocations in untreated vs pretreated patients, whereas 17p- occurred more frequently in treated patients as compared with untreated ones (26% and 6.5% of the cases, respectively).

A total of 79 out of 101 patients successfully analysed with the 5-probe panel (78.2%) showed at least one chromosomal aberration. Using a cytogenetic classification assigning every patient to a single category according to hierarchy 17p- >11q- >14q32 >+12 >13q-, the 11q deletion was the most frequent cytogenetic anomaly accounting for 20% of the cases, followed by 17p- (17%), 14q32/IGH translocation (16%), 13q- (15.8%) an trisomy 12 (11%).

2.3.4.4 Histopathologic subtypes and their association with biologic features

A total of 108/183 cases (59.1%) with scattered PCs were assigned to the typical subtype. A total of 75/183 cases (40.9%) were assigned to the PCs-rich subtype, owing to the presence of confluent PCs. 17p-, + 12 and 14q32/IGH translocation were more frequently encountered in the PCs-rich subtype than in the typical subtype (20/60 cases vs 3/87 for 17p-, p>0.001; 15/52 vs 11/81 for +12, p=0.030; and 22/54 vs 20/82 for 14q32 translocations, p=0.043).

The sections were tested with anti-ZAP70 and anti-Ki-67. Ki-67 was positive in >30% of the cells in 27/164 cases successfully tested: 4/95 Ki-67+ cases belonged to the “typical” subtype as compared with 23/69 Ki-67+ CLL in the PCs-rich group (p<0.0001). ZAP70 was positive in 91/149 (61,1%) and there was no statistically significant difference between cases classified as typical CLL and PCs-rich CLL.

A thorough analysis of existing correlations between histological features and clinico-biologic parameters was performed in 101 patients with successful hybridization with the complete 5-probe panel (Table 2.4).

IGHV usage in correlation with mutational status in 67 cases is shown in Figure 3. Rearranged IGHV families recurrently involved were as follows: IGHV1-69: 19.4%; IGHV3-30: 11.9%; IGHV3-7: 7.4%; IGHV3-23, IGHV3-48, IGHV4-34 and IGHV4-39: 6% each. Others IGHV families were involved in 45% of the cases. As shown in Table 2.4, 17p-, 14q32/IgH translocations, +12 and ‘unfavourable’ FISH abnormalities (that is, 11q- and 17p-) were more frequently encountered in the PCs-rich subtype than in the typical subtype.

TABLE 2.4. Clinical features and demographics of the 101 patients with cytogenetic data obtained with 5-probe panel.

	Typical (no. of cases)	PCs-rich (no. of cases)	P value
Age (mean	63.9 (sd 10.4)	65.0 (sd 12.3)	NS
Sex			
F	24	17	NS
M	40	20	
Stage at time of biopsy			
0-2	27	15	NS
3-4	25	18	
17p-			
No	62	22	<0.001
Yes	2	15	
11q-			
No	49	26	NS
Yes	15	11	
14q32/IgH translocations			
No	50	18	0.002
Yes	14	19	
Trisomy 12			
No	53	25	0.021
Yes	8	12	
13q-			
No	35	27	NS
Yes	29	10	
Unfavourable FISH (11q- and/or 17p-)			
No	48	16	0.001
Yes	16	21	
ZAP-70+ (n=84)			
No	33	17	NS
Yes	21	13	
Ki67 (n=92)			
>30%	4	12	0.001
<30%	53	23	
IgHV (n=67)			
Unmutated	28	19	NS
Mutated	14	6	
Time between diagnosis and biopsy (mean, months)	21.2 (95% CI 0.0-83.0)	32.4 (95% CI 0.0-79.0)	0.006
Pretreated at time of biopsy (n=86)			
Yes	13	20	0.002
No	39	14	

CI, confidential interval; FISH, fluorescence in situ hybridization; NS not significant.

Likewise, Ki-67 positivity in >30% of cells, being pretreated at time of biopsy and longer interval between diagnosis and biopsy, were associated with the PCs-rich subtype, whereas no significant association was noted between ZAP-70+, IGHV mutational status and histological subtypes.

TABLE 2.5. Analysis of factors affecting survival from the time of biopsy.

	No. of patients	Median overall survival (se)	P value
Histology			
Typical	52	64 (1.6)	0.00001
PC-rich	34	11 (2.7)	
Ki67			
>30%	14	4 (0.3)	0.0002
<30%	64	64 (5.6)	
ZAP-70			
No	43	47 (4.5)	NS
Yes	27	64 (16.4)	
Stage at biopsy (n=57)			
0-2	42	64 (1.9)	NS
3-4	43	34 (8.5)	
FISH			
Favourable intermediate	50	64 (1.9)	0.046
Unfavourable (11q- and/or 17p-)	35	24 (3.7	
IgHV			
Unmutated	41	42 (3.3)	NS
Mutated	16	36 (5.1)	
Pretreated at time of biopsy			
Yes	33	18 (2.8)	0.0073
No	53	64 (1.9)	

FISH, fluorescence in situ hybridization; NS not significant.

As shown in Table 2.5, the median survival from the time of tissue biopsy for PCs-rich and typical subtypes in 86 patients for whom clinical data could be obtained was 11 and 64 months, respectively (p=0.00001) (Fig. 2.5). Other variables that influenced negatively the survival in our series were unfavourable cytogenetic abnormalities (17p- and/or 11q-), Ki-67 >30% and being pretreated at the time of biopsy.

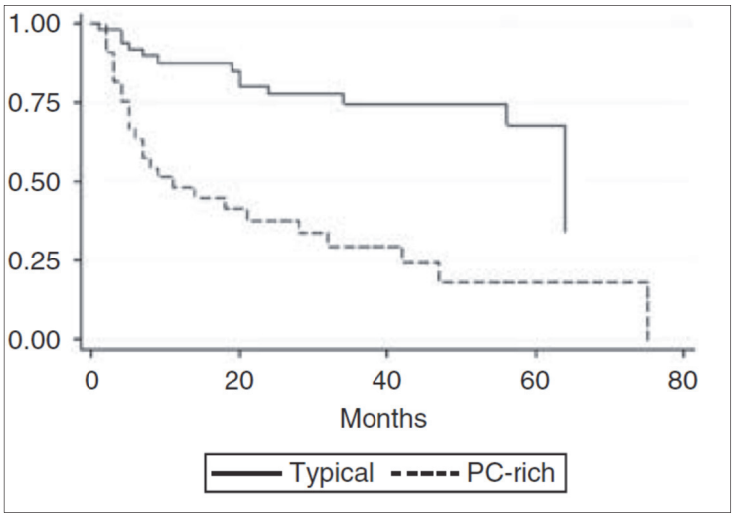


FIGURE 2.5. Survival in patients according to the histological pattern. Confluent PCs (PC-rich) identify patients with inferior outcome.

2.4 DISCUSSION

TOWARDS A BETTER UNDERSTANDING OF THE PATHOGENESIS OF CLL: CELL ACTIVATION AND GENETIC INSTABILITY

In CLL patients, there is evidence that a complex subclonal architecture of the leukemic clone may correlate with a more aggressive course of the disease and that in most cases genomic abnormalities are recurrent non-random events that expand over time due to a Darwinian selective pressure [89, 116]. Clinically, the existence of multiple, genetically distinct, subpopulations that escape therapeutic intervention presents formidable challenges for the development of effective treatment for patients with relapsed or refractory CLL.

CD38 is a marker of unfavourable prognosis and an indicator of cell activation and proliferation that may prelude clonal evolution and worse clinical outcome [117]. Several reports suggest that patients with 7-30% CD38-positive leukemic cells exhibit a more aggressive disease course and have an inferior response to treatment. Grubor et al. [89] found clear evidence of intraclonal heterogeneity within patients, searching for genomic differences between CD38+ and CD38- populations in the same patient. Peppers et al. [91] demonstrated that CD38+ CLL cells possess a distinct gene expression profile when compared with CD38- subclones derived from the same patient. Because clonal members can be heterogeneous in expression of genes and markers related to cellular activation and adhesion, in particular CD38, this intraclonal heterogeneity may translate in differences that determine which cells enter the cell cycle. This finding suggest that the expression of the CD38 antigen defines a subpopulation of CLL cells with a distinct transcriptional profile that may be the cause or effect of an increase in cellular activation.

We therefore studied the biological and clinical significance of the presence of genetic heterogeneity in the minor CD38+ leukemic population, in a cohort of untreated low-risk CLL patients as define by CD38 negativity (CD38+ cells < 7%) and low-risk FISH findings (normal or 13q deletion as sole abnormality). Our data showed that a significant proportion of CD38- CLL patients with low risk FISH findings presented genetic aberrations within CD38+ cells. Most of these abnormalities were high risk lesions (11q deletion in 9 cases, 17p deletion in 8 cases) and in most of the cases these lesions were found in different cells indicating that multiple cytogenetically unrelated minor clones may be present in

the CD38+ cell fraction. The small size of the abnormal C38+ clones precluded the detection of these aberrations when analyzing by FISH the entire neoplastic population consisting of a majority of CD38- cells. Interestingly, the presence of these additional FISH lesions in the small CD38+ cell fraction was associated with shorter TTT. Genetic complexity and heterogeneity in the architecture of the leukemic clone was previously documented in CLL and was associated with disease progression and shorter TTT [118].

To identify biomarkers associated with this phenomenon, we performed miRNA expression analysis because deregulation of miRNA was previously shown to be associated with activation markers [119]. By comparing C1 patients with and without small abnormal clones in the CD38+ fraction, we were thus able to show a deregulated miRNA expression profile in CLL cases with additional FISH lesions in CD38+ cells. In particular, miR-125a-5p was found to be down-regulated both in CD38+ and CD38- cells in patients with FISH abnormal clones as compared to patients without FISH abnormal clones. MiR-125a-5p was therefore chosen as a marker associated with genetic complexity and possibly with a more aggressive clinical behaviour as suggested by the shorter TTT that we observed in these patients. The relevance of miR-125a-5p as a biomarker of inferior outcome and genetic complexity was then validated in a prospective cohort of 71 consecutive untreated CD38- CLL patients with normal FISH or 13q deletion as single abnormality. In this validation cohort, we were able to confirm the predictive role of miR-125a-5p down-regulation in terms of shorter TTT. To our knowledge, this is the first observation linking a deregulated miRNA expression to inferior outcome in a subset of low risk CLL patients. This finding is valuable because the majority of CLL patients present with low risk features at diagnosis and disease progression is difficult to predict in such cases. In addition, in this validation cohort we found, through the use of NGS technology, that CLL patients with lower levels of miR-125a-5p displayed an increased rate of mutations in CLL-related genes. Interestingly most of the mutations found in our patients, including TP53, SF3B1 and ATM, have been associated with a worse clinical outcome and prognosis. This observation further strengthens the association between genetic complexity, miR-125-5p down-regulation and worse outcome. Alterations in miRNA expression are involved in the initiation, progression, and metastasis of several human cancers, by acting both as tumor suppressors and oncogenes in cancer development [120]. In particular, miR-125a-5p was previously found to act as a non-organ specific

tumor suppressor gene that, when down-regulated, is associated, in several solid cancers, with a more aggressive course of the disease and a worse prognosis [121-123]. A germline mutation in mature miR-125a-5p has also been closely associated with breast cancer tumorigenesis [124]. This miRNA primarily achieves its antiproliferative effect through down-regulation of proliferation related genes, involved in the phosphoinositide-3 kinase (PI3K)- AKT and RAS/RAF/mitogen-activated protein kinase signalling [121]. Noteworthy, it was recently shown that in SMZL, an indolent B cell lymphoproliferative disorder like CLL, there is a characteristic deregulation of miRNA expression including down-regulation of miR-125a-5p with a possible implication in its molecular tumorigenesis [125]. By contrast, miR-125a was also found commonly gained and/or overexpressed in DLBCL [126] and in this perspective it would be interesting to look at miR-125a-5p levels in CLL patients that has transformed into Richter's syndrome.

To better understand the pathogenic role of membrane maker CD38, we put our attention on this molecule that supports B cell interactions and differentiation. Several recent studies have shown that CD38 expression is higher in CLL cells in the bone marrow and lymphoid tissues when compared to peripheral blood [93]. This implies that once CLL cells leave these compartments, they begin to lose CD38 expression and by inference, the higher the degree of renewal/turnover from bone marrow and lymph nodes [91]. Indeed CLL is a disease in which the host's microenvironment promotes leukemic cell growth, leading to sequential acquisition and accumulation of genetic alterations [127]. In order to clarify this idea, an increasing body of data suggests that proliferative centers play an important role in the biology of CLL as they constitute its proliferative compartment [66, 128]. However the complexity of the microenvironment of proliferation centers has not been completely elucidated [128]. To better define the significance of proliferation centers (PCs), the morphological hallmark of chronic lymphocytic leukemia (CLL), we studied lymph node biopsies taken from 183 patients by fluorescence in situ hybridization (FISH) studies using a 5-probe panel on tissue microarrays (TMAs). As tissue biopsy is not mandatory for the diagnosis of CLL, few studies addressed the significance of histological findings and the incidence of chromosomal aberrations at the tissue level in this setting. The TMA technology offers the possibility to analyze simultaneously a consistent number of cases thus reducing costs and experimental variability. The patient population was not representative of the entire CLL population seen at the referring centers during the study period,

because we included patients with progressive disease and clinically relevant adenopathy, and FISH analysis was performed in biopsies taken in different phases of the history of the disease. In line with these considerations, the IGHV usage in our cases showed frequent V1-69 gene involvement, mostly in the unmutated configuration, a relatively low VH4-34, frequently found in mutated CLL [129], and very low incidence of other VH subtypes normally associated with indolent CLL or monoclonal B-cell lymphocytosis [129]. An unexpectedly high 12% incidence of V3-30 involvement was seen in our series. Unlike previous reports, describing V3-30 usage in association with mutated CLL running an indolent course [130, 131], 7/8 cases using V3-30 genes in this series lacked IGHV somatic mutations, suggesting that the mutational status might be an important determinant of outcome within specific IGHV families. The 78.2% overall incidence of chromosome lesions in our study using a 5-probe panel is in line with previous analyses performed on PB samples obtained from untreated and pretreated patients [15]. Caraway et al. [132] found abnormal FISH signal patterns in 81% of CLL cases on fine-needle aspirates, and Flanagan et al. [133] described at least one cytogenetic aberration in all 18 patients analyzed on lymph node biopsy. Interestingly, while confirming that the occurrence of 17p- was more common in relapsed CLL [134], other aberrations were found in our series to occur at a similar incidence in untreated and in treated patients. 11q deletion is usually associated with a disease characterized by marked lymphadenopathy [135]. As the incidence of 11q- may be higher in patients with therapy-demanding disease independent by previous treatments [60], the relatively high incidence of 11q- in our series (24.7%) may be accounted for by inclusion in this study of patients with clinically significant adenopathy. A previously unreported finding was represented by a high incidence (30.8%) of 14q32/IgH translocations both in treated and in untreated patients. Three possible explanations may account for this observation: i) 14q32 translocation may appear more frequently at the tissue level than in the PB, as previously suggested for trisomy 12 [136]; ii) 14q32 translocation may be associated with adenopathy and active disease requiring treatment, as suggested in two recent analyses [137, 138]; and iii) 14q32 translocation may represent in some of our cases a secondary anomaly acquired late during the course of the disease. Indeed, karyotype instability was detected in a fraction of CLL [139], and 14q32/ IgH translocation may represent a late event in the progression of lymphoid neoplasias. Interestingly, recent analyses on a limited number of CLL patients

reported a 17–21% incidence for these translocations in lymph node samples [133], and 18 independent lymph node biopsies submitted to FISH analysis on isolated cell suspension in this study gave a 33% incidence for this aberration.
The frequency of PCs-rich CLL in our series was 40.9%, and 14q32/IgH translocations, þ12 and 17p-, were significantly associated with the PCs-rich group. Unfavorable cytogenetic features, such as 11q-/17p-, were more frequently encountered in the PCs-rich group than in the typical group. The presence of prominent PCs, which was associated with ki-67þ in our analysis, is thought to be an index of B-cell proliferation capability, which increases the risk that DNA replication errors could happen, thus predisposing the cell to the acquisition of sequential genetic damage. Likewise, ATM and p53 gene deletions impair the checkpoint cell machinery and render the cell more fragile to antigenic stimuli encountered at PCs level. These considerations may provide a biological basis to explain the strong unfavourable prognostic significance of confluent PC in CLL that was found in our study. Interestingly, Gine et al. [112] recently described a new histological category, that is, “accelerated” CLL, including 23 out of 78 (29.4%) cases with enlarged and confluent PCs, with 42.4 mitosis per PCs and 430% Ki-67 per PCs. This histological subset of CLL was associated with adverse biological features and inferior survival. Our data support and reinforce this view, in that they show that confluent PCs are associated with adverse cytogenetics and represent the strongest prognostic factor.

In conclusion, in order to clarify the pathogenetic process in CLL, we focus our attention on genetic heterogeneity reflected by membrane marker CD38. In our first mentioned study (i) by FISH analysis we disclosed genetic lesions in the minor of CD38+ cell fraction in CD38- CLL with low-risk FISH findings, (ii) we provided evidence supporting an association between cryptic genetic lesions in CD38+ cells and disease progression, (iii) we found that genomic complexity and worse outcome were associated with miR-125a-5p down-regulation and, iv) we validated this finding in an independent cohort of untreated CD38- CLL patients with low risk FISH findings. This set of data show that genetic lesions may appear in CD38+ cells in low-risk CLL and that they may be associated with miR-125a-5p down-regulation, allowing for a more accurate prognostication in low-risk CLL. Aiming to the hypothesis that genetic instability origins into CLL microenvironment, we focused on lymph nodes, and in particular on proliferation centers, correlating

PCs pattern with clinico-biologic and cytogenetic characteristic. The association of PCs-rich pattern with unfavourable cytogenetics and short survival may support the pathogenetic role of proliferation center in CLL. As the patients included in this analysis represented an unselected cohort of CLL undergoing biopsy due to clinically significant adenopathy, we found a high incidence of adverse biologic features, that is, unmutated IGHV configuration, ZAP70, and high-risk cytogenetics. Our study demonstrated that the histopathological pattern defined by the presence of confluent PCs may represent an important feature for risk assessment in this subset of patients. This finding is important when considering that increasing awareness of the possible evolution into Richter's syndrome is prompting clinicians to perform lymph node biopsy more often than in the past and that the therapeutic armamentarium in clinically aggressive CLL may include intensive chemoimmunotherapy and allogenic bone marrow transplantation from HLA-identical siblings and well-matched unrelated donors.

CHAPTER 3

TRANSLATING PATHOGENESIS INTO CLINICAL PRACTICE

3.1 INTRODUCTION

One of the clinical hallmarks of chronic lymphocytic leukemia (CLL) is the high degree of variability in its disease course. Whereas some patients experience an indolent disease course, others succumb to the disease rapidly despite intensive treatment. Over time, most patients develop refractory disease that does not respond to chemotherapy [4, 96]. Although the two major staging systems have provided valuable information in addressing this clinical heterogeneity, they have been unable to predict an indolent or aggressive course within the intermediate risk category [7, 8]. Several clinical and biological prognostic factors have been identified, such as specific cytogenetic alterations [140], mutational status of immunoglobulin (Ig) genes and the expression level of CD38 [67] and ZAP70 [141]. The implication of these biological predictor factors with the molecular pathogenesis of the CLL is still under investigation, and an eventual therapeutic application is not yet developed. The recognition of novel molecular variables identified through the use of high-throughput molecular analytical techniques could contribute to a better knowledge of the disease pathogenesis, the development of more accurate biological predictive factors, the adjustment of therapies to the specific risk, and the identification of new therapeutic targets in CLL.

CLL turned out to be a disease with multiple facets in its pathogenic mechanisms including genetic aberrations, antigen drive and microenvironmental interactions. In the first part of this work, we focused our attention on the role of microenvironment in CLL pathogenesis and, in particular, on the modality by which the cell membrane marker CD38 reflects genetic instability and heterogeneity. At this point we aimed to translate this knowledge in clinical practice focusing i) on the correlation between clonal evolution and development of chromosomal aberrations and ii) on how to stratify patients at diagnosis according to cytogenetic abnormalities and gene mutations.

3.2 CYTOGENETIC ABNORMALITIES IN CLL

The presence of cytogenetic abnormalities is a hallmark of CLL. Indeed, several studies have shown that the type and number of chromosomal aberrations are an independent predictor of prognosis in CLL, and cytogenetic analysis is routinely performed in this disease. No single genetic abnormality responsible for CLL development has been identified. Instead, the disease is characterized by a variety of chromosomal abnormalities. The most common recurrent chromosomal abnormalities include deletion 13q, trisomy 12 and deletions 11q, 17p and 6q [15]. Five prognostic categories have been identified in a hierarchical model, showing poor survival in patients with 17p deletion and 11q (median survival 32 and 79 months, respectively) but better survival for patients with trisomy 12, normal karyotype, and deletion 13q as sole abnormality (114, 11 and 133, respectively) [15] (Fig. 3.1). The current International Workshop on Chronic Lymphocytic Leukemia guidelines consider assessment of genomic aberrations by FISH mandatory in clinical trials and desirable in general practice as a pre-treatment evaluation [4].

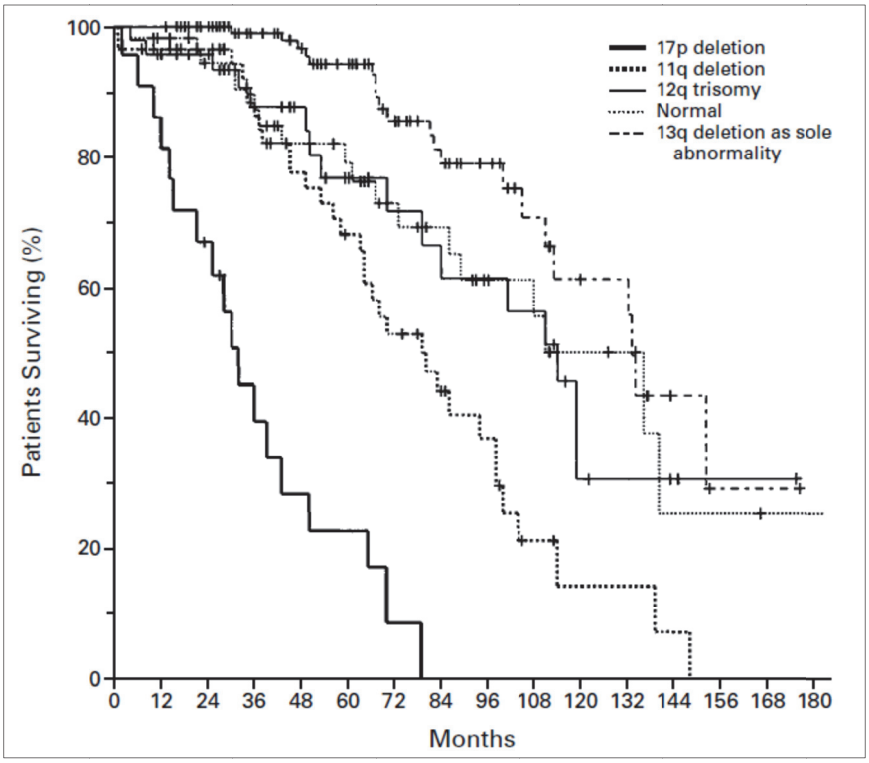


FIGURE 3.1. Probability of survival from the date of diagnosis among the patients in the five genetic categories.

13q-. Loss of 13q14 is the most common chromosome aberrations in CLL, with a prevalence of 40-60% [15, 142, 143]. In contrast with other recurrent

aberrations, 13q14 deletions may be heterozygous (monoallelic in 76% of cases) or homozygous (biallelic in 24% of cases). Studies of serial samples suggest that heterozygous deletion is an early event, whereas deletion of the second copy of this region occurs at later stage [144, 145].

Patients with losses on 13q14 as the only aberration, based on FISH data, show a favourable prognosis; however, recent studies indicate that the situation may be more complex, suggesting that the percentage of interphase nuclei with deletions, as well as the size of the deletion, could influence the outcome [15].

Various groups have attempted to identify a tumor suppressor gene in 13q. No inactivation of candidate genes by mutation has been demonstrated, but a complex epigenetic regulatory tumor suppressor mechanism that controls the expression of the whole region has been described [146]. In a crucial study, Calin and co-workers showed that 13q14 deletion in CLL is associated with down-regulation of miR-15a and miR-16-1, whose genes cluster in the minimally deleted region (MDR) within 13q14 [147]. The miR-15a/16-1 cluster was deleted or down-regulated in the majority of CLL cases (68%) and seems to negatively regulate the expression of BCL2 and several other genes involved in proliferation and apoptosis [97, 148, 149]. Subsequent murine studies in which the specific miR-15a/16-1 cluster was deleted have led to the development of monoclonal B cell lymphocytosis and CLL with low penetrance [149].

Trisomy 12. Trisomy 12 is among the most frequent aberrations in CLL, occurring in 10-20% of cases. It has long been associated with early progression [15]. Initial FISH studies have suggested that the outcome was intermediate for this group. Recent analysis of prospective trials suggests that although progression-free survival (PFS) may be shorter, OS is favourable [49]. Cases with trisomy 12 rarely show TP53 mutations and rarely acquire these over time [150]. Trisomy 12 has been associated with an atypical morphology or immunophenotype [151]. A minimal common gained region has been confined to 12q13, small duplications of 12q have been reported and MDM2 gene, which is located at 12q15, is amplified in CLL [152]. Moreover CLLU1, which is located at 12q22, has been proposed as a prognostic marker in patients <70 years of age, since its higher level of expression has been associated with shorter OS [153], although over-expression of CLLU1 occurs irrespective of trisomy 12 [154]. The critical genes involved in this aberration, therefore, remain unknown.

11q-. Approximately one-fifth of patients with treatment indications exhibit 11q deletions, and ATM mutations have been reported in 30% of patients with del(11q). Patients with an 11q deletion are generally younger, have more B-symptoms and a more rapid progression of the disease, and shorter OS [135]. Furthermore, the aberration is typically associated with extensive lymphoadenopathy [4]; however, it has been recently reported that de novo 11q deleted CLLs can exhibits variable clinical outcome [155]. There is a very strong association between the presence of the deletion 11q and unmutated IgHV mutation status [25]. The biological basis for this association is currently unclear.
The minimal consensus region in bands 11q22.3-q23.1 harbors the ATM gene in almost all cases. The ATM protein kinase is a central component of the DNA damage pathway and mediates cellular responses to DNA double-strand breaks [156]. ATM activates cell cycle checkpoints, can induce apoptosis in response to DNA breaks and functions directly in the repair of DNA double-strand breaks.

17p-. Del(17p) has been described in 3-8% of patients who are naïve to treatment, although higher occurrences of up to 30% have been reported in patients with advanced, relapsed disease [15, 157]. About 80-90% of cases with deletion of one copy of the TP53 locus will have mutation on the remaining copy [158]. Very few cases with 17p deletion will have a functional p53 pathway. This aberration is usually associated with a very aggressive clinical course and is predictive of lower PFS, lack of response to therapy, short response duration and short OS [15, 159].
The deletion always contains the TP53 locus but usually covers most of the short arm of chromosome 17, which has prompted a consideration of other genes that might be targeted by this deletion. The tumor suppressor p53 plays an essential role in inducing apoptosis or cell cycle arrest after DNA damage. Therapy with fludarabine and alkylating agents is based on a p53-dependent mechanism, which could explain why CLL patients with del17 or inactivating mutations of TP53 are refractory to such chemotherapy [143]; however, fludarabine refractoriness is caused by TP53 disruption in approximately 40% of CLL patients who did not respond to treatment, but, in a sizeable fraction, the molecular basis of this aggressive clinical phenotype still remains unknown [158, 160, 161]. Current treatment approaches take advantage of the growing number of drugs with documented effectiveness independent of functional p53. A more specific or

targeted option for this subgroup of patients is the identification of drugs targeting mutant p53, which could be effective in this CLL subgroup.

6q-. Deletion of the long arm of chromosome 6 is a rare cytogenetic abnormality seen in approximately 6% of CLL patients [15, 162, 163]. It is generally considered an intermediate-risk feature, since it is associated with more prominent lymphocytosis with atypical morphology, splenomegaly, higher rates of CD38 positivity, and no association with IGHV mutation status [162].

Translocations. Recurrent balanced translocations are rare in CLL, in contrast to other types of leukemia or B cell lymphomas in which specific and recurrent translocations deregulate known oncogenes [15, 164, 165]. Translocations involving immunoglobulin genes are the most common recurrent translocations in CLL, although they have only been observed in <5% of cases. Recurrent partners include BCL2, BCL3, BCL11A and c-MYC, although unknown partner genes are also frequent [137]. Chromosomal translocations generally have a negative effect on response to therapy and survival, especially when unbalanced. Unbalanced non-reciprocal aberrations are frequent and often seen in complex karyotypes.

Complex karyotype. Although CLL is characterized by relatively stable genome and most CLL cases appear to carry few genomic aberrations, a high number of CNAs (>3 per patient), termed genomic complexity, is detected in a proportion of CLL patients (~20%). Genomic complexity demarcates a CLL subset with progressive and aggressive disease, short survival, and decreased therapeutic efficacy [166, 167].

3.2.1 Clonal evolution in CLL

In 1985, Nowell et al. stated that karyotypic evolution is rare in B-CLL [168]. Its occurrence indicates a poor prognosis, but its rarity suggests that clinical progression in this disease is usually more dependent on other factors. Despite these claims, clonal evolution (CE) was more recently reported in 15-42% of CLLs using conventional karyotyping or fluorescence in situ hybridization [139, 145, 169]. The incidence of this phenomenon is variable, depending on the length of follow up [145] and on the number of probes used for FISH analyses [170]. CE was defined by the late appearance of aberrations of chromosome 17p, 11q, 6q and 12. Shanafelt et al. reported that 27% of CLL patients developed new

cytogenetic abnormalities at chromosome sites of known prognostic importance during the course of their disease [145]. Patients with high ZAP-70 expression seem to be more likely to experience such clonal evolution and to acquire unfavourable cytogenetic abnormalities. In another study, despite the limited case number, clonal evolution was identified as an independent prognostic factor for overall survival and it only occurred in CLL with unmutated IGHV genes indicating to karyotypic instability as a pathomechanism [139]. Basically, CE is associated with markers of active disease, i.e. ZAP70 positivity and unmutated IGHV gene.
Besides the classical CLL-associated aberrations [15, 165], other recurring changes have been described in CLL [171], and attention was recently devoted to 14q32 translocations involving the immunoglobulin heavy chain gene (IGH). This aberrations was found in 6-19% of patients with CLL at diagnosis [165], and was associated with advanced stage disease, unmutated IGHV genes, CD38 positivity and need therapy [137, 138].

The incidence of this aberration at clonal evolution was unknown. To better define the incidence and the significance 14q32/IGH translocations occurring at CE, we performed this study including 105 cases of CLL analysed sequentially over a 10-year period with panel of probes including an IGH break-apart probe.
Details are shown in Appendix III (paper Cavazzini F et al, Leukemia & Lymphoma 2012).

3.2.2 Methods and results

3.2.2.1 Patients

105 cases of CLL, seen at our institution between 1995 and 2004, were included in this study, after making sure that fulfilled the following criteria:

- Diagnosis of CLL based on morphology and immunophenotyping (score Matutes ≥3 [172])
- Successful FISH analysis at diagnosis and during follow-up
- Clinical records available for review.

Patients were submitted to FISH analyses as part of routine diagnostic work-up. FISH was repeated on at least one occasion in all 105 patients. Sequential samples were obtained before each line of treatment and 4-6 years intervals in those not requiring treatment.

Diagnosis was based on the presence of persistent lymphocytosis (>5.000/µl), on examination of peripheral blood (PB) smear and on the results of immunophenotyping. The following markers were tested in all cases by cytofluorimetric analysis, using 30% cut-off for positivity in the lymphocyte gate: CD5, CD19, CD23, CD22, CD10 antigens; the FMC7 monoclonal antibody and the expression of surface immunoglobulins were also tested. The co-expression of CD38 and CD19 antigens was tested with a 30% cut-off for positivity. As a rule, trephine biopsy was performed in young patients (<60 years of age). Histological studies were performed for diagnostic purposes in selected cases. Patients were treated according to guidelines in use at our institutions during the study period. Fludarabine containing regimens were used as front-line treatment in young patients and in refractory or relapsing patients. Intermittent chlorambucil administration was used as first-line therapy in the majority of elderly patients (<70 years).

3.2.2.2 Clonal evolution

Sequential FISH studies were performed on peripheral blood (PB) samples using commercially available probes for the identification of deletions at 13q14/D13S25, 11q23/ATM, 17p13/TP53, trisomy 12 and translocations at 14q32/IGH. Sensitivity limit for the detection of 14q32 translocation, trisomy 12 and deletions were >3%, >3% and >8% interphase cells with split signal, three signals and one signal, respectively. The following probes were used in patients with 14q32/IGH translocation to identify the partner chromosome: 11q14/BCL1, 18q21/BCL2, 3q27/BCL6, 18q21/MALT1 and 8q24/MYC. Non-commercial bacterial artificial clones (BACs) were also used for 2p12/BCL11A, 6p21/CCND3, 19q13/BCL3, 7q21/CDK6 and for the evaluation of the region 5p15.31-33.

Those patients with 11q- and/or 17p- were considered as “high cytogenetic risk” and the remaining patients with 13q-, +12 or 14q32/IGH translocations were considered as “standard cytogenetic risk”.

The median interval between diagnosis and first FISH analysis was 2 months (range 1–12 months). CE was observed in 15/105 patients after 24–170 months, median 64, as detailed in Table 3.1. Recurring aberrations at clonal evolution were 14q32/IGH translocation in seven patients; 17p- in four patients, 11q- in two patients, biallelic 13q- in four cases, hemizygous 13q- in one case and 14q32 deletion in one patient. A 17p deletion was associated with 14q32/IGH

rearrangement in 3/7 patients (patients 1, 2 and 6), one of whom also developed a biallelic 13q14 deletion (patient 6). In two cases with 14q32/IGH translocation at CE (patients 1 and 7), a paired BM or lymph node (LN) sample and PB samples were available for FISH studies. In these patients the appearance of IGH translocation in the BM or the LN sample preceded its appearance in PB samples (Table 3.1) by 13–58 months. The 14q32/IGH translocation persisted at subsequent analyses in both cases. All patients with the 14q32/IGH translocation were assessed by interphase FISH for the detection of possible chromosome partners. Three out of seven cases (cases 2, 5 and 6) showed an IGH–BCL2 fusion signal consistent with a t(14;18)(q32;q21) translocation. In the remaining four cases it was not possible to identify the partner chromosome with our probe panel.

TABLE 3.1. Outcome of FISH investigations in 15 patients with clonal evolution.

Patient	Aberrations at diagnosis (% of cells)	Aberrations at CE	No. of previous lines of treatment	Interval between diagnosis and clonal evolution (months)
1	13q- biallelic (42%)	13q- biallelic (55%); IgH R (18%)*; 17p- (27%)	2	60
2	No aberration	17p- (26%); IgH R (21%) (IGH-BCL2)	1	58
3	11q- (20%)	11q- (26%); IgH R (20%)	1	73
4	13q- (21%)	13q- (53%); IgH R (19%)	3	91
5	+12 (28%)	+12 (42%); IgH R (16%) (IGH-BCL2)	4	64
6	13q- (32%); 11q- (15%)	13q- biallelic (34%);11q- (27%); IgH R (21%) (IGH-BCL2); 17p- (18%)	4	74
7	+12 (30%)	+12 (55%); IgH R (25%)*	3	51
8	No aberration	11q- (42%)	4	41
9	No aberration	17p- (54%)	4	97
10	No aberration	11q- (61%)	3	48
11	13q- (66%); 11q- (65%)	11q- (61%); 13q- biallelic (60%)	5	84
12	+12 (56%)	+12 (51%); 13q- (71%)	3	87
13	No aberration	13q- biallelic (71%)	3	53
14	13q- (78%)	13q- biallelic (67%)	5	170
15	No aberration	14q32 deletion (45%)	2	24

3.2.2.3 Correlation between CE, 14q32/IGH translocations, hematologic and clinical parameters

Forty-seven patients did not require treatment throughout the study period and 58 patients received 1–6 lines of treatment. CE was detected in 15 pre-treated patients, seven of whom had a 14q32/IGH translocation, after 1–4 lines of treatment (median 3). In contrast none of 47 untreated patients developed CE ($p < 0.0001$), as shown in Table 3.2, where the patients' characteristics at initial evaluation are presented in detail.

ZAP70+ and high-risk cytogenetics predicted for the occurrence of CE with borderline statistical significance, $p<0.055$ and 0.07, respectively (Table 3.2); no

other baseline hematologic characteristic predicted for CE and for the late appearance of 14q32/IGH translocations.

TABLE 3.2. Baseline characteristics and clinical data in 105 cases submitted to sequential FISH investigations.

Characteristic	All patients (no. of cases)	Without CE (n=90)	With CE (n=15)	Without 14q32 translocations at CE (n=98)	With 14q32 translocations at CE (n=7)
Median age, years (range)	63 (31-86	63 (31-86)	63 (51-78)	63 (31-86)	60 (51-71)
M/F ratio	66/39	57/33	09-giu	62/36	04-mar
Lymphocytes					
<30 x 10^9/L	89	78	11	84	5
>30 x 10^9/L	16	12	4	14	2
Rai stage					
0-1	84	74	10	80	4
2-4	21	16	5	18	3
CD38+					
Negative	72	64	8	69	3
Positive	30	23	7	26	4
ZAP70					
Negative	46	40	5	42	4
Positive	25	17	8	22	3
FISH aberrations					
Standard risk	92	81	11	87	5
High risk	13	9	4	11	2
Treated before CE					
Yes	58	43	15*	51	7**
No	47	47	0	47	0
Relapsed/refractory					
Yes	27	16	11***	21	6****
No	31	27	4	30	1

*p<0.0001; **p=0.014; ***p=0.016; ****p=0.027.
CLL, chronic lymphocytic leukemia; FISH, Fluorescence in situ hybridization; CE, clonal evolution.

TABLE 3.3. Correlation of outcome measures and development of CE.

Outcome measure	Months	p-Value
TFT		
All patients (n=105)	54	
With CE (n=15)	35	0.0033
Without CE (n=90)	71	
With 14q32 translocations (n=7)	36	0.067
Without 14q32 translocations (n=98)	63	
TTCR		
All patients (n=58)	72	
With CE (n=15)	34	0.0046
Without CE (n=43)	86	
With 14q32 translocations (n=7)	27	0.0002
Without 14q32 translocations (n=51)	75	
Survival form diagnosis		
All patients (n=105)	173	
With CE (n=15)	124	ns
Without CE (n=43)	173	
With 14q32 translocations (n=7)	125	ns
Without 14q32 translocations (n=51)	173	

CE, clonal evolution; TFT, Time to first treatment; TTCR, Time to chemorefractoriness; ns, not significant.

The correlation between the development of CE and measures of clinical outcome is presented in Table 3.3.

Median OS measured from diagnosis was shorter in patients with CE, but the difference was not statistically significant; a shorter TTT was noted in 15 patients with CE as compared to 90 patients without CE ($p<0.0033$) (Fig. 3.2 (A)). A total of 27 patients, including 11/15 with CE (six out of seven with 14q32/IGH translocation) became refractory to fludarabine after a median of 72 months from the start of treatment, and a significant association was noted between short TTCR and CE (Table 3.3, Fig. 3.2 (B)).

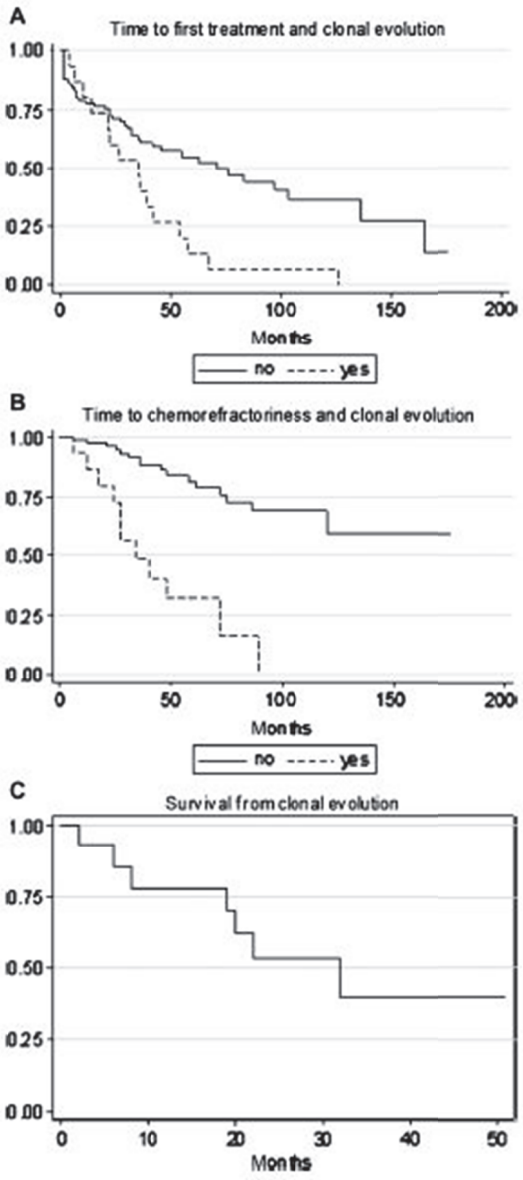


FIGURE 3.2. (A) Time to first treatment in patients with ("yes", n=15) and without CE ("no", n=90); (B) Time to refractoriness in patients with ("yes", n=15) and without CE ("no", n=43); (C) Survival from the development of CE in 15 patients with CE.

Presentation features predicting the development of chemorefractoriness in 58 treated patients were: high-risk cytogenetics ($p<0.0032$) and advanced stage ($p<0.0004$) (Table 3.4), which maintained their predictivity at multivariate analysis. Survival after the development of CE was 32 months (standard error 8.5 months) in 15 patients (Fig. 3.2 (C)) and 32 months (standard error 0.9 months) in seven patients with late-appearing 14q32 translocation.

TABLE 3.4. Impact of baseline characteristics on TTCR in 58 treated patients.

Characteristic	Median TTCR (months	SE	p-Value
Sex			
M (n=38)	72	7.8	0.95
F (n=20)	86	19.7	
Stage			
0-2 (n=47)	86	8.7	0.0004
3-4 (n=11)	27	6.1	
CD38			
Negative (n=34)	75	8.1	0.0857
Positive (n=23)	48	3.5	
ZAP70			
Negative (n=28)	72	15.1	0.34
Positive (n=21)	58	9.5	
Cytogenetics at diagnosis			
Standard (n=46)	86	10.7	0.0032
High (n=12)	36	4.8	

TTCR, Time to chemorefractoriness; SE, standard error.

3.2.3 Conventional karyotyping using novel mitogens

Cytogenetic aberrations play an important role as prognostic factors in CLL. Due to the low mitotic index of CLL B cells in vitro, analysis of a set of the most commonly known aberrations is usually done by FISH on interphase cells, which detects aberrations in about 80% of CLL samples [15]. The use of metaphase cytogenetics, which provides an unsupervised insight into the chromosomal aberrations of a specific sample, has been limited in CLL because of technical issues. Even though 90% of patient samples could be evaluated cytogenetically, aberrations could be detected in only 40% to 50% of cases [140], in contrast to FISH analysis. It is presently not clear whether other aberrations that are not detected by the standard FISH panel have any impact on prognosis and disease progression. Therefore, an approach to generate high-quality metaphases in CLL still would be highly desirable.

The B-cell mitogen CD40-ligand (CD40L) was previously compared to conventional mitogens for metaphase induction in CLL, and these results were compared with those from standard FISH analysis [164, 173]. CD40L stimulation induced metaphases in 93% of cases, versus 78% with conventional methods [173]. Even more important, CD40L stimulation resulted in the detection of aberrations in 89% of cases versus only 22% by conventional methods and confirmed all aberrations detected by FISH [173]. In addition, so-called complex aberrations (that is, 3 or more aberrations), were detected in 41% of cases. Due to

the need for a labor-intensive, cellular coculture system, CD40L-enhanced cytogenetics is hardly applicable for routine diagnostics.
Dicker et al. [174] presented a method for the efficient induction of metaphases in CLL B cells. This method relies on the addition of the immunostimulatory oligonucleotide DSP30 plus IL-2 as a CLL B-cell–specific mitogen to the cultures, which was originally developed for immunotherapeutic applications [175, 176]. In contrast to earlier reports on metaphase cytogenetics in CLL that employed different B-cell mitogens such as TPA, lipopolysaccharide, and others, here they generated metaphases for a successful analysis in 95% of all CLL samples that were subjected to the DSP30/IL-2 culture. More importantly, aberrations could be detected by chromosomal banding in more than 80% of these samples, a number that is similar to FISH analysis and that shows an almost 2-fold increase relative to earlier studies.

In order to better understand the significance of chromosome aberrations in CLL patients with relatively favourable outcome, we designed a study to assess whether karyotypic aberrations in patients without FISH anomalies (here referred to as “normal” FISH) correlate with established clinical and prognostic parameters. The clinical and prognostic significance of karyotypic aberrations in normal FISH CLL was first evaluated in a retrospective single centre series of patients and then validated prospectively in a multicentre series of cases diagnosed and analysed for karyotype with DPS30/IL2 stimulation.
Details are shown in Appendix IV (paper Rigolin GM et al, Blood 2012).

3.2.4 Methods and results

3.2.4.1 Patients

We first evaluated a retrospective series of patients (learning cohort, LC) that consisted of 65 out of 70 unselected “normal” FISH CLL patients for whom a successful cytogenetic analysis was available, derived form a series of 218 consecutive CLL diagnosed between 1998 and 2006. Then we validated in prospective series of 85 normal FISH patients, with successful cytogenetic analysis and derived from a series of 274 consecutive CLL diagnosed at 4 centres between 2007 and 2011. Only patients with a score Matutes ≥3 [172] were

included. The expression of CD38 and ZAP-70 were tested on fresh peripheral blood cells with a 20% cut-off for positivity.

3.2.4.2 Comparison between conventional kayotyping and FISH analyses

In the LC cytogenetic analyses was performed using traditional mitogens. In the VC cytogenetic analyses was centralized in the Ferrara centre and performed using CpG-oligonucleotide DSP30 plus IL2. In all cases cytogenetic analysis was conducted on the same samples used for FISH studies. Interphase FISH was performed on peripheral blood samples obtained at diagnosis or before therapy using probes for the following regions: 13q14, 12q13, 11q22/ATM, 17p13/TP53.

An abnormal karyotype was observed in 14/65 (21.5%) and 30/84 (35.7%) patients of LC and VC, respectively. A significant proportion of CLL cases with normal FISH carry chromosome aberrations in regions not covered by the 4-probe FISH panel commonly used [15]. An abnormal karyotype was found in 6.4% and 10.9% of normal FISH cases in the LC and in the VC, respectively. Recurring aberrations were: 14q deletions, 7q deletions, 6q deletions, 14q32 translocations, 3q deletions. A complex karyotype (≥3 aberrations) was found in 14 patients, 12 of which in the VC.

This study confirms previous observations that in CLL patients stimulation with DSP30/IL2 improves the rate of metaphase generation (65/70 vs 85/85 cases in the LC and VC, respectively, p=0.017)

3.2.4.3 Correlation between karyotype and clinicobiological parameters, and TFT analyses

The correlations between karyotype and clinicobiological parameters are reported in Table 3.5. Interestingly, the abnormal karyotype did not correlate with known molecular prognostic parameters, including CD38 and ZAP-70 positivity (in both LC and VC), and IgHV mutational status in the VC. By mutational analysis we observed no TP53 mutations in the VC (0 out of 23 patients with abnormal karyotype). Time to first treatment (TTT) was calculated as the interval between diagnosis and the start of first line therapy. Overall survival (OS) was calculated form the date of diagnosis until death due to any cause or until the last patient follow-up. In CLL patients with “normal” FISH the abnormal karyotype significantly correlated with a shorter TTT in univariate analysis in both the LC and the VC (Table 3.5, Figure 3.2). At multivariate analysis, the factors independently

predictive of shorter TTT were: in the LC: CD38 positivity (HR 2.82, 95% CI 1.19-6.69, p=0.018) and abnormal karyotype (HR 2.54, 95% CI 1.07-6.07, p=0.034); in the VC: advanced Binet stage (HR 2.77, 95% CI 1.05-7.29, p=0.039,) and abnormal karyotype (HR 6.39, 95% CI 2.44-16.86, p<0.001). The abnormal karyotype also correlated at univariate analysis with OS in both the LC and the VC (HR 5.87, 95% CI 2.08-62.68, p=0.005, and HR 9.41, 95% CI 1.61-47.35, p=0.0119 respectively, Figure 3.3)

TABLE 3.5. Learning and validation cohorts: clinical correlations according to karyotype and analysis of factors affecting TFT.

Clinical correlations		Learning cohort			Validation cohort		
Parameter		Normal karyotype (51 cases)	Abnormal karyotype (14 cases)	p	Normal karyotype (54 cases)	Abnormal karyotype (30 cases)	p
Age							
Mean	(range)	64 (31-84)	69 (50-87)	ns	63 (41-90)	63 (32-84)	ns
Sex							
M/F	n. of pts	31/20	8/6	ns	34/20	23/7	ns
Matutes score							
3/4/5	n. of pts	3/25/23	4/3/7	0.030	4/24/25	7/6/12	0.035
Binet Stage							
A/B/C	n. of pts	47/3/1	10/3/1	0.089	45/9/0	20/7/3	0.040
CD38							
pos/neg	n. of pts	21/29	8/6	ns	11/41	12/18	0.079
ZAP70							
pos/neg	n. of pts	15/6	5/4	ns	16/31	15/11	0.083
IgHV							
unmutated/mutated	n. of pts	nd	nd		11/38	10/17	0.191
Therapy							
yes/no	n. of pts	16/35	11/3	0.002	6/48	19/11	<0.0001

TTT analysis	Learning cohort			Validation cohort		
Parameter	N. of cases	HR (95% CI)	p	N. of cases	HR (95% CI)	p
Karyotype						
Abnormal vs Normal	14 vs 51	3.33 (2.03-15.66)	0.0009	34 vs 50	7.80 (4.36-25.12)	<0.0001
Binet Stage						
B-C vs A	8 vs 57	4.56 (4.43-89.97)	<0.0001	19 vs 65	3.66 (2.47-26.83)	0.0006
CD38						
pos vs neg	29 vs 35	3.26 (1.60-7.78)	0.0018	23 vs 59	2.00 (0.91-5.73)	0,54583333
ZAP70						
pos vs neg	10 vs 20	2.31 (0.81-5.78)	ns	32 vs 41	1.52 (0.70-3.41)	ns
IgHV						
unmutated vs mutated	nd	nd	nd	21 vs 55	2.40 (1.11-7.64)	0.0297

nd, not done; ns, not significant; HR, hazard ratio.

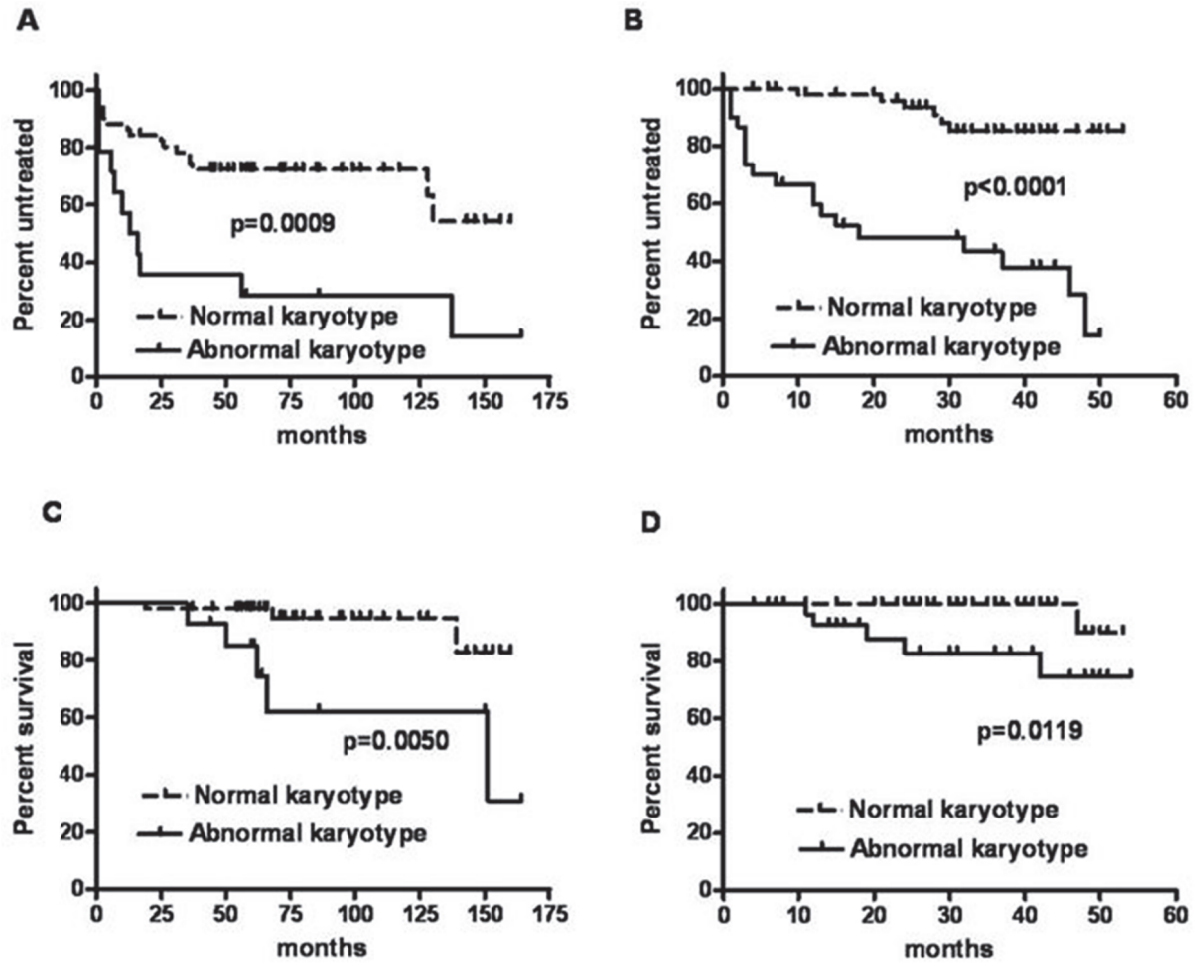


FIGURE 3.3. TTT and OS respectively in the learning (A, C) and the validation (B, D) cohorts.

3.3 NEXT-GENERATION SEQUENCING IN CLL

The detection of recurrent chromosomal aberrations has been of key importance for understanding the biology of CLL and the mechanisms driving the variable clinical phenotype of this disease [15, 60]. However, cytogenetic lesions do not entirely explain the molecular pathogenesis and the clinical heterogeneity of CLL. The advent of next-generation sequencing (NGS) technologies, coupled with gene copy number analysis, has enabled exploration of the CLL genome, uncovering genetic lesions that recurrently target this leukemia. NGS studies have further elucidated the genomic complexity of CLL and have shown that the average number of non-silent mutations per case is 10-12 at diagnosis, whereas the average number of copy number abnormalities is approximately two [26, 27]. Whole genome/exome sequencing of more than 200 CLL cases has disclosed the genetic landscape of CLL, providing comprehensive catalogs of somatic mutations and new insights into the genes that contribute to cellular transformation [29]. NOTCH1, SF3B1, BIRC3 and MYD88 are the most recurrently mutated genes that

have been identified through the application of whole genome/exome sequencing to CLL, and, beside their contribution to leukemic transformation, a number of observations point to mutations of these genes as attractive biomarkers of potential clinical relevance. Alterations of these genes occur in approximately 5-10% of CLL patients at diagnosis and have shown significant correlations with survival [28, 30, 177].

NOTCH1. Among the genes mutated in CLL, NOTCH1 emerged as a recurrently targeted by genetic lesions in specific phases of the disease. NOTCH1 encodes a ligand-activated transcription factor that regulates several down-stream pathways important for cell growth control and is affected by activating mutations in 60% of T-lineage acute lymphoblastic leukemia [178]. In CLL, the frequency of NOTCH1 mutations at the time of diagnosis is approximately 10% [26-28]. NOTCH1 mutations at CLL diagnosis preferentially occur among unmutated CLL and cluster with trisomy 12 [26-28, 179, 180] . NOTCH1 mutations identify a high-risk subgroup of patients showing poor survival comparable to that associated with TP53 abnormalities and exert a prognostic role that is independent of widely accepted risk factors, as confirmed in multiple consecutive series from different institutions [26-28]. One recurrent mutation (c.7544_7545delCT) accounts for approximately 80% of all NOTCH1 mutations [26-28] and can be rapidly detected by a simple PCR-based approach, providing a potential strategy for a first level screening of NOTCH1 alterations [28]. Although the precise role of NOTCH1 activation in CLL pathogenesis is still unclear, the relevance of these mutations is highlighted by the distinctive GEP signature of CLL carrying NOTCH1 mutations [26, 180].

SF3B1. SF3B1 is a core component of the splicing machinery, which catalyses the removal of introns from precursor messenger RNA (mRNA) to produce mature mRNA [181]. At diagnosis, SF3B1 is mutated in 5-10% of CLLs [29, 177, 182]. The pathogenicity of SF3B1 mutations in CLL is supported by the clustering of these mutations in evolutionary hotspots localized within HEAT domain (codon 662, 66, 700, 704 and 742) [29, 177, 182]. Although the precise mechanistic aspects of SF3B1 mutations are still under investigation, the observation that SF3B1 regulates the alternative splicing program of genes controlling cell cycle progression and apoptosis points to a potential contribution of SF3B1 mutations in modulating tumor cell proliferation and survival [29, 182]. At

CLL diagnosis, SF3B1 mutations predict reduced survival independent of other clinical and biological risk factors, and show a preferential association with CLL showing 11q22-q23 deletion and ATM mutations [29, 177, 182].

BIRC3. BIRC3, along with TRAF2 and TRAF3, cooperates in the same protein complex that negatively regulates MAP3K14, an activator of the non-canonical pathway of NF-kB signalling [183]. The BIRC3 gene is recurrently disrupted in CLL by mutations, deletions or a combination of mutations and deletions [30]. BIRC3 lesions are rare in the early phases of the disease, where they mark poor prognosis, while they accumulate in progressive and refractory CLL [30]. Identification of BIRC3 involvement in CLL may be important for elucidating the molecular genetics of 11q22-q23 deletion. In fact, although ATM has been regarded as the relevant gene of this cytogenetic lesion, biallelic inactivation of ATM does not exceed 30% of CLL with 11q22-q23 deletion. On the basis, a second tumor suppressor in the 11q22-q23 region has been postulated along with ATM. The BIRC3 gene, that maps on 11q22.2 might represent an attractive candidate.

MYD88. In B-cells, Toll-like receptors (TLRs) are central to the BCR-independent response to antigens by sensing a variety of pathogen associated molecular patterns [184]. After stimulation of TLRs, MYD88 is recruited, as cytoplasmic adaptor, to the activated receptor complex as a homodimer and form proteins complexes that trigger activation of NF-kB [184]. The most prevalent mutation in CLL is the L265P missense substitution that occurs in ~3% of cases [26, 29, 182]. Among biological subgroups of CLL, MYD88 mutations are enriched among cases harboring mutated IgHV genes and 13q14 deletion as the sole cytogenetic abnormality [185].

TP53. The tumor suppressor gene TP53 maps on the short arm of chromosome 17 (17p13) and codes for a central regulator of the DNA-damage-response pathway. Activation of TP53 leads to cell-cycle arrest, DNA repair, apoptosis, or senescence via both transcription-dependent and transcriptional-independent activities. Consistently, TP53 plays a central role in mediating the pro-apoptotic and antiproliferative action of several DNA-damaging chemotherapeutic agents, including alkylators and purine analogs [186].

In CLL, the TP53 gene may be inactivated by deletion and/or somatic mutations [23, 150]. Most cases with 17p13 deletion also carry TP53 mutations on the second allele (~60%), while the remaining cases have monoallelic 17p13 deletion

in the absence of TP53 mutations (~10%), or TP53 mutations in the absence of 17p13 deletion (~30%) [23, 150]. TP53 abnormalities frequently couple with complex karyotype [23, 150]. At the molecular level, approximately 75% of all TP53 mutations are missense substitutions, while the remaining lesions are represented by truncating events, including frameshift insertions or deletions, non-sense substitutions and splice-site mutations [150]. From a clinical standpoint, genetic lesions affecting the TP53 gene are significantly enriched in high risk CLL, and represent the only established biomarker of chemorefractoriness in this leukemia [187].

FIGURE 3.4. NOTCH1, SF3B1, BIRC3, TP53 mutation type and distribution in CLL. Schematic representation of the human NOTCH1 (A), SF3B1 (B), BIRC3 (C), and TP53 (D) proteins, with their key functional domains. Color-coded symbols indicate the type and position of the mutations.

Advancing the knowledge of CLL molecular genetics may have important therapeutic implications including: (a) therapeutic stratification based on molecular prognosticators; and (b) targeted therapy aimed at correcting the genetic defect or its functional consequences.

3.3.1 Ion Torrent PGM (Personal Genome Machine)

Over the course of the 10 years that have passed since the publication of the first human genome sequence, the landscape of cancer research has changed with

remarkable speed [188]. Massive parallel sequencing technology provides a means of systematically discovering the genetic alterations that underlie disease and identifying new therapeutic targets and clinically predictive biomarkers. Since the launch of first NGS platform, a number of technological advances have been made, such as improved sequencing chemistry and novel signal detection methodologies [189]. This has resulted in the availability of the new small NGS systems as well as Ion Torrent Personal Genome Machine (PGM). Ion Torrent is a sequencer that uses semiconductor sequencing technology. It bases the sequencing process on the detection of the change in pH resulting from H+ ion release upon nucleotide incorporation. PGM uses a high-density array of micro wells to perform sequencing and beneath the wells is an ion sensitive layer followed by a proprietary ion sensor which detects changes in pH resulting from release of hydrogen ion following nucleotide incorporation. Fragments to be sequenced are captured on beads and amplified by emulsion PCR. The beads are then deposited in the microwell such that each well has only one bead carrying a unique amplified fragment. Nucleotides are then added in a predetermined sequence to the wells. A nucleotide complementary to the nucleotide in the fragment being sequenced gets incorporated in the strand being synthesized and a hydrogen ion is released. Upon release of hydrogen ion, the voltage of the solution changes in that well and is detected by the ion sensor. If two nucleotides are incorporated in a cycle, then the voltage is doubled and the sensor records two nucleotides added. If a nucleotide is not added during a cycle then no voltage change is recorded. It is the first commercial sequencing machine that does not require fluorescence and camera scanning, resulting in higher speed of analysis and lower cost [190].

In order to improve understanding of genetic basis of CLL and to apply NGS by Ion Torrent technology to CLL, we sequenced DNA samples from 28 untreated patients affected by chronic lymphocytic leukemia and we correlated mutational status with clinicobiological parameters.
Details are shown in Appendix V (paper Saccenti E et al, in submission).

3.3.2 Methods and results

3.3.2.1 Patients

Twenty-eight untreated cases of CLL seen at our institution between 2006 and 2008 were included in this study. The principal clinical characteristics of the patients are reported in Table 3.6. Diagnosis was made according to standard NCI criteria [4]. Indications for treatment included: increased WBC count with <6 month lymphocyte doubling time, anemia or thrombocytopenia due to bone marrow infiltration or autoimmune phenomena not responding to steroids, disease progression in the Binet staging system. Fludarabine-containing regimens were used as first-line treatment; chlorambucil was used in some elderly and unfit patients. At diagnosis, immunophenotypic analysis was performed according to NCI criteria [4]. Immunophenotyping with a standard diagnostic panel including anti CD38 monoclonal antibodies was performed as previously reported [137, 191]. The expression of CD38 were tested, as described [192], on fresh peripheral blood (PB) cells with a 20% cut-off for positivity.

TABLE 3.6. Baseline characteristics and clinical data in 28 cases submitted to NGS sequencing.

CLINICAL AND BIOLOGICAL CHARACTERISTICS	
N of patients	28
Age mean yrs (range)	62 (49-75)
M/F	18/10
Stage (Binet) A/B/C	22/4/2
CD38 (>20%) neg/pos	20/7
IgVH Mutated/Unmutated	17/11
FISH Low /Intermediate/High	21/4/3
Karyotype Low /Intermediate/High	15/8/5

The patients were classified in 3 cytogenetic risk categories: 17p-, 11q- or complex karyotype defined the high risk group, +12 or 1-2 cytogenetic aberrations defined the intermediate risk group, isolated 13q- or absence of detectable aberrations defined the low risk group.

3.3.2.2 Cytogenetic analysis

FISH studies were performed as previously reported [137, 191]. Conventional karyotyping was performed using ODN and IL2 as previously reported [192]. Results of FISH and cytogenetic analysis are reported in Table 3.7. In 14/28 cases, FISH studies revealed genetic lesions. 17p deletion was seen in 2 cases, 11q deletion in 1 case, trisomy 12 in 4 cases and 13q deletion in 9 cases.

In two cases, the 13q14 deletion was associated respectively to the deletion of chromosome 11 and chromosome 17.

Case	Mutated genes	% mutation	FISH results	Conventional kayotyping results
Emato Unife-942	TP53	6,43	del(13q), del(17p)	46,XY,add(8)(p23) [19] / 46,XY [1]
	TP53	18,89		
Emato Unife-121	Nessuna		neg	46,XY,t(14;18)(q32;q21) [5] / 46,XY, [12] / 46,XY,del(1)(q32),add(9)(q34),-14,+mar [4] CNC
Emato Unife-662	MYD88	10,83	neg	46,XY,t(4;11)(q21;q24),add(22)(p13) [8] / 46,XY,dic(15;15)(p11;p11) [3] / 46,XY [9]
Emato Unife-83	Nessuna		neg	47,X,der(X)(p11),+del(3)(p11p14) [4] / 46,XX [16]
Emato Unife-884	FBXW7	32,94	neg	46,XY [20]
Emato Unife-658	MAPK1	18,84	neg	46,XY [20]
Emato Unife-874	Nessuna		neg	46,XX [20]
Emato Unife-84	Nessuna		neg	46,XY[20]
Emato Unife-1572	TP53	23,1	neg	46,XY,del(7)(q32) [4] / 46,XY [21]
	POT1	22,28		
Emato Unife-288	POT1	19,92	trisomy 12	47,XX,+12 [16] / 46,XX [4]
Emato Unife-437	Nessuna		del(13q)	46,XY,del(13)(q14q22) [6] / 46,XY [14]
Emato Unife-455	TP53	39,37	trisomy 12	47,XY,+12 [7] / 47,XY,+12,add(19)(p13) [3] / 46,XY [10]
	DDX3X	66,81		
Emato Unife-656	Nessuna		trisomy 12	47,XX,+12 [17] / 46,XX [3]
Emato Unife-676	KLHL6	33,1	del(11q), del(13q)	46-47,XX,add(2)(p25), add(2)(q36), add(3)(p26), add(4)(p16) ,del(11)(q21q23), del(13)(q14q21), add(14)(p13), add(15)(p13), add(18)(p11), +mar [21] / 46,XX [1]
	ATM	76,51		
Emato Unife-974	Nessuna		neg	46,XY[20]
Emato Unife-978	Nessuna		del(13q)	46,XY,del(13)(q14q22) [11] / 46,XY [10]
Emato Unife-1184	Nessuna		del(13q)	46,XY,del(13)(q14q22)[11] / 46,XY [9]
Emato Unife-1214	Nessuna		del(13q)	46,XY,t(14;18)(q32;q21)[13]/46,XY,del(13)(q14q22),t(14;18)(q32;q21) [5] / 46,XY [2]
Emato Unife-194	TP53	16,96	del(17p)	45-46,XX,der(2)(p23),add(16)(p13),der(14)(q32),der(17)(p12),+19,-21 [cp10] / 46,XX [10]
Emato Unife-1343	Nessuna		del(13q)	46,XX,del(13)(q14q22) [3] / 46,XX [17]
Emato Unife-1353	Nessuna		del(13q)	46,XY,del(13)(q14q22)[4]/46,XY,del(10)(q22q24),del(13)(q14q22) [5] / 46,XY [11]
Emato Unife-1363	Nessuna		neg	46,XX [20]
Emato Unife-1451	POT1	29,3	trisomy 12	47,XY,+12 [20]
	KRAS	8,17		
Emato Unife-1455	TP53	46,81	neg	46,XY[20]
Emato Unife-1465	Nessuna		del(13q)	46,XX,del(13)(q14q22) [5] / 46,XX [15]
Emato Unife-1473	Nessuna		neg	46,XY[20]
Emato Unife-1495	SF3B1	29,21	neg	46,XX,der(7)t(7;?)(q32;?) [7] / 46,XX [19] sospetto 6q-CNC [3]
Emato Unife-1496	Nessuna		neg	46,XY[20]

TABLE 3.7. Outcome of NGS sequencing, FISH investigations and conventional karyoptyping analysis.

19/28 cases showed an abnormal karyotype by conventional cytogenetic analysis, including 5/15 cases without detectable lesions by FISH (see Table 3.7). A complex karyotype (≥3 aberrations) was found in 4 cases.

3.3.2.3 Mutational analysis by Next Generation Sequencing (NGS)

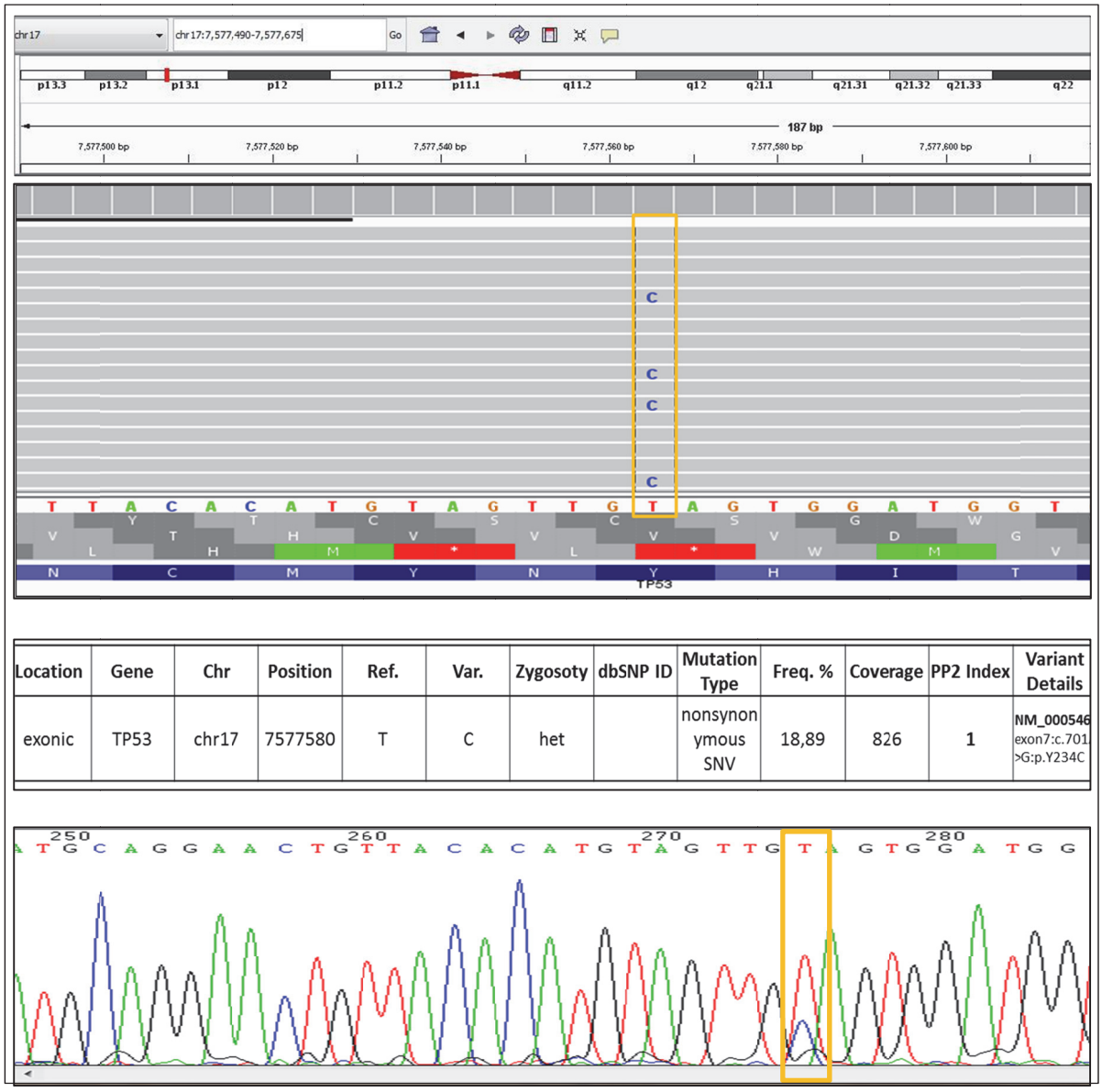
NGS analysis was performed using Ion Torrent PGM. Libraries of spot exonic regions of 20 genes (ATM, BIRC3, BRAF, CDKN2A, CTNNB1, DDX3X, FBXW7, KIT, KLHL6, KRAS, MAPK1, MYD88, NOTCH1, NRAS, PIK3CA, POT1, SF3B1, TP53, XPO1, ZMYM3) were constructed using Agilent Haloplex Target Enrichment kit starting from genomic DNA. Enriched libraries linked to Ion Sphere Particles were loaded in one Ion chip and sequenced using Ion Torrent PGM, according to manufacturer instructions. Genomic segments analysed are reported in Table 3.8.
Using Ion Torrent technology, somatic mutations were identified in 12 (42.8%) cases, as reported in Table 3.7. Mutations were found in a range from 6.4 to 76.5% of the reads analysed. Each segment had a number of reads > 500.
5 cases showed mutations in the TP53 gene in 6.4%-46. 8% of the reads, 3 cases presented POT1 mutations, while mutations of MYD88, FBXW7, MAPK1, DDX3X, KLHL6, SF3B1, KRAS and ATM were detected in 1 case each. 8 cases presented one mutated gene and 4 cases 2 mutated genes (TP53 in association with POT1 or DDX3X, ATM in association with KLHL6 and KRAS with POT1) (Table 3.7). Gene mutations found were validated using capillary Sanger sequencing (Fig. 3.5)

3.3.2.4 Correlation between mutational status and clinicobiological parameters

The time to first treatment (TTT) was calculated as the interval between diagnosis and the start of first-line treatment. Survival curves were compared by using the log-rank test. A P value <.05 was used as a criterion for statistical significance. The correlations between mutational status and clinico-biological parameters are reported in Table 3.9.

TABLE 3.8. Genomic segments analysed with NGS by Ion Torrent.

Chromosome	Gene	Start genomic position	End genomic position	Analyzable region
chr2	XPO1	61719173	61719706	"NC_000002.11:61719173-61719706"
chr2	SF3B1	198267115	198267848	"NC_000002.11:198267115-198267848"
chr2	SF3B1	198266411	198267036	"NC_000002.11:198266411-198267036"
chr2	SF3B1	198265215	198265916	"NC_000002.11:198265215-198265916"
chr15	CHD2	93499516	93499959	"NC_000015.9:93499516-93499959"
chr15	CHD2	93510384	93511033	"NC_000015.9:93510384-93511033"
chr15	CHD2	93534475	93535000	"NC_000015.9:93534475-93535000"
chr15	CHD2	93540268	93540784	"NC_000015.9:93540268-93540784"
chr15	CHD2	93555303	93555804	"NC_000015.9:93555303-93555804"
chr15	CHD2	93552180	93552765	"NC_000015.9:93552180-93552765"
chrX	DDX3X	41196436	41196991	"NC_000023.10:41196436-41196991"
chrX	DDX3X	41204135	41205040	"NC_000023.10:41204135-41205040"
chrX	ZMYM3	70461788	70462292	"NC_000023.10:70461788-70462292"
chrX	ZMYM3	70460768	70461442	"NC_000023.10:70460768-70461442"
chrX	ZMYM3	70472711	70473246	"NC_000023.10:70472711-70473246"
chrX	ZMYM3	70469561	70470208	"NC_000023.10:70469561-70470208"
chr17	TP53	7576768	7577268	"NC_000017.10:7576768-7577268"
chr17	TP53	7577349	7577687	"NC_000017.10:7577349-7577687"
chr17	TP53	7577851	7578801	"NC_000017.10:7577851-7578801"
chr17	TP53	7579055	7579834	"NC_000017.10:7579055-7579834"
chr4	KIT	55595386	55595769	"NC_000004.11:55595386-55595769"
chr4	KIT	55602365	55602872	"NC_000004.11:55602365-55602872"
chr4	FBXW7	153249048	153249593	"NC_000004.11:153249048-153249593"
chr4	FBXW7	153245138	153245696	"NC_000004.11:153245138-153245696"
chr4	FBXW7	153246985	153247682	"NC_000004.11:153246985-153247682"
chr4	FBXW7	153258767	153259117	"NC_000004.11:153258767-153259117"
chr3	MYD88	38182079	38182867	"NC_000003.11:38182079-38182867"
chr3	CTNNB1	41265794	41266469	"NC_000003.11:41265794-41266469"
chr3	PIK3CA	178951808	178952299	"NC_000003.11:178951808-178952299"
chr3	KLHL6	183272884	183273632	"NC_000003.11:183272884-183273632"
chr22	MAPK1	22126949	22127493	"NC_000022.10:22126949-22127493"
chr22	MAPK1	22159919	22160421	"NC_000022.10:22159919-22160421"
chr9	NOTCH	139390287	139391047	"NC_000009.11:139390287-139391047"
chr1	NRAS	115256330	115256815	"NC_000001.10:115256330-115256815"
chr1	NRAS	115258445	115258986	"NC_000001.10:115258445-115258986"
chr7	POT1	124498740	124499390	"NC_000007.13:124498740-124499390"
chr7	POT1	124510738	124511207	"NC_000007.13:124510738-124511207"
chr7	POT1	124532174	124532633	"NC_000007.13:124532174-124532633"
chr7	POT1	124538031	124538662	"NC_000007.13:124538031-124538662"
chr7	POT1	124536926	124537435	"NC_000007.13:124536926-124537435"
chr7	BRAF	140452990	140453495	"NC_000007.13:140452990-140453495"
chr11	BIRC3	102201521	102202174	"NC_000011.9:102201521-102202174"
chr11	BIRC3	102207344	102207768	"NC_000011.9:102207344-102207768"
chr11	ATM	108119490	108120023	"NC_000011.9:108119490-108120023"
chr11	ATM	108117519	108118136	"NC_000011.9:108117519-108118136"
chr11	ATM	108123199	108123716	"NC_000011.9:108123199-108123716"
chr11	ATM	108137735	108138170	"NC_000011.9:108137735-108138170"
chr11	ATM	108154862	108155488	"NC_000011.9:108154862-108155488"
chr11	ATM	108173355	108173950	"NC_000011.9:108173355-108173950"
chr11	ATM	108170161	108170733	"NC_000011.9:108170161-108170733"
chr11	ATM	108172302	108172703	"NC_000011.9:108172302-108172703"
chr11	ATM	108180579	108181241	"NC_000011.9:108180579-108181241"
chr11	ATM	108200697	108201254	"NC_000011.9:108200697-108201254"
chr11	ATM	108205463	108206084	"NC_000011.9:108205463-108206084"
chr11	ATM	108206442	108206939	"NC_000011.9:108206442-108206939"
chr11	ATM	108204378	108204828	"NC_000011.9:108204378-108204828"
chr11	ATM	108217938	108218252	"NC_000011.9:108217938-108218252"
chr11	ATM	108225292	108225744	"NC_000011.9:108225292-108225744"
chr11	ATM	108235751	108236396	"NC_000011.9:108235751-108236396"
chr12	KRAS	25397964	25398541	"NC_000012.11:25397964-25398541"



Location	Gene	Chr	Position	Ref.	Var.	Zygosoty	dbSNP ID	Mutation Type	Freq. %	Coverage	PP2 Index	Variant Details
exonic	TP53	chr17	7577580	T	C	het		nonsynonymous SNV	18,89	826	1	NM_000546 exon7:c.701 >G:p.Y234C

FIGURE 3.5. Sanger validation of a TP53 mutation detected by Ion Torrent PGM.

The presence of somatic mutation did not correlate with sex, age, Binet stage, CD38 or IgHV status. A positive mutational status correlated with the presence of cytogenetic abnormalities detected by FISH and the relative risk status (favourable, intermediate and high) (p=0.0198) and unfavourable karyotype findings (p=0.0322). Mutated patients showed a significant (p=0.0002) shorter median time to first treatment in comparison to those without mutations (20 months vs not reached at 76 months) (Figure 3.6).

TABLE 3.9. Clinical correlations according to presence or absence of somatic mutation by NGS sequencing.

CLINICAL CORRELATIONS				
Parameter		**Mutation positive (12 cases)**	**Mutation negative (16 cases)**	**P**
Age				
Mean	(range)	62 (49-70)	63 (51-75)	ns
Sex				
M/F	n. of pts	8/4	10/6	ns
Binet Stage				
A/B,C	n. of pts	8/4	14/2	ns
CD38				
Pos/Neg	n. of pts	4/7	3/13	ns
IgVH				
Mutated/Unmutated	n. of pts	6/6	11/5	ns
FISH				
Low /Intermediate/High	n. of pts	6/3/3	15/1/0	0.0198
Karyotype				
Low /Intermediate/High	n. of pts	3/5/4	12/3/1	0.0322
Therapy				
Yes/No	n. of pts	7/5	2/14	0.014
Time to First Treatment				
	months	20.0	Not reached	0.0002

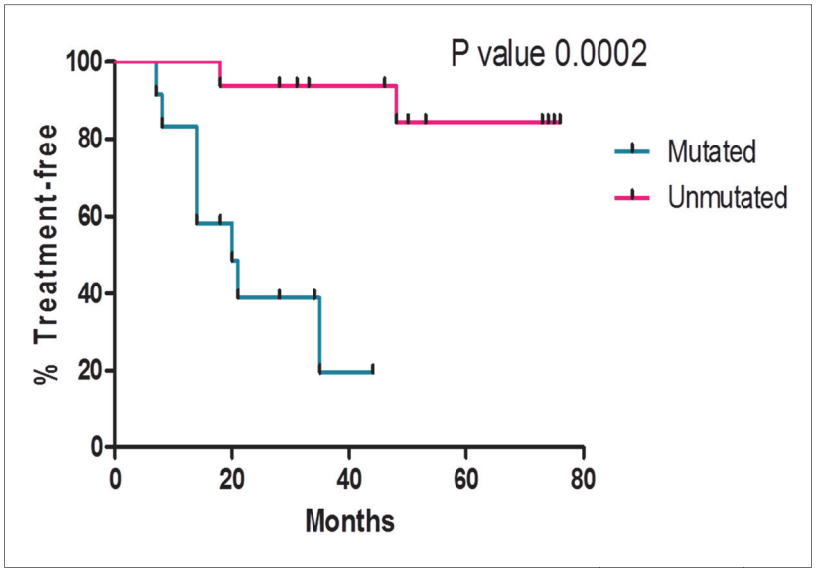


FIGURE 3.6. Time to first treatment in patients with (Mutated, n=12) and without somatic mutations (Unmutated, n=16).

3.4 DISCUSSION

ASSESSING PROGNOSIS BY MODERN MOLECULAR CYTOGENETIC STUDIES

The presence of cytogenetic abnormalities is a hallmark of CLL but the acquisition of new genomic aberrations during the disease course (clonal evolution) is

thought to be an infrequent phenomenon. CLL may undergo CE, with late appearance of 11q deletion, 17p deletion and 6q deletion in 16–17% of cases [139, 145]. Although recent evidence has been provided by several groups that the 14q32/IGH translocation may identify a cytogenetic group of patients with CLL characterized by therapy-demanding disease [137, 138], scant information is available as to its possible appearance as a secondary abnormality in CLL. Fifteen out of 105 cases (14.3%) studied by FISH showed CE in our study, and seven of these 15 cases showed a 14q32/IGH translocation. The presence of 14q32/IGH translocation was previously documented to involve a minority of interphase cells in some patients with CLL [137], and one case was described as harboring IGH translocations with different partners [193]. These findings argue in favor of a possible secondary nature of this translocation in some patients. Interestingly, in our series the appearance of a 14q32 translocation in BM and LN samples preceded its appearance in the PB in two cases. Recent studies, included our work on proliferation centers, indicated that CLL cells in lymph nodes and bone marrow may have an important role in tumor proliferation [83, 92] and, interestingly, a relatively high incidence of 14q32/IGH translocation (17–19%) was noted by Flanagan and co-workers who performed FISH studies on paraffin-embedded lymphoid tissue from CLL/small lymphocytic lymphoma (SLL) [133]. Because our patients were seen over a 10-year period, treatment was heterogeneous, and a comparison of treatment regimens in patients with and without CE was not performed; nevertheless, it is worth noting that in our seven patients with 14q32/IGH translocations at CE the median number of previous treatment lines was 3 (range 1–4), and that all patients received ≥1 cycle containing alkylating agents. Interestingly, IGH translocation accounted for 20% of the interphase cells (range 16–25%) in our study, and was associated with a minor clone carrying 17p deletion in three cases and 13q14 biallelic deletion in one. These data taken together suggest that genetic instability deriving from previous treatment might have played a role in the emergence of minor clones carrying a 14q32/IGH translocation in our series. The late appearance of loss of chromosome material at 14q32 in one patient in this study parallels an observation by Shanafelt and co-workers [145]. Deletion affecting the chromosome 14q32 locus was previously described in CLL at a frequency of approximately 2–8% [194, 195]. Clonal evolution in CLL was demonstrated to be associated with unfavourable biological features in previous analyses, although no difference in overall survival

was noted between patients with and without CE [139]. In our series the development of CE occurred only in previously treated patients. By analyzing possible associations between CE and measures of clinical outcome we were able to show that TTT was shorter in patients who developed CE. This finding may be accounted for by the consideration that CE may reflect genetic instability, a feature normally associated with disease evolution prompting the appearance of resistant clones.

In order to better understand the significance of chromosome aberrations in CLL patients and in particular in those patients with relatively favourable outcome, we designed a study to assess whether karyotypic aberrations in patients without FISH anomalies correlate with established clinical and prognostic parameters.

Our data show that a significant proportion of CLL cases with normal FISH carry chromosome aberrations in regions not covered by the 4-probe FISH panel used in most clinical trials [15]. This novel cytogenetic category (i.e. abnormal karyotype with “normal” FISH) accounted for 8.9% in the pooled series of 492 patients of this study, thus representing the 4th most common cytogenetic group, following deletions at 13q and 11q, and trisomy 12. Recurring aberrations were 14q deletions, 7q deletions, 6q deletions, 14q32 translocations and 3q deletions. A complex karyotype (≥ 3 aberrations) was found in 14 patients. This study confirms previous observations that in CLL patients stimulation with DSP30/IL2 improves the rate of metaphase generation [174]. Interestingly, the abnormal karyotype did not correlate with known molecular prognostic parameters, including CD38 and ZAP 70 positivity, and IGHV mutational status. Most important we could demonstrate, for the first time, that among CLL patients with “normal” FISH the abnormal karyotype significantly correlated with a shorter TTT in univariate analysis. At multivariate analysis, the factors independently predictive of shorter TTT were: CD38 positivity, abnormal karyotype and advanced Binet stage. Recently it was demonstrated that, in CLL, TP53 mutations [158] represent a strong prognostic marker. By mutational analysis we observed no TP53 mutations in our patients. This finding is not surprising if we consider that patients with 17p- were excluded from this analysis and that, among untreated CLL without 17p-, the incidence of TP53 mutations is around 3% [20, 158, 160]. The abnormal karyotype also correlated at univariate analysis with OS. This observation needs to be validated in clinical trials with homogeneous treatment on larger series of patients.

The advent of next-generation sequencing (NGS), with its unprecedented ability to systematically discover key genetic alterations that underlie cancer, offers the tantalizing possibility of unraveling the genetic basis of this heterogeneity [196]. We applied this knowledge to CLL, developing a next-generation sequencing approach to screen 28 CLL patients at diagnosis in a custom-made panel of 20 genes. The panel was designed to cover coding sequence of CLL involved genes according to recent literature [26, 28-30, 182]. We found somatic mutations in 12 cases. TP53 was most frequently mutated (5 cases) and the frequency of mutations involving the other genes investigated was in line with data published in literature using whole exome sequencing [26, 29]. The percentage of reads showing mutation ranged from 6.4 to 76.5%. From the variability in the reads where mutation was found, we deduce that both clonal and subclonal mutations were present. The mutational profile of CLL can be characterized in two main group: clonal mutations, which are present in all tumor cells and represent early events in the leukemogenesis, and subclonal mutations, usually present in a small fraction of leukemic cells and possibly may represent progression events. This finding may explain the basis of the great heterogeneity within the CLL population [118]. ATM mutation present in the 76.5% of the reads analyzed is probably a predominantly clonal, whereas mutation with rates of 6-20% are probably subclonal mutations, that could expand over the time and lead to disease progression.

Among our 19 cases with genetic lesions by FISH and/or cytogenetic analysis NGS showed gene mutations in 9 cases, documenting that cytogenetic, FISH and sequencing may represent complementary methods that allow to detect genetic heterogeneity within CLL. NGS could also provide information in a significant proportion of CLL patients with favorable prognostic features. Our method allow for the identification of genetic lesions in 25% of patients with non-informative cytogenetic analysis (FISH and karyotype) and 50% of FISH negative cases. In the three cases without cytogenetic and FISH abnormalities, we found mutated FBXW7, MAPK1 and TP53, respectively. Data are not surprising, in fact TP53 mutations in the absence of 17p13 deletion are detected in 30% of CLL at diagnosis [150].

We then correlated mutational status with clinico-biological parameters. Interestingly, the presence of mutations correlated with high risk FISH (11q- and/or 17p-) and unfavourable cytogenetic (11q-, 17p- or complex karyotype) findings. No

correlations were instead observed with other parameters like sex, age, Binet stage, CD38 and IGHV. The mutational status also correlated at univariate analysis with time to first treatment, that was significantly shorter in patients harboring somatic mutations. At multivariate analysis, no variable independently predictive of shorter TTT was statistically significant. This data is probably due to small number of patients analysed. Definitely a future goal will be to expand the series of patients.

In conclusion, our data show that the 14q32/IGH translocation may represent one of the most frequent aberrations acquired during the natural history of CLL, and that it may be detected earlier in BM or LN samples. CE occurs in pretreated patients with short TTT, and survival after the development of CE with and without 14q32 translocation is relatively short.

To reassess the importance of cytogenetic analysis, we studied by conventional karyotyping using DSP30/IL2 stimulation a series of patients without FISH lesions. We were able to confirm that DSP30/IL2 stimulation is an effective method for the detection of chromosome aberrations in CLL, by documenting the presence of chromosome aberrations in approximately one third of CLL with “normal” FISH on a conventional 4-probe panel. This set of data also showed that, in CLL patients with “normal” FISH, conventional cytogenetic analysis identifies a subset of cases with adverse clinical and prognostic features to be considered for the design of risk-adapted treatment strategies. After examining these CLL cases with a novel technique of conventional karyotyping, we wanted to deepen our understanding of the molecular abnormalities by analyzing a CLL series with a next-generation sequencing technique. NGS by Ion Torrent might represent an important tool for the characterization of CLL genetic heterogeneity and clinical prognostic outcome. Ion Torrent is surely feasible due to the low cost and the short time for reaction. This techniques expanded our knowledge on the molecular mechanisms involved in the pathogenesis of the disease and offer new perspectives for prognostic stratification and clinical management of the CLL patients.

CONCLUSIONS

In order to better understand the pathogenetic process in CLL, we focused our attention on genetic heterogeneity in the disease. In our first study we provided evidence supporting an association between cryptic genetic lesions in CD38+ cells and disease progression and we found that genomic complexity and worse outcome were associated with miR-125a-5p down-regulation. This set of data show that genetic lesions may appear in CD38+ cells in low-risk CLL and that they may be associated with miR-125a-5p down-regulation, allowing for a more accurate prognostication in low-risk CLL. To assess whether genetic instability is more pronounced in microenvironment, we studied sequential lymph nodes samples, and we established a correlation between the extension of PCs and clinico-biologic as well as cytogenetic characteristic. The association of PCs-rich pattern with unfavourable cytogenetics and short survival may support the pathogenetic role of proliferation center in CLL. We found a high incidence of adverse biologic features, that is, unmutated IGHV configuration, ZAP70, and high-risk cytogenetics in those patients with enlarged PCs. Our study demonstrated that the histopathological pattern defined by the presence of confluent PCs may represent an important feature for risk assessment in this subset of patients.

To establish a correlation between genetic lesions and outcome we then focused on the correlation between clonal evolution (CE) and development of chromosomal aberrations. Our data showed that the 14q32/IGH translocation may represent one of the most frequent aberrations acquired during the natural history of CLL, and that it may be detected earlier in BM or LN samples. CE occurs in pretreated patients with short TTT, and survival after the development of CE with and without 14q32 translocation is relatively short.

To improve the prognostic predictivity of cytogenetic analysis in the clinical practice, we studied by conventional karyotyping using novel mitogens (i.e. DSP30 + IL2 stimulation) a series of patients without detectable FISH lesions. We were able to confirm that DSP30/IL2 stimulation is an effective method for the detection of chromosome aberrations in CLL, by documenting the presence of chromosome aberrations in approximately one third of CLL with “normal” FISH. This set of data also showed that, in CLL patients with “normal” FISH, conventional cytogenetic analysis identifies a subset of cases with adverse clinical and prognostic features

to be considered for the design of risk-adapted treatment strategies. After examining these CLL cases with a novel technique of conventional karyotyping, we elected to further improve our capability to detect genetic lesions by analyzing a CLL series with a next-generation sequencing technique. Mutated patients showed a significant shorter median time to first treatment in comparison to those without mutations. This techniques expanded our knowledge on the molecular mechanisms involved in the pathogenesis of the disease and offer new perspectives for prognostic stratification and clinical management of the CLL patients.

In conclusion, in this work we tried to arrive to a better understanding of the pathogenesis of CLL, through the study of cell activation and genetic instability. We have documented that genetic lesions may appear in a small fraction of leukemic clones in low-risk CLL and that they may be associated with a microRNA down-regulation. Then we have identified some genetic lesions associated more frequently with CE and with CLL showing adenopathy and confluent PCs. Finally, to translate this knowledge in clinical practice, we assessed prognosis with modern molecular techniques and we identified cytogenetic and molecular (somatic mutations) markers associated with disease progression and shorter time to treatment.

APPENDIX I

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Genetic subclonal complexity and miR125a-5p down-regulation identify a subset of patients with inferior outcome in low-risk CLL patients

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ABSTRACT:

The majority of patients with chronic lymphocytic leukemia (CLL) and favorable prognostic features live for long periods without treatment. However, unexpected disease progression is observed in some cases. In a cohort of untreated CD38- CLL patients with normal FISH or isolated 13q- we found that, by fluorescence in situ hybridization (FISH), 16/28 cases presented, within immunomagnetic sorted CD38+ cells, genetic lesions undetectable in the CD38- fraction. These patients showed a shorter time to first treatment (TTFT, p=0.0162) in comparison to cases without FISH lesions in CD38+ cells. Patients with FISH abnormalities in CD38+ cells showed a distinctive microRNA profile, characterized by the down-regulation of miR-125a-5p both in the CD38- and CD38+ populations. In an independent cohort of 71 consecutive untreated CD38- CLL with normal FISH or isolated 13q-, a lower miR125a-5p expression was associated with a shorter TTFT both in univariate and multivariate analysis (p=0.003 and 0.016, respectively) and with a higher prevalence of mutations (7/12 vs 0/8, p=0.015) as assessed by next-generation sequencing. In conclusion, our data showed previously unrecognized subclonal heterogeneity within the CD38+ fraction of CD38- CLL patients with low-risk FISH findings and suggested an association between down-regulated miR-125a-5p expression, genetic complexity and worse outcome.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease [1-3]. Several adverse prognostic features have been identified including stage [4], CD38 positivity [5], the unmutated configuration of the variable region of the immunoglobulin heavy chain gene (*IGHV*) [5], ZAP70 positivity [6], chromosome aberrations [7, 8] and molecular abnormalities [9].

Even though most of CLL patients with favorable prognostic features, i.e. CD38-, mutated IGHV, absence of chromosome lesions or isolated 13q-, live for long periods without any treatment, some cases may show progression to a more aggressive leukemia. The biologic and molecular characteristics predicting disease progression in these patients are unknown.

CD38 is considered as a dynamic indicator of cell activation and proliferation that may prelude clonal evolution and worse clinical outcome [10]. Interestingly, by representational oligonucleotide microarray analysis (ROMA), copy number differences between CD38+ and CD38- cells were documented in some patients [11] while the heterogeneity in the subclonal architecture of the leukemic cells was suggested to correlate with a poor

clinical outcome [12]. Moreover, in CLL distinct gene expression profiles were associated to CD38 expression [13] and a unique microRNA expression signature was shown to be associated with activation markers and unfavorable prognostic factors [14].

In order to better understand the biologic and molecular features predicting disease progression in CLL patients with favorable prognostic features we designed a two-phase study having the following aims: phase 1, a) to assess whether genetic lesions may be present in the minority of CD38+ cells in a series of untreated low-risk CLL patients as defined by CD38 negativity (CD38+ cells < 7%) and favorable genetic findings, b) to identify biologic factors associated with genetic lesions in the small CD38+ fraction of CD38- CLL patients and predicting for disease progression; phase 2 to validate our findings in an independent cohort of consecutive untreated CD38- CLL patients with favorable FISH findings.

Table 1: Principal clinical and biologic characteristics of the patients of the cohort 1 and cohort 2.

	Cohort 1	Cohort 2
N of patients	28	71
M/F	16/12	49/22
Age mean yrs (range)	65 (50-91)	64 (38-86)
Stage (Binet) a/b/c	28/0/0	63/8/0
FISH neg/13q deletion	14/14	40/31
ZAP70 (>30%) neg/pos	22/5	61/10
IGHV mut/unmut	20/2	60/11
TP53 mut/unmut	0/18	0/69

Table 2:FISH results in CLL patients with detectable genetic lesions in CD38+ cells (*bilallelic 13q deletion).

	FISH results on CD38- cells (%)	FISH results on CD38+ cells (% of positive cells)						
case		del(13q)	del(11q)	Trisomy 12	14q32 rearr	del(17p)	Number of additional lesions in CD38+ cells	Cohybridization
56	13q del (20%)	25	20	Neg	32	24	3	Different cells
58	13q del (30%)	28	30	Neg	28	18	3	Different cells
41	Neg	23	24	Neg	Neg	40	3	Different cells
50	13q del (60%)	37	21	15	Neg	33	3	ND
46	13q del (18%)	42	22	Neg	Neg	38	2	ND
49	13q del (60%)	37	23	Neg	21	20	3	Different cells
61	Neg	33	26	Neg	22	23	3	Different cells
43	Neg	34	Neg	Neg	27	Neg	2	Same cells
56	Neg	19	Neg	Neg	49	Neg	2	Different cells
63	Neg	20 (69*)	Neg	Neg	Neg	21	2	ND
45	13q del (69%)	58	33	17	Neg	Neg	2	Different cells
54	Neg	62*	Neg	Neg	18	Neg	2	Same cells
60	Neg	18	Neg	Neg	Neg	Neg	1	NA
48	13q del (38%)	45	20	Neg	Neg	Neg	1	Different cells
57	13q del (30%)	37	Neg	19	Neg	Neg	1	ND
64	Neg	34	Neg	33	Neg	Neg	1	Same cells

RESULTS

Patients

In this study 2 cohorts of patients have been considered. Cohort one (C1) included 28 untreated CLL patients seen between 2005 and 2006. Cohort two (C2) consisted of 71 consecutive untreated CLL patients diagnosed between 2007 and 2011. The principal clinical characteristics of C1 and C2 are reported in Table 1.

FISH analysis on immunomagnetically sorted cells in patients of C1

Results of FISH analysis in CD38- cells and CD38+ cells in C1 are reported in table 2. In 16/28 patients, genetic aberrations were detected in CD38+ cells and not in C38- cells. 11q deletion was seen in 9 cases, 13q deletion in 8 cases, 17p deletion in 8 cases, 14q32 rearrangements in 7 cases, trisomy 12 in 4 cases, biallelic 13q deletion in 2 cases (table 2). In the remaining

12 patients no additional genetic lesions were found in the CD38+ population as compared to the CD38- cells. Hybridization patterns with the control probes were within the expected normal range.

To assess whether the genetic lesions were on different clones, cohybridization experiments using appropriate probes were performed in 11 cases with >1 aberration in CD38+ cells. In these experiments it was shown that the genetic lesions involved different CD38+ cells in 8 cases and involved the same cells in the remaining 3 cases (Figure 1 supplemental).

miRNA profiling on immunomagnetically sorted cells in C1

We evaluated the global miRNA expression profile of 19 patients by considering CD38+ and CD38- cell

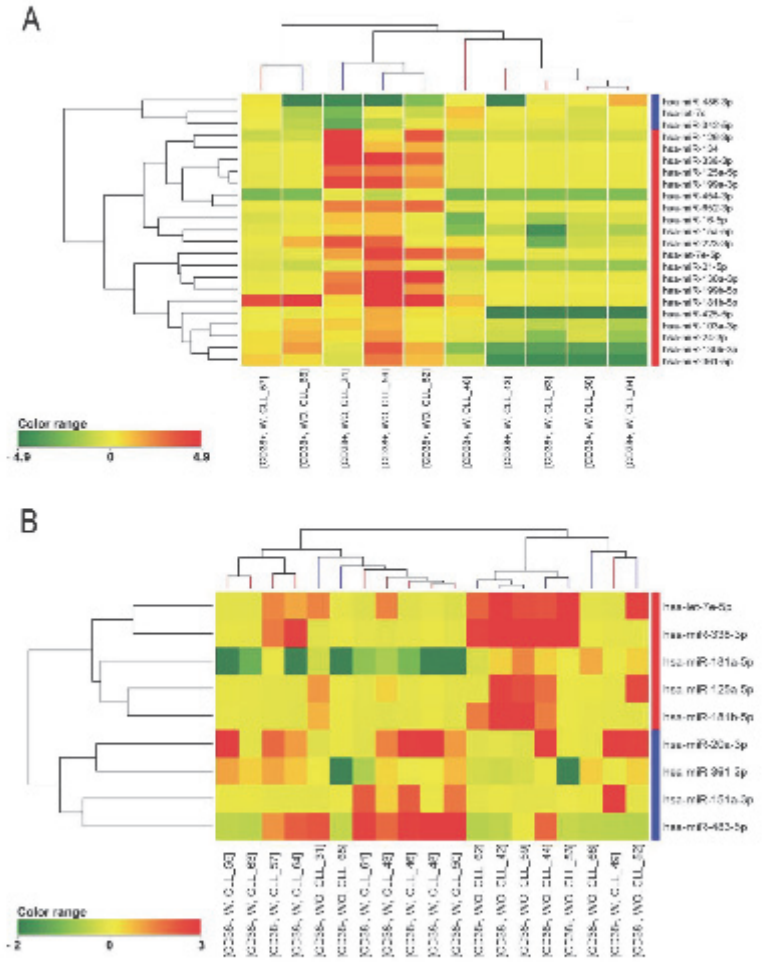

Figure 1:Cluster analysis of patients with (W) and without (WO) lesions in the CD38+ fraction. miRNA profiling of CD38+ (a) and CD38- (b) cells from CD38- CLL patients with (red) and without (blue) FISH lesions in the CD38+ fraction. A distinctive miRNA profile characterized patients with and without FISH lesions both in the CD38+ (23 microRNAs) and CD38- cells (9 microRNAs). The colors of the genes represented on the heatmap correspond to the expression values normalized on miRNA mean expression across all samples: green indicates down-regulated; red indicates up-regulated.

populations separately. We found that at diagnosis most of the patients with genetic lesions in CD38+ cells (W) had a distinctive miRNA profile when compared to those without genetic lesions (WO), both in the CD38+ (Figure 1A) and CD38- subpopulation (Figure 1B). Twenty-three miRNAs were found to be differentially expressed in CD38+ population (corrected $p<0.05$, Table 1 supplemental) and 9 miRNAs were found to be differentially expressed in CD38- population (corrected $p<0.05$, Table 2 supplemental). Four miRNAs were found to be down-regulated in patients with vs. without genetic aberrations in CD38+ cells both in the CD38+ and CD38- populations: let-7e-5p, miR-125a-5p, miR-181b-5p and miR-338-3p. Interestingly, miR-125a-5p showed the higher degree of significance both in CD38+ and CD38- subpopulations and was therefore chosen for further clinical correlations. The down-regulation of miR-125a-5p was confirmed by RT-qPCR analysis (Figure 2 supplemental).

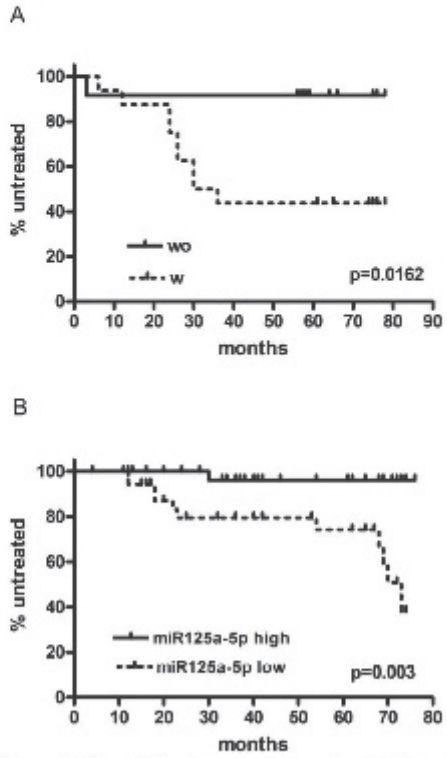

Figure 2: Time to first treatment in cohort 1 (A) and cohort 2 (B) respectively, according to the presence (W) or not (WO) of FISH lesions in CD38+ cells and to the level of expression of miR125a-5p (low or high), respectively.

Clinical outcome

In C1, the presence of additional FISH abnormalities in the CD38+ cells correlated with a more aggressive course of the disease that was characterized by a shorter TTFT (HR 8.052, range 1.332-16.760, p=0.0162, Figure 2a). No difference was instead observed between the 2 groups of patients concerning the principal clinical and biological characteristics (Table 3).

Having shown that miR-125a-5p down-regulation was strongly associated with additional FISH lesions on CD38+ cells and with shorter TTFT, we investigated in an independent cohort of 71 consecutive untreated low-risk CLL the clinical relevance of miR125a-5p expression. For the purposes of this analysis patients were subdivided into 2 groups based on the 50th percentile of miR125a-5p distribution (range 0.00276 – 6.57599 $\Delta\Delta$Cq, 50th percentile 0.85082 $\Delta\Delta$Cq). The median follow up was 43 months and no difference was observed between the 2 groups of patients in terms of clinical and hematologic characteristics (Table 3). However, patients with a lower miR125a-5p expression were characterized, by a more aggressive course of the disease and a shorter TTFT both in univariate and in multivariate analysis (Table 4 and figure 2b).

Mutational analysis by next generation sequencing (NGS)

Because down-regulation of miR125a-5p was associated with the presence of genetic lesions in a minor CD38+ fraction of the total neoplastic cells population in C1, we screened 20 consecutive patients in C2 by mutational analysis using next-generation sequencing detecting somatic mutations in minor cell fractions (sensitivity 5%) [15]. Seven out of 12 patients with low miR-125a-5p expression displayed mutations in the CLL population (table 5) as compared with no patients out of 8 cases with high miR-125a-5p expression (7/12 vs 0/8, p=0.015).

DISCUSSION

In CLL patients, there is evidence that a complex subclonal architecture of the leukemic clone may correlate with a more aggressive course of the disease and that in most cases genomic abnormalities are recurrent non-random events that expand over time due to a Darwinian selective pressure [11, 16].

CD38 is a marker of unfavorable prognosis and an indicator of cell activation and proliferation that may prelude clonal evolution and ultimately a worse clinical outcome [10]. Indeed CLL is a disease in which the host's microenvironment promotes leukemic cell growth, leading to sequential acquisition and accumulation of genetic

Table 3: Clinical and biological characteristic of the patients in cohort 1 and cohort 2 according to the presence/absence of additional FISH lesions in CD38+ cells and the level of miR-125a-5p expression, respectively.

	Cohort 1			Cohort 2		
	Patients with FISH lesions	Patients without FISH lesions	p	miR-125a low	miR-125a high	p
N of patients	16	12	-	35	36	-
M/F	9/7	7/5	ns	26/9	23/13	ns
Age mean yrs (range)	64 (50-91)	66 (52-80)	ns	64 (41-83)	64 (38-86)	ns
Binet stage a/b/c	16/0/0	12/0/0	ns	30/4/1	33/3/0	ns
ZAP70 (>30%) pos/neg	4/12	1/10	ns	6/29	4/32	ns
FISH neg/13qdeletion	8/8	6/6	ns	16/19	16/20	ns
IGHV mut/unmut	11/2	9/0	ns	30/5	30/6	ns
Treated /untreated	9/7	1/11	0.0159	11/24	1/35	0.001

Table 4: Factors affecting TTFT in univariate and multivariate analysis in the cohort 2.

variable	N of patients	Univariate analysis		Multivariate analysis	
		HR (95% CI)	p	HR (95% CI)	P
IGHV unmut/mut	11 vs 60	3.939 (1.66-66.97)	0.012	5.51 (1.40-21.63)	0.015
MiR-125a-5p high/low	36 vs 35	11.45 (1.80-17.60)	0.003	13.17 (1.40-21.63)	0.016
Stage b-c/a	8 vs 63	3.84 (1.29-87.40)	0.028	3.52 (0.88-14.131)	0.076
ZAP70 pos/neg	10 vs 61	1.45 (0.27-8.98)	ns	-	-
FISH normal /13q deletion	32 vs 39	0.97 (0.30-3.13)	ns	-	-

Table 5: NGS mutations as assessed by IonTorrent technology in 7 patients with low miR125a-5p expression (in patients 4 and 6, 2 different clones, a and b, were observed).

pat	Gene	Chr	Position	Location	Ref sequence	Var sequence	Mutation Type	Freq. %
1	DDX3X	chrX	41.204.747	exonic	GAAAGTAGTTT GGGTGGAAGA	GAAAGTAGTT---- TGGAAGA	Frame shift deletion	39,1
2	SF3B1	chr2	198.267.491	exonic	C	G	Non synonymous SNV	52,69
3	MYD88	chr3	38.182.641	exonic	T	C	stoploss SNV	10,83
4a	TP53	chr17	7.577.575	exonic	A	C	Non synonymous SNV	6,43
4b	TP53	chr17	7.577.580	exonic	T	C	Non synonymous SNV	18,89
5	XPO1	chr2	61.719.472	exonic	C	T	Non synonymous SNV	26,33
6a	ATM	chr11	108.138.003	exonic	T	C	Non synonymous SNV	64,06
6b	FBXW7	chr4	153.249.384	exonic	C	T	Non synonymous SNV	32,94
7	TP53	chr17	7.578.536	exonic	T	C	Non synonymous SNV	28,59

alterations [11]. Interestingly, there is also evidence that proliferation centers are enriched in CD38+ cells and that a high frequency of genetic lesions may accumulate at these sites [17].

In a cohort of untreated low-risk CLL patients as defined by CD38 negativity (CD38+ cells < 7%) and low-risk FISH findings (normal or 13q deletion as single aberration), we therefore studied the biological and clinical significance of the presence of genetic heterogeneity in the minor CD38+ leukemic population.

Our data showed that a significant proportion of CD38- CLL patients with low risk FISH findings presented genetic aberrations within CD38+ cells. Most of these abnormalities were high risk lesions (11q deletion in 9 cases, 17p deletion in 8 cases) and in most of the cases these lesions were found in different cells indicating

that multiple cytogenetically unrelated minor clones may be present in the CD38+ cell fraction. The small size of the abnormal C38+ clones precluded the detection of these aberrations when analyzing by FISH the entire neoplastic population consisting of a majority of CD38- cells. Interestingly, the presence of these additional FISH lesions in the small CD38+ cell fraction was associated with shorter TTFT.

Genetic complexity and heterogeneity in the architecture of the leukemic clone was previously documented in CLL and was associated with disease progression and shorter TTFT [18]. Moreover, Grubor et al [11] in 3 out of 4 CLL patients found genetic imbalances between CD38+ cells and CD38- cells, some of which involved loci of clinical relevance including ATM and TP53. However, it is noteworthy that our findings apply to a the subset of CLL patients with "favorable" prognostic features in terms of CD38 expression and cytogenetic data on the entire cell population.

To identify biomarkers associated with this phenomenon, we performed miRNA expression analysis because deregulation of miRNA was previously shown to be associated with activation markers [14]. By comparing C1 patients with and without small abnormal clones in the CD38+ fraction, we were thus able to show a deregulated miRNA expression profile in CLL cases with additional FISH lesions in CD38+ cells. In particular, miR-125a-5p was found to be down-regulated both in CD38+ and CD38- cells in patients with FISH abnormal clones as compared to patients without FISH abnormal clones. miR-125a-5p was therefore chosen as a marker associated with genetic complexity and possibly with a more aggressive clinical behavior as suggested by the shorter TTFT that we observed in these patients.

The relevance of miR-125a-5p as a biomarker of inferior outcome and genetic complexity was then validated in a prospective cohort of 71 consecutive untreated CD38- CLL patients with normal FISH or 13q deletion as single abnormality. In this validation cohort we were able to confirm the predictive role of miR-125a-5p down-regulation in terms of shorter TTFT. To our knowledge, this is the first observation linking a deregulated miRNA expression to inferior outcome in a subset of low risk CLL patients [19]. This finding is valuable because the majority of CLL patients present with low risk features at diagnosis and disease progression is difficult to predict in such cases.

In addition, in this validation cohort we found, through the use of NGS technology, that CLL patients with lower levels of miR-125a-5p displayed an increased rate of mutations in CLL-related genes. Several recent reports have correlated the presence of specific mutations, detected by NGS, to prognosis [9, 20]. Interestingly most of the mutations found in our patients, including TP53, SF3B1 and ATM, have been associated with a worse clinical outcome and prognosis. This observation further strengthens the association between genetic complexity, miR-125-5p down-regulation and worse outcome.

Alterations in miRNA expression are involved in the initiation, progression, and metastasis of several human cancers, by acting both as tumor suppressors and oncogenes in cancer development [21]. In particular, miR-125a-5p was previously found to act as a non-organ specific tumor suppressor gene that, when down-regulated, is associated, in several solid cancers, with a more aggressive course of the disease and a worse prognosis [22-26]. A germline mutation in mature miR-125a-5p has also been closely associated with breast cancer tumorigenesis [27]. This miRNA primarily achieves its antiproliferative effect through down-regulation of proliferation related genes, involved in the phosphoinositide-3 kinase (PI3K)-AKT and RAS/RAF/mitogen-activated protein kinase signaling [23]. Noteworthy, it was recently shown that in SMZL, an indolent B cell lymphoproliferative disorder like CLL, there is a characteristic deregulation of miRNA expression including down-regulation of miR-125a-5p with a possible implication in its molecular tumorigenesis [28]. By contrast, miR-125a was also found commonly gained and/or overexpressed in DLBCL [29] and in this perspective it would be interesting to look at miR-125a-5p levels in CLL patients that has transformed into Richter's syndrome.

In conclusion, (i) by FISH analysis we disclosed genetic lesions in the minor of CD38+ cell fraction in CD38- CLL with low-risk FISH findings, (ii) we provided evidence supporting an association between cryptic genetic lesions in CD38+ cells and disease progression, (iii) we found that genomic complexity and worse outcome were associated with miR-125a-5p down-regulation and, iv) we validated this finding in an independent cohort of untreated CD38- CLL patients with low risk FISH findings.

This set of data show that genetic lesions may appear in CD38+ cells in low-risk CLL and that they may be associated with miR-125a-5p down-regulation, allowing for a more accurate prognostication in low-risk CLL.

METHODS

Patients

In this study 2 cohorts of patients have been considered. Cohort one (C1) included 28 untreated CLL patients seen between 2005 and 2006. Cohort two (C2) consisted of 71 consecutive untreated CLL patients diagnosed between 2007 and 2011. Inclusion criteria were the following: diagnosis of CLL according to NCI criteria [30], CD38 negativity (CD38 percentage < 7%), good prognosis genetic lesions as defined as isolated

13q deletion or absence of genetic lesions by FISH, here referred to as FISH negativity, using a standard 4-probe FISH panel [31] (vide infra).

Indications for treatment included: increased WBC count with <6 month lymphocyte doubling time, anemia or thrombocytopenia due to bone marrow infiltration or autoimmune phenomena not responding to steroids, disease progression in the Binet staging system. Fludarabine-containing regimens were used as first-line treatment; chlorambucil was used in some elderly and unfit patients.

This study was approved by the local ethics committee and informed consent was obtained from the patients.

Immunophenotypic analysis

At diagnosis, immunophenotypic analysis was performed according to NCI criteria [30] as previously described [32]. The Matutes immunophenotypic score [33] was calculated giving 1 point each to CD5 positivity, CD23 positivity, CD22 weak positivity, sIg weak positivity and FMC7 negativity. Only patients with a score ≥3 (i.e. typical CLL) were included. The expression of CD38 and ZAP-70 were tested, as described [34], on fresh peripheral blood (PB) cells with a 7% and 30% cut-off for positivity, respectively.

Cell isolation by immunomagnetic sorting

In the 28 patients in C1, CD38+ and CD38- CLL cells were isolated by immunomagnetic sorting as previously described [35]. Briefly, PB mononuclear cells (PBMCs) were isolated by density gradient separation (Lympholyte-H Cedarlane, Cellbio. Milan Italy). In order to eliminate monocytes, T and NK cells and to obtain a cell fraction enriched in B-cells we first performed a negative selection with Dynabeads PanMouse IgG (Dynal A.S., Oslo, Norway) coated with anti-CD14, anti-CD3, and anti-CD16 monoclonal antibodies (clones UCHM1, UCHT1 and B-E16, respectively, all provided by Denamed, Ferrara, Italy). CD3, CD14 and CD16 negative cells (i.e. B cells) were subsequently subjected to a positive selection with Dynabeads coated with anti-CD38 antibody (clone T16, Denamed Ferrara, Italy). At the end of this procedure we obtained CD38+ and CD38- B cells. The purity of sorted CD38- and CD38+ B lymphocytes was > 98% as determined by flow cytometric analysis.

FISH analysis

For CLL risk assessment, interphase FISH was performed in all patients on PB samples obtained at diagnosis using probes for the following regions: 13q14, 12q13, 11q22/ATM, 17p13/TP53 (Vysis/Abbott Co, Downers Grove, IL, USA) as described [32]. Cut-off points for positivity were previously reported [32].

In C1 patients, FISH analysis was performed on both CD38+ and CD38- immunomagnetic sorted cells, as previously described [35], and the following regions were investigated: 13q14, 12q13, 11q22/ATM, 17p13/TP53 and 14q32 (LSI IGH) (Vysis/Abbott Co, Downers Grove, IL, USA). The following probes were used as controls: CEP10, LSI PDGFRB, LSI RUNX1/RUNX1T1, LSI BCR/ABL (Vysis/Abbott Co, Downers Grove, IL, USA). Co-hybridization experiments were performed in order to evaluate the coexistence on the same cells of more genetic lesions. The sensitivity limits for FISH analysis on sorted cells were calculated on 5 normal healthy controls as median values + 3 standard deviations and were set at 10% for translocations and trisomies, and at 14% for deletions.

IGHV and *TP53*

IGHV genes were amplified from genomic DNA and sequenced according to standard methods and the cut-off of 98% homology to the germline sequence was chosen to discriminate between mutated (<98% homology) and unmutated (≥98% homology) cases, as reported previously [8]. TP53 mutational analysis was performed as described elsewhere [8].

MicroRNA microarray

MiRNA expression was investigated using the Agilent Human miRNA microarray v.2 (#G4470B, Agilent Technologies). as previously described [36]. Microarray results were analyzed by using the GeneSpring GX v.12 software (Agilent Technologies). Data transformation was applied to set all the negative raw values at 1.0, followed by a Quantile normalization and a log2 transformation. Filters on gene expression were used to keep only the miRNAs expressed (Detected) in at least one sample. Then, samples were grouped according to the presence or not of genetic lesions by FISH analysis in the CD38 positive cells. Differentially expressed miRNAs were identified by using a 2 fold-change filter followed by a moderated t-test, with $p<0.05$ using a moderated t-test with Benjamini-Hochberg correction. Microarray experiments have been submitted to ArrayExpress database (accession number to be received).

Quantitative Real time RT-PCR (qRT-PCR)

Mature miRNAs expression was evaluated by Taqman MicroRNA assays (Applied Biosystem, Life Technologies, Foster City, CA, USA) specific for miR-125a-5p and normalized on 18S ribosomal RNA. Briefly,

5 ng of total RNA was reverse transcribed using the specific looped primer and quantitative real-time reverse transcription PCR (qRT-PCR) was conducted using the standard TaqMan MicroRNA assay protocol on a Bio-Rad-CFX thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Each sample was analyzed in triplicate. qRT-PCR for 18S rRNA was performed using 500 ng of total RNA for each sample according to the instructions of the manufacturer (M-MLV Reverse Transcriptase; Promega, Madison, WI, USA) and the real-time reaction using EvaGreen® Dye (Biotium, Hayward, CA, USA) on the Bio-Rad-CFX instrument. Each sample was analyzed in triplicate. The level of miRNA and mRNA was measured by the use of the quantitation cycle (Cq). The amount of target, normalized on 18S rRNA amount, was calculated using $2-\Delta Cq$ (comparative Cq) method as implemented by Biorad CFX Manager Software (Bio-Rad Laboratories, Hercules, CA, USA). Significance in qRT-PCR results was determined by t-test.

IonTorrent Personal Genome Machine (PGM) analysis

Agilent HaloPlex Target Enrichment kit (Agilent Technologies, Santa Clara, CA, USA) was used to construct libraries of spot exonic regions of 20 genes (ATM, BIRC3, BRAF, CDKN2A, CTNNB1, DDX3X, FBXW7, KIT, KLHL6, KRAS, MAPK1, MYD88, NOTCH1, NRAS, PIK3CA, POT1, SF3B1, TP53, XPO1, ZMYM3) starting from genomic DNA from peripheral blood samples, according to HaloPlex Target Enrichment System (Agilent Technologies, Santa Clara, CA, USA). Diluted libraries were linked to Ion Sphere Particles, clonally amplified in an emulsion PCR and enriched using Ion OneTouch emulsion PCR System (Life technologies, Foster City, CA, USA). Exon-enriched DNA was precipitated with magnetic beads coated with sptrptavidin. Enriched, template-positive Ion Sphere Particles were loaded in one Ion chip and sequenced using Ion Torrent PGM (Life technologies, Foster City, CA, USA). Sequencing data were aligned to the human reference genome (GRCh37). Data analysis and variants identification were performed using Torrent Suite 3.4 and Variant Caller plugin 3.4.4 (Life technologies, Foster City, CA, USA).

Statistical analysis

Quantitative variables were reported as mean values with standard deviations (SDs) and were compared using the Mann-Whitney test. The Fisher exact test was used for categorical variables. All tests were 2-sided. The time to first treatment (TTFT) was calculated as the interval between diagnosis and the start of first-line treatment. Survival curves were compared by using the log-rank test. A P value <.05 was used as a criterion for statistical significance. Proportional hazards regression analysis was used to identify the most significant independent prognostic variables on TTFT. Statistical analyses were performed using Stata software release 8.0 (Stata Corporation, College Station, TX, USA) and Prism 4.00 for Windows, GraphPad Software (San Diego California USA).

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Authorship contributions

G.M.R., M.N. and A.C. created and designed the study; G.M.R., F. Cibien, F. Cavazzini, M.C., G.D, and O.S. provided study materials and patients; L.R. and M.C. performed the FISH analysis; E.S., L.R., L.L., C.B., B.Z. and M.F. performed the molecular studies; G.M.R. S.M., L.F., M.C, and E.L. collected and assembled data; all authors assisted in the analysis and/or interpretation of the data; all authors critically revised the manuscript; and all authors gave final approval of the manuscript.

Conflict of interest disclosure

The authors declare no competing financial interests.

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APPENDIX II

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ORIGINAL ARTICLE

Proliferation centers in chronic lymphocytic leukemia: correlation with cytogenetic and clinicobiological features in consecutive patients analyzed on tissue microarrays

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To better define the significance of proliferation centers (PCs), the morphological hallmark of chronic lymphocytic leukemia (CLL), lymph node biopsies taken from 183 patients were submitted to histopathologic and fluorescence *in situ* hybridization (FISH) studies using a 5-probe panel on tissue microarrays. Seventy-five cases (40.9%) with confluent PCs were classified as 'PCs-rich' and 108 cases (59.1%) with scattered PCs were classified as 'typical'. Complete FISH data were obtained in 101 cases (55.1%), 79 of which (78.2%) displayed at least one chromosomal aberration. The incidence of each aberration was: 13q- 36,7%, 14q32 translocations 30.8%, 11q- 24.7%, trisomy 12 19.5% and 17p- 15.6%. Five cases showed extra copies of the 14q32 region. The 'PCs-rich' group was associated with 17p-, 14q32/IgH translocation, +12, KI-67 > 30%. The median survival from the time of tissue biopsy for PCs-rich and typical groups was 11 and 64 months, respectively (*P* = 0.00001). The PCs-rich pattern was the only predictive factor of an inferior survival at multivariate analysis (*P* = 0.022). These findings establish an association between cytogenetic profile and the amount of PC in CLL, and show that this histopathologic characteristic is of value for risk assessment in patients with clinically significant adenopathy.

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Keywords: FISH; chronic lymphocytic leukemia; small lymphocytic lymphoma; proliferation centers; 14q32 translocation

Introduction

Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) is a B-cell clonal lymphoproliferative disorder characterized by the accumulation of small lymphocytes in the peripheral blood, bone marrow and lymph nodes deriving from the transformation of a CD5+ B-cell. Recurrent molecular cytogenetic abnormalities may identify specific disease subtypes in CLL/SLL.[1,2] The most frequent aberrations include[3] 13q14 deletion (40–50% incidence); 11q22-23 deletion (10–20% incidence); total or partial trisomy 12 (10–15% incidence); and 17p13 deletion (2–7% incidence). The 17p/p53 deletion and the 11q- deletion involving the *ATM* gene were associated with an unfavorable outcome.[4,5] Evidence was also provided that 6q deletion may be found in a subset of SLL[6] and CLL with hyperleukocytosis and intermediate outcome[7,8] and that 14q32 translocations involving the immunoglobulin heavy (IgH) chain gene may be associated with unfavorable prognosis.[9,10]

A characteristic histopathologic feature of SLL/CLL is represented by 'proliferation centers' (PCs), which consist of regularly distributed pale areas without a mantle with numerous prolymphocytes and paraimmunoblasts, resulting in a pseudofollicular pattern.[11] Unlike reactive germinal centers, the PCs are CD10-, Bcl6-, IRF4+ and bcl2+,[12,13] and contain large Ki-67+ cells, admixed to typical B-CLL cells. They are visible on bone marrow and lymph node sections, and probably represent the sites where the T-dependent immune response to an unknown antigen promotes the selection and the clonal expansion of B-cells.[14] Although the size of PCs and the amount of paraimmunoblasts in lymph node sections did not show a correlation with clinical course,[15,16] the presence of more extensive PCs in follow-up biopsies compared with diagnostic biopsies has been reported.[17] Interestingly, a recent study suggested that an association may exist between the expanded and highly active PCs and an aggressive variant of CLL.[18]

Recently, techniques were developed that allow for the application of fluorescence *in situ* hybridization (FISH) on paraffin-embedded fixed tissues.[19,20] The preparation of tissue microarray (TMA) for FISH analysis[21] made this technique a potentially ideal method for the screening of large number of cases in a single hybridization experiment.

As—to the best of our knowledge—only few data are available on the cytogenetic profile of lymph nodes in CLL/SLL and the presence of chromosome lesions in correlation with the extension of PCs has never been previously tested, we designed the present study aiming to:

(i) analyze the sensitivity and reproducibility of FISH on paraffin-embedded fixed tissues arranged on TMA from 183 consecutive lymph node biopsies corresponding to histologically ascertained CLL/SLL,
(ii) estimate the frequency of chromosome lesions on lymph node samples and
(iii) analyze the possible correlation of specific chromosome lesions and the extension of PCs, and how these features may impact on clinical outcome.

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Patients and methods

Patients

A total of 183 consecutive patients with CLL/SLL diagnosed according to NCI criteria,[22] submitted to excisional lymph node biopsy for diagnostic purposes, were included in this study. All the patients had a clonal B-cell population with CD5+, CD19+, CD23+ and surface kappa/lambda light chain restriction.

Lymph node biopsy in these patients was performed in the presence of progressive disease[23] requiring treatment, with adenopathies ⩾3 cm. In all, 49% of these patients were untreated, 51% had received 1–3 lines of treatment (median 2 lines).

These patients were referred for histological diagnosis at the Hemolymphopathology Service of S.Orsola-Malpighi Hospital, Bologna University, Bologna, Italy between 2002 and 2008. The only selection criterion was represented by the availability of sufficient amount of paraffin-embedded tissue.

Histopathologic studies

Each biopsy had been fixed in formalin. Hematoxylin–eosin, Giemsa, Gomori and immunohistochemical stains performed at the time of diagnosis were always available. All cases were reviewed by three observers (SP, CA and ES). CLL diagnosis was confirmed in all instances based on cell morphology and phenotype.[11] The latter resulted: $CD3^-$, $CD20^+$ (the staining ranging from weak in the small B-cell component to moderate in PCs), $CD79a^+$, $CD5^+$, $CD23^+$, $IRF4^+$ in PCs, Cyclin $D1^-$, $CD10^-$, $BCL6^-$ and $BCL2^+$. Details of the antibodies and techniques applied are reported in Table 1.

The cases were classified into two categories according to the previously reported criteria[16]: 'PCs-rich' and 'typical'. The former included those cases with confluent PCs whereas the latter showed scattered, small, ill-defined PCs in a monotonous background of small, relatively round lymphocytes (Figure 1). No histological shift to DLBCL (Diffuse Large B-Cell Lymphoma) was recorded.[11]

Tissue microarrays

A slide stained with Giemsa was prepared from each paraffin block, and representative tumor regions including scattered or confluent PCs were morphologically identified and marked on every slide. Tissue cylinders with a diameter of 1.0 mm were punched from the marked areas of all blocks and brought into a recipient paraffin block using a precision instrument, as previously described.[24] The biopsies were arranged in seven TMAs, each containing material from 32–46 cases. In addition, a number of normal samples (spleen, thymus, tonsil or lymph node) were included in all TMAs as internal controls. Different copies of each TMA were prepared to allow immunohistochemistry and fluorescence *in-situ* hybridization FISH with a 5-probe panel (see below).

Immunohistochemistry

The 4-μm-thick sections were cut from each recipient block and tested with anti-ZAP70 and anti-Ki-67. Technical details are provided in Table 1.

The immunohistochemical results were independently scored by three pathologists (SP, CA and ES) as follows: +, >75% positive cells; +/−, 50–75% positive cells; −/+, 25–50% positive cells; rare, 10–25% positive cells; and −, <10%

Table 1 Details of immunohistochemistry

Antibodies	*Source*	*Clone*	*Dilution*	*Antigen retrieval method*	*Detection technique*
CD3	NeoMarkers, Fremont, CA, USA	SP7	1:300	Microwaving (5′ × 3 cycles at 750 W) in1 mM EDTA (pH 8.0)	APAAP/3 layers
CD20	Dako, Glostrup, Denmark	L26	1:500	Microwaving (5′ × 3 cycles at 750 W) in1 mM EDTA (pH 8.0)	APAAP/3 layers
CD79a	Dako, Glostrup, Denmark	JCB117	1:10	Microwaving (5′ × 3 cycles at 750 W) in1 mM EDTA (pH 8.0)	APAAP/3 layers
CD5	Dako, Glostrup, Denmark	54F6	1:5	Microwaving (5′ × 3 cycles at 900 W) in1 mM EDTA (pH 8.0)	APAAP/3 layers
CD23	Novocastra, Menarini Diagnostics, Grassina, Italy	1B12	1:30	Microwaving (5′ × 3 cycles at 900 W) in1 mM EDTA (pH 8.0)	APAAP/3 layers
IRF4	Professor Falini, Perugia, Italy	MUM-1p	1:4	Microwaving (5′ × 3 cycles at 900 W) in1 mM EDTA (pH 8.0)	APAAP/3 layers
Cyclin D1	NeoMarkers, Fremont, CA, USA	SP4	1:10	Microwaving (5′ × 3 cycles at 750 W) in1 mM EDTA (pH 8.0)	APAAP/3 layers
CD10	Novocastra, Menarini Diagnostics, Grassina, Italy	56C6	1:5	Microwaving (5′ × 3 cycles at 750 W) in1 mM EDTA (pH 8.0)	APAAP/3 layers
BCL6	Professor Falini, Perugia, Italy	PG-B6p	Undiluted	Microwaving (5′ × 3 cycles at 900 W) in1 mM EDTA (pH 8.0)	APAAP/3 layers
BCL2	Dako, Glostrup, Denmark	124	1:3	Microwaving (5′ × 3 cycles at 750 W) in1 mM EDTA (pH 8.0)	APAAP/3 layers
KI-67	Dako, Glostrup, Denmark	Mib-1	1:20	Microwaving (5′ × 3 cycles at 900 W) in1 mM EDTA (pH 8.0)	APAAP/3 layers
CD38	Novocastra, Menarini Diagnostics, Grassina, Italy	SPC32	1:10	Microwaving (5′ × 3 cycles at 750 W) in1 mM EDTA (pH 8.0)	APAAP/3 layers
Zap70	Upstate, Millipore, Billerica, MA, USA	2F3.2	1:80	PTLink (5′at 92 °C) in EnVision Flex Target Retrival Solution high PH	Dako REAL Detection System, Alkaline Phosphatase/RED Rabbit/Mouse

Abbreviations: APAAP, alkaline phosphatase anti-alkaline phosphatase complex; EDTA, ethylendiaminetetraacetic acid.

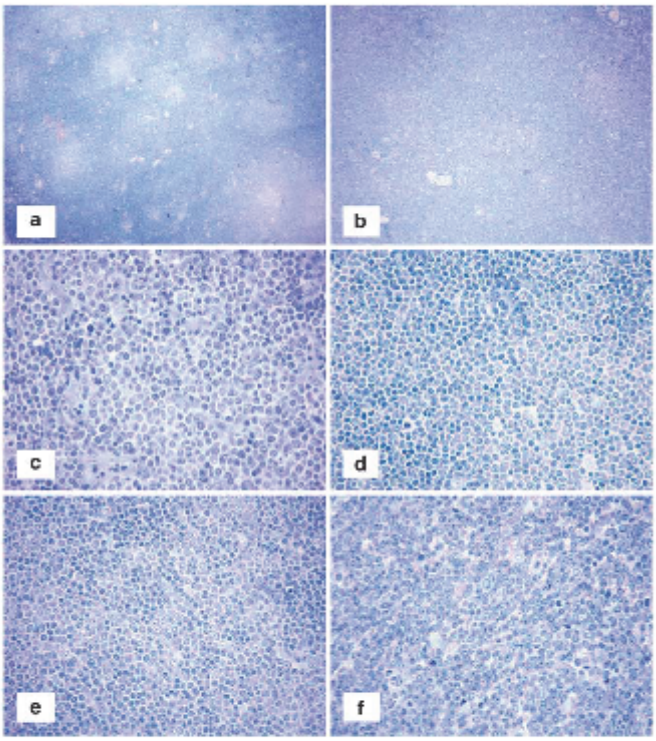

Figure 1 (a) Typical PC pattern at low magnification (Giemsa, ×40); (b) PC-rich pattern at low power: please note that PCs tend to merge together (Giemsa, ×40); (c) Cellular composition of a typical PC with a mixture of small lymphocytes, prolymphocytes and paraimmunoblasts (Giemsa, ×400); (d) An example of PC-rich case with predominant prolymphocytic composition (Giemsa, ×400); (e) In a case with PC-rich pattern, there is a mixture of prolymphocytes and paraimmunoblasts (Giemsa, ×400); (f) In another case with similar pattern, paraimmunoblasts predominate (Giemsa, ×400).

positive cells. The staining intensity was also assessed and recorded as strong, moderate or weak by comparison with the strong positivity of normal T lymphocytes and plasma cells for ZAP70. Those cases with moderate or strong intensity in at least 10% of the cells were classified as positive. Ki-67 staining was quantified on at least 10 40× high power fields in the tissue section that included both PCs and surrounding areas with a predominant small cell component. The cutoff point for positivity was set at 30%.

Fluorescence in situ *hybridization*

Preparation of the slides and hybridization. The 4-μm-thick sections were preferred for FISH assays, as recommended by Ventura *et al*.[19] to limit the number of nuclei truncation during sectioning and consequent loss of probe signals. Each TMA was submitted to hybridization using the following 5-probe panel in dual-colour hybridization tests using a chromosome 10 centromeric probe as internal control in each experiment: Spectrum orange LSI ATM DNA probe to the band 11q22.3; Spectrum orange LSI D13S25 DNA probe (band 13q14.3); Spectrum orange LSI p53 probe to the band 17p13.1; Spectrum orange CEP 12 alpha satellite probe to the centromere band of chromosome 12 and Spectrum green CEP 10 alpha satellite probe to the centromere of chromosome 10; and LSI IGH Dual Color, break-apart rearrangement probe (*14q32/IgH* gene breakpoint). These probes were purchased by Vysis Co (Downers Grove, IL, USA), distributed by Abbott Co (Rome, Italy). As previously suggested,[25] the slides were submitted to a dewaxing step consisting of heating at 55 °C for 10 or more minutes followed by washing in xilene for 10 min ×3 times at room temperature. Subsequently, the slides were dehydrated twice in ethanol 100% for 5 min. The demasking step was performed combining chemical treatment and high temperature: thus the slides were immersed in a 1 mmol ethylendiaminetetraacetic acid solution in pressure cooker at boiling point temperature for 4 min. To reduce background noise and enhance signal intensity, the slides were bathed in a proteolytic solution containing chloride acid and pepsin for 14 min at 37 °C. Finally, the slides were fixed in 10% formalin solution, re-hydratated in an inverse ethanol alcohol series (100, 85 and 70%) and dried at room temperature. Each probe (10 μl) was added to each slide and covered with a coverslip. Rubber cement was used to seal the edges, and the slides were placed in the co-denaturation/hybridization device (ThermoBrite, Abbott Co).

DNA denaturation was obtained at a melting temperature of 80 °C for 22 min followed by overnight hybridization with each probe by incubation at 38 °C. Post-hybridization washes included baths at 75 °C in 0.4 × SSC/0.3% NP-40 (Vysis Co) for 2 min, and at a room temperature in 2 × SSC/0.1% NP-40 for 1 min. A 10 µl of DAPI II counterstain (Vysis Co) was applied to the target area and a coverslip was added.

FISH signal screening. Evaluation of FISH results was performed on a fluorescence-equipped microscope (Nikon Italia, Milan, Italy) with a charge-coupled black and white camera, and appropriate hardware and software. The results of FISH labeling were interpreted and scored by one investigator (MC) unaware of the histological data. Depending on the amount of cells for each core section, at least 100 and usually 200–300 nuclei were manually scored. To ensure accuracy of the analysis, scoring was performed on almost the entire core section, and both cores were screened for each case. Signal screening was performed in those areas without excessive overlapping, avoiding areas where nuclei borders were not clearly shaped. For the break-apart probe, a nucleus with a fusion signal (normal signal), one red and one green signal (split signal), was scored as translocated. The presence of one fusion signal and one single-color signal (either red or green) was considered as indicative of deletion of part of the Ig gene, a phenomenon reflecting physiologic rearrangement of the IgH gene.[26]

Cut-off for positivity. To establish the cutoff, we used 10 cases of reactive tonsil, spleen and thymus tissues arranged in the five TMAs plus two reactive lymph nodes on two separate slides. The cutoff point for positivity was set at 43% for probes detecting deletions, at 4.1% for probes detecting trisomy 12 and at 6.1% for the 14q32/IgH translocation probe, corresponding to the mean false-positive values obtained on the 12 control–cases plus 3 s.d.'s[19] A case was classified as carrier of deletion or trisomy provided that a concomitant normal hybridization pattern was observed with the chromosome-10-centromeric probe. As previously described by Meiner *et al*.[27] amplification was defined as the presence of tumor cell nuclei with three or more signals exceeding the mean plus 3 s.d. of tri-/polysome nuclei in the reference cases.

To further verify the specificity and accuracy of our method, FISH was performed on tissue sections of 18 cases of CLL/SLL with known cytogenetic lesions, detected by FISH on cell suspensions obtained from the same lymph node biopsy.

Immunoglobulin heavy chain variable region (IGHV) mutational status

IGHV mutations were studied in 67 patients with a complete set of clinicobiologic data for whom sufficient material was available. Genomic DNA was isolated from paraffin-embedded lymph nodes using QIAmp DNA mini kit (Qiagen, Milan, Italy), and subjected to amplification of the VDJ (variable/diversity/joining) gene rearrangements by polymerase reaction (PCR). In particular, IGHV rearrangements were amplified with family-specific primers hybridizing to sequences in the framework region 1 or framework region 2 in conjunction with JH primers, in separate reactions for each VH family.[28] The PCR products, after ethidium bromide stained agarose gel electrophoresis, were spin column purified (Promega, Milan, Italy) and directly sequenced. IGHV sequences were aligned to the international ImMunoGeneTics database (IGMT http://imgt.cines.fr; initiator and coordinator: Marie-Paule Lefranc, Montpellier, France) using the IgBLAST software (http://www.ncbi.nlm.nih.gov/igblast/). IGHV sequences were considered mutated if deviation from the corresponding germline gene was >2%.

Statistical analysis

Quantitative variables were reported as mean values with s.d.'s and were compared using Mann–Whiney test. The Fisher exact test was used for categorical variables. All tests were two-sided. The overall survival was calculated as the interval between biopsy and the last follow-up. Survival curves were compared using the log-rank test. A *P*-value < 0.05 was used as a criterion for statistical significance. Proportional hazards regression analysis was used to identify the most significant independent prognostic variables on overall survival. Statistical analyses were obtained using Stata software release 8.0 (Stata Corporation, College Station, TX, USA).

Results

FISH studies

Efficiency of hybridization. Hybridization with each single probe was successful in 61.7–80.3% of the cases (Figure 2), as shown in Table 2. Assessable data for the complete 5-probe panel were obtained in 101/183 cases (55,1%). In 58 cases (31.6%) assessable data were obtained with 1–4 probes, whereas no data were obtained in 24 cases (13.1%). The efficiency of hybridization was comparable in 114 cases biopsied between 2002 and 2005 (successful analysis in 54.3% of the cases) and in 69 cases biopsied between 2006 and 2008 (56.5% success rate). Technical failures (see Table 2) were accounted for by the absence of adequate number of cells on TMA corresponding cores in 12.5–26.2% of the cases; inefficient hybridization and overlapping nuclei precluded accurate signal screening in 7.1–13.2% of the cases.

In 18 cases with known cytogenetic aberrations on conventional cell suspensions obtained from fresh lymph node biopsy, FISH analysis on paraffin-embedded tissues confirmed the presence of 11q- (8/18 cases), +12 (5/18 cases), 13q- (7/18 cases), 14q32/IgH translocation (6/18 cases) and of 17p- (6/18 cases), in the absence of false-positive results.

Incidence of chromosome lesions. The incidence of each single aberration was as follows: 13q14 deletion 36.7% (54/147 cases), 14q32 translocations 30.8% (42/136), 11q deletion 24.7% (28/113), trisomy 12 19.5% (26/133) and 17p deletion 15.6% (23/147). Five cases showed amplification of the *14q32*/IgH gene (that is, 3–7 fusion signals). Concomitant aberrations in these five cases were: 17p- in two cases, 14q32 translocation, +12 and 11q- in one case each.

There was no significant difference in the incidence of 13q-, +12, 11q- and 14q32 translocations in untreated vs pretreated patients, whereas 17p- occurred more frequently in treated patients as compared with untreated patients (26% and 6.5% of the cases, respectively).

A total of 79 out of 101 patients successfully analyzed with the 5-probe panel (78.2%) showed at least one chromosomal aberration, as shown in Table 3. Using a cytogenetic classification assigning every patient to a single category according to the hierarchy 17p- >11q- >14q32 >+12 >13q-,[3,9] the 11q deletion was the most frequent cytogenetic anomaly accounting for 20% of the cases, followed by 17p- (17%), 14q32/IgH translocation (16%), 13q- (15.8%) and trisomy 12 (11%).

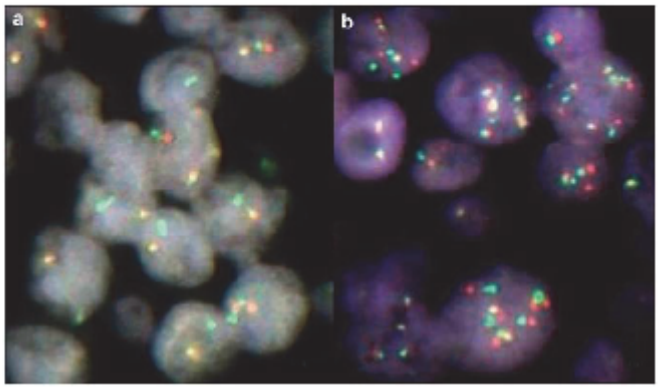

Figure 2 (a) The presence of one fusion signal (yellow) and of one green and one red signal (split signals), is indicative of 14q32/IgH translocation. (b) 14q32/IgH amplification documented by the presence of multiple nuclei with ⩾3 fusion signals; the presence of red and green split signals indicates concomitant 14q32/IgH translocation.

Table 2 Efficiency of FISH studies on samples arranged on TMA

Type of probe	*No. of cases successfully analyzed (%)*[a]	*Reason for failures (%)*	
		Lack of cells	*Other*[b]
17p-	147/183 (80.3)	23/183 (12.5)	13/183 (7.2)
11q-	113/183 (61.7)	48/183 (26.2)	22/183 (12.1)
14q32/IgH	136/183 (74.3)	23/183 (12.5)	24/183 (13.2)
+12	133/183 (72.7)	28/183 (15.3)	22/183 (12)
13q-	146/183 (79.8)	24/183 (13.1)	13/183 (7.1)
No. of probes			
5	101/183 (55.1)		
4	26/183 (14.2)		
3	15/183 (8.2)		
2	8/183 (4.3)		
1	9/183 (4.9)		

Abbreviations: FISH, fluorescence *in situ* hybridization; PC, proliferation center; TMA, tissue microarray.
[a]24/183 cases (11 cases 'PC-rich' and 13 cases 'typical') not evaluable.
[b]'Other' include a majority of cases showing poor hybridization and some cases with overlapping nuclei precluding accurate interpretation of the results.

Histopathologic subtypes and their association with biologic features

A total of 108/183 cases (59.1%) with scattered PCs were assigned to the typical subtype. A total of 75/183 cases (40.9%) were assigned to the PCs-rich subtype, owing to the presence of confluent PCs. In 35 cases (19.1%) paraimmunoblasts represented the predominant cell population within the PCs, in 14 cases (7.6%) the vast majority of cells were prolymphocytes and in 26 cases (31.3%) a mixture of paraimmunoblasts and prolymphocytes was seen. Notably, none of the cases with predominant paraimmunoblastic cell population within confulent PCs had the clinical characteristics of Richter's syndrome.

When considering those cases with successful hybridization with each single probe, 17p-, +12 and 14q32/IgH translocation were more frequently encountered in the PCs-rich subtype than in the typical subtype (20/60 cases vs 3/87 for 17p-, $P<0.001$; 15/52 vs 11/81 for +12, $P=0.030$; and 22/54 vs 20/82 for 14q32 translocations, $P=0.043$).

Ki67 was positive in >30% of the cells in 27/164 cases successfully tested: 4/95 Ki-67+ cases belonged to the 'typical'

Table 3 Frequency of chromosome aberrations according to a hierarchical classification[a] in 101 cases successfully analyzed with the complete 5-probe panel

Abnormality		*No. of cases*[b] *(%)*	*% Abnormal cells, range (median)*
17p-		17/101 (17)	46–94 (82)
	17p- single	1	
Additional aberrations	11q-	2	
	+12	2	
	14q/IgH	2	
	13q-	2	
	11q-, +12	1	
	+12, 14q32/IgH	1	
	14q32/IgH, 13q-	3	
	11q-, 14q32/IgH, 13q-	2	
	11q-, +12, 13q-	1	
11q-		20/101(20)	47.5–96 (66)
	11q- single	5	
Additional aberrations	+12	1	
	13q-	4	
	14q32/IgH, 13q-	5	
	+12, 13q-	1	
	14q32/IgH	4	
14q32/IgH		16/101 (16)	24–72 (50)
	14q32/IgH single	9	
Additional aberrations	+12	2	
	13q-	5	
+12		11/101 (11)	7.2–48 (31)
	+12 single	10	
Additional aberrations	13q-	1	
13q-	13q- single	15/101 (15.8)	48.4–89.8 (81)

[a]Hierarchical classification as follows: 17p- >11q- >14q32/IgH >+12 >13q-.
[b]22 cases (21.8%) did not show any abnormality.

subtype as compared with 23/69 Ki-67+ CLL in the PCs-rich group ($P<0.0001$). ZAP70 was positive in 91/149 (61,1%) and there was no statistically significant difference between cases classified as typical CLL PCs-rich CLL (vide infra).

A thorough analysis of existing correlations between histological features and clinicobiologic parameters was performed in 101 patients with successful hybridization with the complete 5-probe panel (Table 4). These 101 patients were representative of the entire series in terms of incidence of confluent PCs (37% vs 40.9% in the total series, $P=$NS) and of scattered PCs (64% vs 59.1% in the total series, $P=$NS). IGHV usage in correlation with mutational status in 67 cases is shown in Figure 3. Rearranged IGHV families recurrently involved were as follows: IGHV1-69: 19.4%; IGHV3-30: 11.9%; IGHV3-7: 7.4%; IGHV3-23, IGHV3-48, IGHV4-34 and IGHV4-39: 6% each. Others IGHV families were involved in >5% of the cases.

As shown in Table 4, 17p-, 14q32/IgH translocations, +12 and 'unfavorable' FISH abnormalities (that is, 11q- and 17p-) were more frequently encountered in the PCs-rich subtype than in the typical subtype. Likewise, Ki-67 positivity in >30% of cells, being pretreated at time of biopsy and longer interval between diagnosis and biopsy, were associated with the PCs-rich subtype, whereas no significant association was noted between ZAP-70+, IGHV mutational status and histological subtypes.

Table 4 Clinical features and demographics of the 101 patients with cytogenetic data obtained with the 5-probe panel

	Typical (No. of cases)	*PCs- rich (No. of cases)*	*P-value*
Age (mean)	63.9 (sd 10.4)	65.0 (sd 12.3)	NS
Sex			
F	24	17	NS
M	40	20	
Stage at time of biopsy			
0–2	27	15	NS
3–4	25	18	
17p-			
No	62	22	<0.001
Yes	2	15	
11q-			
No	49	26	NS
Yes	15	11	
14q32/IgH translocations			
No	50	18	0.002
Yes	14	19	
+12			
No	53	25	0.021
Yes	8	12	
13q-			
No	35	27	NS
Yes	29	10	
Unfavorable FISH (11q- and/or 17p-)			
No	48	16	0.001
Yes	16	21	
ZAP-70+ (n=84)			
Positive	33	17	NS
Negative	21	13	
Ki67 (n=92)			
⩾30%	4	12	0.001
<30%	53	23	
IGHV (n=67)			
Unmutated	28	19	NS
Mutated	14	6	
Time between diagnosis and biopsy (mean, months) (n=72, typical: 39; PCs-rich: 33)	21.2 (95% CI 0.0-83.0)	32.4 (95% CI 0.0-79.0)	0.006
Pre-treated at time of biopsy (n=86)			
Yes	13	20	0.002
No	39	14	

Abbreviations: CI, confidence interval; F, female; FISH, fluorescence *in situ* hybridization; IGHV, immunoglobulin heavy chain variable region; M, male; NS, not significant; PCs, proliferation centers.

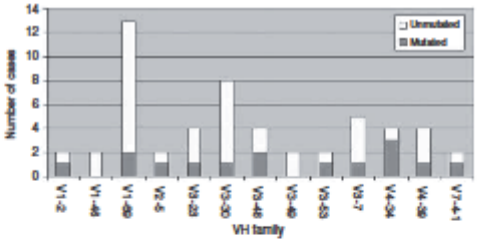

Figure 3 Recurrent usage of VH families and mutational status in 67 cases.

Table 5 Analysis of factors affecting survival from the time of biopsy

Univariate analysis variable	*No. of patients*	*Median overall survival (se)*	P
Histology			
Typical	52	64 (1.6)	0.00001
PC-rich	34	11 (2.7)	
Ki67			
⩾30%	14	4 (0.3)	0.0002
<30%	64	64 (5.6)	
ZAP70			
Positive	43	47 (4.5)	NS
Negative	27	64 (16.4)	
Stage at biopsy (n=57)			
0–2	42	64 (1.9)	NS
3–4	43	34 (8.5)	
FISH			
Favorable Intermediate	50	64 (1.9)	0.046
Unfavorable (11q- and/or 17p-)	35	24 (3.7)	
IGHV			
Unmutated	41	42 (3.3)	NS
Mutated	16	36 (5.1)	
Pre-treated at time of biopsy			
Yes	33	18 (2.8)	0.0073
No	53	64 (1.9)	

Abbreviations: FISH, fluorescence *in situ* hybridization; IGHV, immunoglobulin heavy chain variable region; NS, not significant; PC, proliferation center.

As shown in Table 5, the median survival from the time of tissue biopsy for PCs-rich and typical subtypes in 86 patients for whom clinical data could be obtained was 11 and 64 months, respectively ($P=0.00001$) (Figure 4a). Other variables that influenced negatively the survival in our series were unfavorable cytogenetic abnormalities (17p- and/or 11q-), Ki-67>30% and being pretreated at the time of biopsy.

Survival in 47 patients, not included in the multiparameter analysis due to incomplete FISH data, was shorter in 24 PC-rich cases than in 23 typical cases ($P=0.04$) (Figure 4b).

A Cox proportional-hazards analysis showed that only PCs-rich pattern retained predictive value of poor outcome (HR 2.74, 95% CI 1.16–6.51, $P=0.022$), while the presence of high-risk cytogenetic abnormalities, treatment status and Ki67>30% did not have an independent impact on survival.

Discussion

As tissue biopsy is not mandatory for the diagnosis of CLL, few studies addressed the significance of histological findings[15,16] and the incidence of chromosomal aberrations at the tissue level in this setting.[29,30]

The TMA technology offers the possibility to analyze simultaneously a consistent number of cases thus reducing costs and experimental variability.[23,24] To the best of our knowledge, the present study represents the most extensive analysis conducted on biopsies of patients affected by CLL/SLL. A preliminary methodological problem with FISH on histological preparations is represented by the accuracy of signal screening, in relation to the thickness of the section. On the one hand, a thick section may influence the cutoff point because overlapping nuclei would increase both the false-positive rate for trisomies and the false-negative rate for deletions; on the other hand, a thin section would cause major truncation artefacts, rendering more probable the loss of one or more signals, yielding false deletions and falsely normal results in the presence of trisomy. To ensure the accuracy of FISH data in our study, we elected (i) to adopt in all sections the same 4-μm thickness, which proved to be the most reasonable compromise in our experience, in line with previous studies,[29] (ii) to calculate the cutoff for positivity in a large number of controls (12 cases) with various reactive conditions, (iii) to use an internal control probe in each hybridization to ensure accuracy of signal screening, (iv) to use the same criteria for the evaluation of all cases, selecting the best area of the core section (that is, minimal nuclei overlapping and truncation) for signal screening and (v) to confirm the reproducibility of our method by comparing the results on tissue samples and on fresh cell suspensions in 18 patients with known cytogenetic aberrations.

In this study, hybridization efficiency was very good, with a 55.1% overall success rate using a 5-probe panel in an unselected series of 183 cases.[30] Excluding those cases in which failure was a consequence of lack of tissue, assessable results were obtained in 86.8 to 92.8% of the cases with each single probe (Table 1). Interestingly, there was similar hybridization efficiency in biopsies performed between 2002–2005 and 2006–2008, providing evidence that FISH on TMA under these stringent methodological conditions is an efficient screening tool to identify archival material-specific chromosome lesions in lymphoproliferative disorders.

A second methodological consideration should be raised concerning patient selection for this study. As in previous studies,[23] patients with progressive disease and clinically relevant adenopathy were included in our analysis. Therefore, the patient population was not representative of the entire CLL population seen at the referring centers during the study period, and FISH analysis was performed in biopsies taken in different phases of the history of the disease.

In line with these considerations, the IGHV usage in our cases showed frequent *V1-69* gene involvement, mostly in the unmutated configuration, a relatively low VH4-34, frequently found in mutated CLL,[31] and very low incidence of other VH subtypes normally associated with indolent CLL or monoclonal B-cell lymphocytosis.[31] An unexpectedly high 12% incidence of V3-30 involvement was seen in our series. Unlike previous reports, describing V3-30 usage in association with mutated CLL running an indolent course,[32,33] 7/8 cases using *V3-30* genes in this series lacked IGHV somatic mutations (Figure 3), suggesting that the mutational status might be an important determinant of outcome within specific IGHV families.

The 78.2% overall incidence of chromosome lesions in our study using a 5-probe panel is in line with previous analyses performed on PB samples obtained from untreated and pretreated patients.[3,8] Caraway *et al.*[30] found abnormal FISH signal patterns in 81% of CLL/SLL cases on fine-needle aspirates, and Flanagan *et al.*[29] described at least one cytogenetic aberration in all 18 patients analyzed on lymph node biopsy.

Interestingly, while confirming that the occurrence of 17p- was more common in relapsed CLL,[34] other aberrations were found in our series to occur at a similar incidence in untreated and in treated patients. 11q deletion is usually associated with a disease characterized by marked lymphadenopathy.[5] As the incidence of 11q- may be higher in patients with therapy-demanding disease independent by previous treatments,[35] the relatively high incidence of 11q- in our series

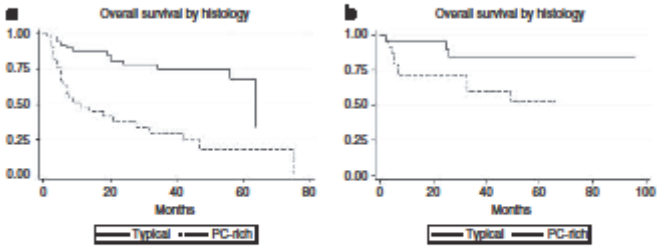

Figure 4 Survival in patients according to the histological pattern. Confluent PCs (i.e. PC-rich) identify patients with an inferior outcome. (a) 86 patients with a complete set of clinicobiologic data; (b) 47 patients were not included in the multiparameter analysis owing to insufficient information in the clinical charts.

(24.7%) may be accounted for by inclusion in this study of patients with clinically significant adenopathy.

A previously unreported finding was represented by a high incidence (30.8%) of 14q32/IgH translocations both in treated and in untreated patients. Three possible explanations may account for this observation: i) 14q32 translocation may appear more frequently at the tissue level than in the PB, as previously suggested for trisomy 12.[36] It is worth noting that cores for microarray construction were obtained from those areas with PC in the original lymph node section, and that PC may be viewed as a genetically unstable proliferation compartment releasing small lymphocytes in the accumulation compartment and in the PB[34]; ii) 14q32 translocation may be associated with adenopathy and active disease requiring treatment, as suggested in two recent analyses[9,10]; and iii) 14q32 translocation may represent in some of our cases a secondary anomaly acquired late during the course of the disease. Indeed, karyotype instability was detected in a fraction of CLL,[37–39] and 14q32/IgH translocation may represent a late event in the progression of lymphoid neoplasias.[40] Interestingly, recent analyses on a limited number of CLL patients reported a 17–21% incidence for these translocations in lymph node samples,[29,41] and 18 independent lymph node biopsies submitted to FISH analysis on isolated cell suspension in this study gave a 33% incidence for this aberration.

Furthermore, we detected five cases with a minority of cells showing IgH amplification as additional aberration, a finding previously unreported in molecular cytogenetic studies conducted on PB samples.[42,43] Interestingly, the 14q32/IgH region was recently found to be involved in the genesis of complex rearrangements, referred to as 'complicons', including multiple rearrangements and gene amplification.[44] Further study is required to clarify how and at what extent gains of chromosome material surrounding the Ig genes at 14q32 could contribute to the pathogenesis of B-derived lymphoproliferative diseases.

To our knowledge, this is the first study analyzing the correlation between the histological features and the cytogenetic profiles in CLL.

The frequency of PCs-rich CLL in our series was 40.9%, and 14q32/IgH translocations, +12 and 17p-, were significantly associated with the PCs-rich group.

Unfavorable cytogenetic features, such as 11q-/17p-, were more frequently encountered in the PCs-rich group than in the typical group. The presence of prominent PCs, which was associated with ki-67 + in our analysis, is thought to be an index of B-cell proliferation capability, which increases the risk that DNA replication errors could happen, thus predisposing the cell to the acquisition of sequential genetic damage. Likewise, *ATM* and *p53* gene deletions impair the checkpoint cell machinery and render the cell more fragile to antigenic stimuli encountered at PCs level. These considerations may provide a biological basis to explain the strong unfavorable prognostic significance of confluent PC in SLL/CLL that was found in our study. Interestingly, Gine *et al*.[18] recently described a new histological category, that is, 'accelerated' CLL, including 23 out of 78 (29.4%) cases with enlarged and confluent PCs, with >2.4 mitosis per PCs and >30% Ki-67 per PCs. This histological subset of CLL was associated with adverse biological features and inferior survival. Our data support and reinforce this view, in that they show that confluent PCs are associated with adverse cytogenetics and represent the strongest prognostic factor.

In conclusion, we have demonstrated that FISH on TMA is a convenient tool for the detection of cytogenetic aberrations in CLL/SLL. The association of PCs-rich pattern with unfavorable cytogenetics and short survival may support the pathogenetic role of 'proliferation center' in CLL. As the patients included in this analysis represented an unselected cohort of CLL/SLL undergoing biopsy due to clinically significant adenopathy, we found a high incidence of adverse biologic features, that is, unmutated IGHV configuration, ZAP70+, and 'high risk' cytogenetics. While the latter features did not have prognostic predictivity at multivariate analysis in this clinically aggressive population, our study demonstrated that the histopathological pattern defined by the presence of confluent PCs may represent an important feature for risk assessment in this subset of patients. This finding is important when considering that increasing awareness of the possible evolution into Richter's syndrome is prompting clinicians to perform lymph node biopsy more often than in the past,[45] and that the therapeutic armamentarium in clinically aggressive CLL may include intensive chemoimmunotherapy[46] and allogeneic bone marrow transplantation from HLA-identical siblings and well-matched unrelated donors.[47,48]

Conflict of interest

The authors declare no conflict of interest.

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APPENDIX III

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ORIGINAL ARTICLE: RESEARCH

Clonal evolution including 14q32/*IGH* translocations in chronic lymphocytic leukemia: analysis of clinicobiologic correlations in 105 patients

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Abstract

To better define the significance of clonal evolution (CE) including 14q32 translocations involving the immunoglobulin heavy chain gene (*IGH*) in chronic lymphocytic leukemia (CLL), 105 patients were analyzed sequentially by fluorescence *in situ* hybridization (FISH) with the following panel of probes: 13q14/D13S25, 11q22/*ATM*, 17p13/*TP53*, #12-centromere and 14q32/*IGH* break-apart probe. CE was observed in 15/105 patients after 24–170 months (median 64). Recurring aberrations at CE were 14q32/*IGH* translocation in seven patients; other aberrations were 17p −, 11q −, biallelic 13q − and 14q32 deletion. CE was detected in 15/58 pre-treated patients; in contrast, none of 47 untreated patients developed CE ($p < 0.0001$). In two cases the appearance of 14q32/*IGH* translocation was first detected in the bone marrow (*BM*) or in the lymph node (LN) and 13–58 months later in the peripheral blood (PB). ZAP70 + and high-risk cytogenetics predicted for the occurrence of CE with borderline statistical significance ($p = 0.055$ and 0.07, respectively). Shorter time to first treatment (TTT) and time to chemorefractoriness (TTCR) were noted in 15 patients with CE when compared to patients without CE (TTT: 35 vs. 71 months, $p = 0.0033$ and TTCR: 34 vs. 86 months, 0.0046, respectively). Survival after the development of CE was 32 months (standard error 8.5). We arrived at the following conclusions: (I) 14q32/*IGH* translocation may represent one of the most frequent aberrations acquired during the natural history of CLL and (II) it may be detected earlier in BM or LN samples; (III) CE including 14q32/*IGH* translocation occurs in pre-treated patients with short TTT and TTCR; (III) survival after CE is relatively short.

Keywords: Chronic lymphocytic leukemia, cytogenetics, clonal evolution, *IGH* translocation, chemorefractory disease

Introduction

At diagnosis, up to 80% of chronic lymphocytic leukemias (CLLs) can be shown to harbor clonal chromosome aberrations [1,2], some of which may have important clinical implications [1–3].

Despite previous claims that CLL is a genetically stable disease [4], clonal evolution (CE) was more recently reported in 15–42% of CLLs using conventional karyotyping [5] or fluorescence *in situ* hybridization (FISH) methods [6–9]. The incidence of this phenomenon is variable, depending on the length of follow-up [8] and on the number of probes used for interphase FISH analysis [7]. In previous studies, CE was defined by the late appearance of aberrations of chromosomes 17p, 11q, 6q and 12 [9,10], and was associated with markers of active disease, i.e. ZAP70 positivity and unmutated *IGHV* gene [8,9].

Besides the classical CLL-associated aberrations, i.e. 13q − (40% of cases), + 12 and 11q − (10–15% of cases) and 17p − (2–5% the cases) [1,2], other recurring chromosome changes have been described in CLL [11], and attention was recently devoted to 14q32 translocation involving the immunoglobulin heavy chain gene (*IGH*). This aberration was found in 6–19% of patients with CLL at diagnosis [2,12], and was associated with therapy-demanding disease and inferior outcome [13,14].

The incidence of this aberration at clonal evolution is currently unknown. To better define the incidence and significance of CE, including the late appearance of 14q32/*IGH* translocations, we performed this study including 105 cases of CLL analyzed sequentially over a 10-year period with a panel of probes including an *IGH* break-apart probe.

Patients and methods

Patient population

One-hundred and five cases of CLL, forming the basis of the present report, were seen at our institution between 1995 and 2004. These 105 patients were submitted to FISH

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analysis as part of routine diagnostic work-up and were submitted to sequential FISH studies. FISH was repeated on at least one occasion in all 105 patients; sequential samples were obtained before each line of treatment and at 4–6-year intervals in those patients not requiring treatment. These 105 patients fulfilled the following criteria:

1. Diagnosis of "bona fide" CLL based on morphology and immunophenotyping (score according to Matutes *et al.* ≥3) [15]. Those cases with t(11;14)(q13;q32)/*BCL1–IGH* were excluded from this study.
2. Successful FISH analysis at diagnosis and during follow-up.
3. Clinical records available for review.

Cytogenetic and FISH studies

Sequential FISH studies were performed on peripheral blood (PB) samples using commercially available probes for the identification of deletions at 13q14/D13S25, 11q23/*ATM* and 17p13/*TP53*, as well as for the detection of trisomy 12 and translocations at 14q32/*IGH*. Methods are detailed elsewhere, and the sensitivity limit for the detection of 14q32 translocation, trisomy 12 and deletions were >3%, >3% and >8% interphase cells with split signal, three signals and one signal, respectively [13]. All probes were purchased from Vysis Co., distributed by Abbott Molecular (Rome). In 10 patients bone marrow (BM) aspiration and/or lymph node biopsy were performed for diagnostic purposes (i.e. cytopenia or suspected Richter syndrome). These samples were referred to the cytogenetics laboratories and processed for FISH studies. Those patients with 11q − and/or 17p − were considered as "high cytogenetic risk," and the remaining patients with 13q −, + 12, 14q32/*IGH* translocations were considered as "standard cytogenetic risk."

The following probes were used, as previously reported, in patients with 14q32/*IGH* translocation to identify the partner chromosome [13]: 11q14/*BCL1*, 18q21/*BCL2*, 3q27/*BCL6*, 18q21/*MALT1* and 8q24/*MYC* (Vysis/Abbott Co., Downers Grove, IL). Non-commercial bacterial artificial clones (BACs) were also used: RP11158I21 and 440P5 mapping at 2p12/*BCL11A*, RP11696P19/298I23/533O20/7K24 for 6p21/*CCND3*, RP1121J15/CTB129P6/CTB179K24 for 19q13/*BCL3* (kindly provided by Dr. I. Wlodarska, Catholic University of Leuven, Belgium); RP11888H2/771F6 for 7q21/*CDK6* (provided by Prof. M. Rocchi, University of Bari, Italy); a contig of six BACs (RP1146O23/137P5/773M18/44H 14/811I15) used for the evaluation of region 5p15.31-33, and BAC RP11145B1 used as a centromeric control for sub-region 5p15.2 (provided by Prof. M. Rocchi, University of Bari, Italy).

Hematologic studies

Diagnosis was based on the presence of persistent lymphocytosis (>5000/μL), on examination of the peripheral blood (PB) smear and on the results of immunophenotyping. The following markers were tested in all cases by cytofluorimetric analysis, as previously described [16], using a 30% cut-off for positivity in the lymphocyte gate: CD5, CD19, CD23, CD22, CD10 antigens; the FMC7 monoclonal antibody and the expression of surface immunoglobulins were also tested. The co-expression of CD38 and CD19 antigens was tested using commercially available reagents (Becton Dickinson, San Jose, CA), with a 30% cut-off for positivity. CD38 positivity was tested at diagnosis and during follow-up before administration of treatment. ZAP70 was assessed using the ZAP70 Alexa Fluor 488 monoclonal antibody (Caltag/Valter Occhiena, Torino, Italy) [17]. All patients underwent physical examination, chest X-radiography and abdomen ultrasonography as part of the diagnostic work-up. Routine laboratory investigations including blood count and serum biochemical profile were performed in all cases. As a rule, trephine biopsy was performed in young patients (<60 years of age). Histological studies were performed for diagnostic purposes in selected cases.

Indications for treatment according to the National Cancer Institute (NCI) criteria [18] included a rise of the white blood cell (WBC) count with a <6-month lymphocyte doubling time (LTD), the development of anemia, neutropenia or thrombocytopenia due to BM infiltration, autoimmune phenomena non-responsive to steroid drugs and disease progression in the Rai staging system [19]. Patients were treated according to guidelines in use at our institutions during the study period. Fludarabine containing regimens were used as front-line treatment in young patients and in refractory or relapsing patients. Intermittent chlorambucil administration was used as first-line therapy in the majority of elderly patients (>70 years).

Statistical analysis

Time to first treatment (TTT) was calculated from the date of diagnosis to the initiation of treatment. Refractory disease was defined by stable disease or progressive disease during treatment or disease progression within 6 months from antileukemic treatment using fludarabine alone or in combination with other agents. Time to chemorefractoriness (TTCR) was measured from the date of first-line treatment to the date of refractoriness to the fludarabine-containing regimen or date of last follow-up. Overall survival (OS) was measured from diagnosis to the date of last follow-up or death.

Results

Clonal evolution

The median interval between diagnosis and first FISH analysis was 2 months (range 1–12 months). The number of FISH investigations in each patient was 2–6 (median 3). The median follow-up of the entire series was 73 months (range 12–180 months).

CE was observed in 15/105 patients after 24–170 months, median 64, as detailed in Table I. Recurring aberrations at clonal evolution were 14q32/*IGH* translocation in seven patients; 17p − in four patients, 11q − in two patients, biallelic 13q − in four cases, hemizygous 13q − in one case and 14q32 deletion in one patient. A 17p deletion was associated with 14q32/*IGH* rearrangement in 3/7 patients (patients 1, 2 and 6), one of whom also developed a biallelic 13q14 deletion (patient 6).

In two cases with 14q32/*IGH* translocation at CE (patients 1 and 7), a paired BM or lymph node (LN) sample and PB samples were available for FISH studies. In these patients the appearance of *IGH* translocation in the BM or the LN sample

Table I. Outcome of FISH investigations in 15 patients with clonal evolution.

Patient	Aberration at diagnosis (% of cells)	Aberrations at CE	No. of previous lines of treatment	Interval between diagnosis and clonal evolution (months)
1	13q− biallelic (42%)	13q− biallelic (55%); IgH R (18%)*; 17p− (27%)	2	60
2	No aberration	17p− (26%); IgH R (21%) (*IGH–BCL2*)	1	58
3	11q− (20%)	11q− (26%); IgH R (20%)	1	73
4	13q− (21%)	13q− (53%); IgH R (19%)	3	91
5	+12 (28%)	+12 (42%); IgH R (16%) (*IGH–BCL2*)	4	64
6	13q− (32%); 11q− (15%)	13q− biallelic (34%); 11q− (27%); IgH R (21%) (*IGH–BCL2*); 17p− (18%)	4	74
7	+12 (30%)	+12 (55%); IgH R (25%)*	3	51
8	No aberration	11q− (42%)	4	41
9	No aberration	17p− (54%)	4	97
10	No aberration	11q− (61%)	3	48
11	13q− (66%); 11q− (65%)	11q− (61%); 13q− biallelic (60%)	5	84
12	+12 (56%)	+12 (51%); 13q− (71%)	3	87
13	No aberration	13q− biallelic (71%)	3	53
14	13q− (78%)	13q− biallelic (67%)	5	170
15	No aberration	14q32 deletion (45%)	2	24

*Detected in BM/LN sample before its appearance in PB.
FISH, fluorescence *in situ* hybridization; CE, clonal evolution; IgH R, rearrangement of IgH with split signal by FISH (14q32/*IGH* translocation).

preceded its appearance in PB samples (Table I) by 13–58 months. The 14q32/*IGH* translocation persisted at subsequent analyses in both cases.

All patients with the 14q32/*IGH* translocation were assessed by interphase FISH for the detection of possible chromosome partners. Three out of seven cases (cases 2, 5 and 6) showed an *IGH–BCL2* fusion signal consistent with a t(14;18)(q32;q21) translocation. In the remaining four cases it was not possible to identify the partner chromosome with our probe panel.

Correlation between CE, 14q32/*IGH* translocations, hematologic and clinical parameters

Forty-seven patients did not require treatment throughout the study period and 58 patients received 1–6 lines of treatment. CE was detected in 15 pre-treated patients, seven of whom had a 14q32/*IGH* translocation, after 1–4 lines of treatment (median 3). In contrast none of 47 untreated patients developed CE ($p < 0.0001$), as shown in Table II, where the patients' characteristics at initial evaluation are presented in detail. ZAP70 + and high-risk cytogenetics predicted for the occurrence of CE with borderline statistical significance, $p = 0.055$ and 0.07, respectively (Table II); no other baseline hematologic characteristic predicted for CE and for the late appearance of 14q32/*IGH* translocations.

The correlation between the development of CE and measures of clinical outcome is presented in Table III. Median OS measured from diagnosis was shorter in patients with CE, but the difference was not statistically significant; a

Table II. Baseline characteristics and clinical data in 105 cases of CLL submitted to sequential FISH investigations.

Characteristics	All patients (no. of cases)	Without CE ($n = 90$)	With CE ($n = 15$)	Without 14q32 translocation at CE ($n = 98$)	With 14q32 translocation at CE ($n = 7$)
Median age, years (range)	63 (31–86)	63 (31–86)	63 (51–78)	63 (31–86)	60 (51–71)
M/F ratio	66/39	57/33	9/6	62/36	4/3
Lymphocytes					
$< 30 \times 10^9$/L	89	78	11	84	5
$> 30 \times 10^9$/L	16	12	4	14	2
Rai stage					
0–1	84	74	10	80	4
2–4	21	16	5	18	3
CD38 +					
Negative	72	64	8	69	3
Positive	30	23	7	26	4
ZAP70					
Negative	46	40	6	42	4
Positive	25	17	8	22	3
FISH aberrations					
Standard risk	92	81	11	87	5
High risk	13	9	4	11	2
Treated before CE					
Yes	58	43	15*	51	7**
No	47	47	0	47	0
Relapsed/refractory					
Yes	27	16	11***	21	6****
No	31	27	4	30	1

*$p < 0.0001$; **$p = 0.014$; ***$p = 0.016$; ****$p = 0.027$.
CLL, chronic lymphocytic leukemia; FISH, fluorescence *in situ* hybridization; CE, clonal evolution.

Table III. Correlation of outcome measures and development of CE.

Outcome measure	Months	*p*-Value
TTT		
All patients ($n = 105$)	54 (SE 11.1)	
With CE ($n = 15$)	35 (SE 3.6)	0.0033
Without CE ($n = 90$)	71 (SE 14.0)	
With 14q32 translocation ($n = 7$)	36 (0.40)*	0.067
Without 14q32 translocation ($n = 98$)	63 (13.90)	
TTCR		
All patients ($n = 58$)	72 (SE 8.7)	
With CE ($n = 15$)	34 (SE 5.2)	0.0046
Without CE ($n = 43$)	86 (SE 12.1)	
With 14q32 translocation ($n = 7$)	27 (SE 0.2)	0.0002
Without 14q32 translocation ($n = 51$)	75 (SE 6.8)	
Survival from diagnosis		
All patients ($n = 105$)	173 (SE 35.1)	
With CE ($n = 15$)	124 (SE 2.03)	NS
Without CE ($n = 43$)	173 (SE 18.80)	
With 14q32 translocation ($n = 7$)	125 (SE 2.0)	NS
Without 14q32 translocation ($n = 51$)	173 (SE 33.0)	

*

CE, clonal evolution; TTT, Time to first treatment; TTCR, time to chemorefractoriness; SE, standard error; NS, not significant.

shorter TTT was noted in 15 patients with CE as compared to 90 patients without CE ($p = 0.0033$) [Fig. 1(A)]. A total of 27 patients, including 11/15 with CE (six out of seven with 14q32/*IGH* translocation) became refractory to fludarabine after a median of 72 months from the start of treatment, and a significant association was noted between short TTCR and CE [Table III, Fig. 1(B)]. Presentation features predicting the development of chemorefractoriness in 58 treated patients were: high-risk cytogenetics ($p = 0.0032$) and advanced stage ($p = 0.0004$) (Table IV), which maintained their predictivity at multivariate analysis (Table V).

Survival after the development of CE was 32 months (standard error 8.5 months) in 15 patients [Fig. 1(C)] and 32 months (standard error 0.9 months) in seven patients with late-appearing 14q32 translocation.

Discussion

CLL may undergo CE, with late appearance of 11q deletion [6], 17p deletion and 6q deletion in 16–17% of cases [8,9,20]. Although recent evidence has been provided by several groups that the 14q32/*IGH* translocation may identify a cytogenetic group of patients with CLL characterized by therapy-demanding disease [13,14,21,22], scant information is available as to its possible appearance as a secondary abnormality in CLL.

Fifteen out of 105 cases (14.3%) studied by FISH showed CE in our study, and seven of these 15 cases showed a 14q32/*IGH* translocation. This is the first report identifying the 14q32/*IGH* translocation as late event in the natural history of CLL; however, the late appearance of a 14q32/*IGH* translocation was previously described in a subset of splenic marginal zone B-cell lymphomas characterized by relatively aggressive behavior and therapy-demanding disease [23]. The presence of 14q32/*IGH* translocation was previously

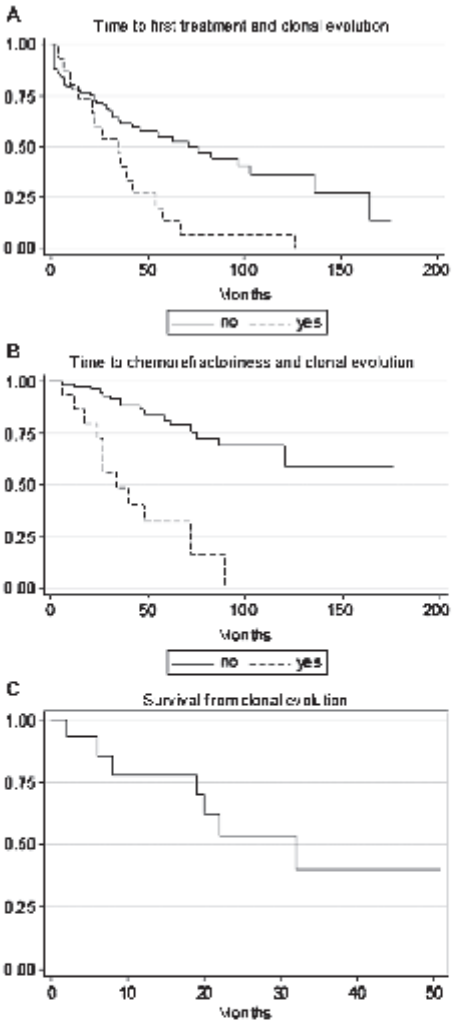

Figure 1. (A) Time to first treatment in patients with ("yes," $n = 15$) and without CE ("no," $n = 90$); (B) time to chemorefractoriness in patients with ("yes," $n = 15$) and without CE ("no," $n = 43$); (C) survival from the development of CE in 15 patients with CE.

documented to involve a minority of interphase cells in some patients with CLL [13], and one case was described as harboring *IGH* translocations with different partners [24]. These findings argue in favor of a possible secondary nature of this translocation in some patients.

Interphase FISH studies of the seven cases in our series with late appearance of a 14q32/*IGH* translocation were performed using a large panel of probes in order to detect some of the possible translocation partners. An *IGH-BCL2* fusion was found in 3/7 cases and the translocation partner was not identified in the remaining cases. This is consistent with previous observations that t(14;18)(q32;q21)/*IGH-BCL2* is

Table IV. Impact of baseline characteristics on TTCR in 58 treated patients.

Baseline characteristics	Median TTCR (months)	SE	*p*-Value
Sex			
M ($n = 38$)	72	7.8	0.95
F ($n = 20$)	86	19.7	
Stage			
0–2 ($n = 47$)	86	8.7	0.0004
3–4 ($n = 11$)	27	6.1	
CD38			
Negative ($n = 34$)	75	8.1	0.0857
Positive ($n = 23$)	48	3.5	
ZAP70			
Negative ($n = 28$)	72	15.1	0.34
Positive ($n = 21$)	58	9.5	
Cytogenetics at diagnosis			
Standard ($n = 46$)	86	10.7	0.0032
High ($n = 12$)	36	4.8	

TTCR, time to chemorefractoriness.

the most frequently occurring translocation involving the *IGH* locus in CLL [13,24,25].

Interestingly, in our series the appearance of a 14q32 translocation in BM and LN samples preceded its appearance in the PB in two cases. Recent studies indicated that CLL cells in lymph nodes and bone marrow may have an important role in tumor proliferation [26–28] and, interestingly, a relatively high incidence of 14q32/*IGH* translocation (17–19%) was noted by Flanagan and co-workers who performed FISH studies on paraffin-embedded lymphoid tissue from CLL/small lymphocytic lymphoma (SLL) [29].

In two previous analyses, 14q32/*IGH* translocation was not observed at CE [8,9], nor was this aberration detected at Richter transformation [20]. Because our patients were seen over a 10-year period, treatment was heterogeneous, and a comparison of treatment regimens in patients with and without CE was not performed; nevertheless, it is worth noting that in our seven patients with 14q32/*IGH* translocations at CE the median number of previous treatment lines was 3 (range 1–4), and that all patients received ≥1 cycle containing alkylating agents. Interestingly, *IGH* translocation accounted for 20% of the interphase cells (range 16–25%) in our study, and was associated with a minor clone carrying 17p deletion in three cases and 13q14 biallelic deletion in one. These data taken together suggest that genetic instability deriving from previous treatment might have played a role in the emergence of minor clones carrying a 14q32/*IGH* translocation in our series.

The late appearance of loss of chromosome material at 14q32 in one patient in this study parallels an observation by Shanafelt and co-workers [8]. Deletion affecting the chromosome 14q32 locus was previously described in CLL at a frequency of approximately 2–8% [30–33]. Clonal evolution in CLL was demonstrated to be associated with unfavorable biological features in previous analyses, although no difference in overall survival was noted between patients with and without CE [9]. In our series the development of CE occurred only in previously treated patients. Among baseline hematologic parameters, ZAP70-positivity and the presence of 11q and/or 17p deletion predicted for CE with borderline statistical significance. By analyzing possible associations between CE and measures of clinical outcome we were able to show that TTT and TTCR were shorter in patients who developed CE. This association held when considering separately the patients with 14q32/*IGH* translocation and TTCR. This finding may be accounted for by the consideration that CE may reflect genetic instability, a feature normally associated with disease evolution prompting the appearance of resistant clones. Predictive factors for short TTCR at baseline evaluation were advanced stage and high-risk cytogenetic features. In our series a short median survival of 32 months was recorded after the development of CE (Fig. 1).

Table V. Cox analysis of factors predictive for TTCR.

Variable	Hazard ratio	95% Confidence interval	*p*-Value
Cytogenic risk group	2.53	1.08–3.92	0.033
Stage at first treatment	2.72	1.06–7.01	0.038

TTCR, time to chemorefractoriness.

In conclusion, our data show that the 14q32/*IGH* translocation may represent one of the most frequent aberrations acquired during the natural history of CLL, and that it may be detected earlier in BM or LN samples. CE occurs in pretreated patients with short TTT and TTCR, and survival after the development of CE with and without 14q32 translocation is relatively short.

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APPENDIX IV

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Chromosome aberrations detected by conventional karyotyping using novel mitogens in chronic lymphocytic leukemia with "normal" FISH: correlations with clinicobiological parameters

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Chromosome aberrations detected by conventional karyotyping using novel mitogens in chronic lymphocytic leukemia with "normal" FISH: correlations with clinicobiological parameters

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Key words: Chronic lymphocytic leukemia, cytogenetics, FISH

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ABSTRACT

It is unclear whether karyotype aberrations occurring in regions uncovered by the standard fluorescence in situ hybridization (FISH) panel, have prognostic relevance in chronic lymphocytic leukemia (CLL). We evaluated the significance of karyotypic aberrations in a learning cohort (LC, n=84) and in a validation cohort (VC, n=84) of CLL with "normal" FISH. An abnormal karyotype was found in 21.5% and 35,7% of cases in the LC and VC, respectively, and was associated with lower immunophenotypic score (p=0.030 in the LC, 0.035 in the VC), advanced stage (p=0.040 in the VC) and need of treatment (p=0.002 in the LC, <0.0001 in the VC). The abnormal karyotype correlated with shorter time to first treatment and shorter survival in both the LC and the VC, representing the strongest prognostic parameter. In CLL patients with "normal" FISH, karyotypic aberrations by conventional cytogenetics using novel mitogens identify a subset of cases with adverse prognostic features.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease[1-3]. Adverse prognostic parameters include stage[4], CD38 and the unmutated configuration of the variable region of the immunoglobuline heavy chain gene (IGHV)[5], ZAP70[6] and chromosome aberrations[7,8].

By fluorescence in situ hybridization (FISH) analysis detecting trisomy 12 and deletions at 13q14, 11q22-23/*ATM* and 17p13/*p53*, 60-80% of the cases carry an abnormality[9]. Patients without FISH aberrations (here referred to as "normal" FISH) have a relatively favourable outcome[9].

In vitro stimulation with CpG-oligonucleotide DSP30 plus interleukin-2 (DSP30/IL2) improves the proliferation of CLL cells, yielding assessable metaphases in most patients[10]. By this method, chromosomal aberrations are detected in 80% of CLL with some patients showing aberrations in regions not covered by the classical 4-probe panel[11].

This study was designed to assess whether karyotypic aberrations in CLL with "normal" FISH correlate with established clinical and prognostic parameters. The clinical and prognostic significance of karyotypic aberrations in normal FISH CLL was first evaluated in a retrospective single centre series of patients (learning cohort, LC) diagnosed between 1998 and 2000 and then validated prospectively in a multicentre series of cases (validation cohort, VC) diagnosed and analysed for karyotype with DSP30/IL2 stimulation between 2007 and 2011.

METHODS

The LC consisted of 65 out of 70 unselected "normal" FISH CLL patients for whom a successful cytogenetic analysis was available. These patents were derived from a series of 218 consecutive CLL diagnosed between 1998 and 2000 at the Hematology Section of the University of Ferrara. The VC consisted of 85 out of 85 "normal" FISH patients, with successful cytogenetic analysis, and were derived from a series of 274 consecutive CLL diagnosed according to the NCI criteria[12] at 4 GIMEMA CLL group centres between 2007 and 2011.The Matutes immunophenotypic score[13] was calculated giving 1 point each to CD5 positivity, CD23 positivity, CD22 weak positivity, sIg weak positivity and FMC7 negativity. Only patients with a score ≥3 (i.e. typical CLL) were included. The expression of CD38 (since 2000) and ZAP-70 (since 2003) were tested

on fresh peripheral blood (PB) cells with a 20% cut-off for positivity, as described[14]. This study was approved by the University of Ferrara ethics committee. Indications for treatment included: increased WBC count with <6 month lymphocyte doubling time, anemia or thrombocytopenia due to bone marrow infiltration or autoimmune phenomena not responding to steroids, disease progression in the Binet staging system. Fludarabine-containing regimens were used as first-line treatment; chlorambucil was used in some elderly and unfit patients.

In the LC cytogenetic analysis was performed using traditional mitogens, as reported [8]. In the VC cytogenetic analysis was centralized in the Ferrara centre and performed using CpG-oligonucleotide DSP30 (2µmol/L TibMolBiol Berlin, Germany) plus IL2 (100U/mL Stem Cell Technologies Inc) as described [10, 15]. In all cases cytogenetic analysis was conducted on the same samples used for FISH studies. Interphase FISH was performed on PB samples obtained at diagnosis or before therapy using probes for the following regions: 13q14, 12q13, 11q22/ATM, 17p13/TP53 (Vysis/Abbott Co, Downers Grove, IL, USA) as described[16]. IGHV genes were amplified from genomic DNA and sequenced according to standard methods and the cut-off of 98% homology to the germline sequence was chosen to discriminate between mutated (<98% homology) and unmutated (≥98% homology) cases, as reported previously[14]. TP53 mutational analysis was performed as described elsewhere[17].

The Mann–Whitney test and the Fischer's exact test were applied for quantitative and categorical variables, respectively. Time to first treatment (TFT) was calculated as the interval between diagnosis and the start of first line treatment. Overall survival (OS) was calculated from the date of diagnosis until death due to any cause or until the last patient follow-up. Survival curves were compared by the log-rank test. Proportional hazards regression analysis was used to identify the significant independent prognostic variables on TFT. Statistical analysis was performed using Stata 8.0 (Stata Corp., College Station, TX, USA) and Prism 4.00 for Windows, GraphPad Software (San Diego California USA).

RESULTS AND DISCUSSION

Our data show that a significant proportion of CLL cases with normal FISH carry chromosome aberrations in regions not covered by the 4-probe FISH panel used in most clinical trials[3, 9]. An abnormal karyotype was observed in 14/65 (21.5%) and 30/84 (35,7%) patients of the LC and VC, respectively (p=ns). This novel cytogenetic category (i.e. abnormal karyotype with "normal" FISH) accounted for 6.4%, and 10.0% of the cases in the LC and in the VC, respectively, and for 8.0% in the pooled series of 402 patients of this study, thus representing the 4th most common cytogenetic group, following deletions at 13q and 11q, and trisomy 12.

Recurring aberrations were (Table S1, S2): 14q deletions in 8 cases (1 in the LC, 7 in the VC), 7q deletions in 5 cases (1 in the LC, 4 in the VC), 6q deletions in 5 cases (4 in the LC, 1 in the VC), 14q32 translocations in 4 cases (2 in the LC, 2 in the VC), 3q deletions, distal to the q14 band, in 3 cases (all in the VC). A complex karyotype (≥ 3 aberrations) was found in 14 patients, 12 of which in the VC.

This study confirms previous observations that in CLL patients stimulation with DSP30/IL2 improves the rate of metaphase generation (65/70 vs. 85/85 cases in the LC and VC, respectively, p=0.017)[10].

The correlations between karyotype and clinicobiological parameters are reported in Table 1. Interestingly, the abnormal karyotype did not correlate with known molecular prognostic parameters, including CD38 and ZAP 70 positivity (in both the LC and VC), and IGHV mutational status in the VC.

Most important we could demonstrate, for the first time, that among CLL patients with "normal" FISH the abnormal karyotype significantly correlated with a shorter TFT in univariate analysis in both the LC and the VC (Table 1, Figure 1). At multivariate analysis, the factors independently predictive of shorter TFT were: in the LC; CD38 positivity (HR 2.82, 95% CI 1.10-6.00, p=0.018) and abnormal karyotype (HR 2.54, 95% CI 1.07-6.07, p=0.034): in the VC; advanced Binet stage (HR 2.77, 95% CI 1.05-7.20, p=0.030,) and abnormal karyotype (HR 6.30, 95% CI 2.44-16.80, p<0.001).

Recently it was demonstrated that, in CLL, *TP53* mutations[18, 19] represent a strong prognostic marker. By mutational analysis we observed no *TP53* mutations in the VC (0 out of 23 patients with abnormal karyotype). This finding is not surprising if we consider that patients with 17p- were excluded from this analysis and that, among untreated CLL without 17p-, the incidence of *TP53* mutations is around 3%[17-19].

The abnormal karyotype also correlated at univariate analysis with OS in both the LC and the VC (HR 5.87, 95% CI 2.08-62.08, p=0.005, and HR 9.41, 95% CI 1.61-47.35, p=0.0110 respectively, Figure 1). This observation needs to be validated in clinical trials with homogeneous treatment on larger series of patients.

In conclusion, conventional karyotyping using DSP30/IL2 stimulation is an effective method for the detection of chromosome aberrations in approximately one third of CLL with "normal" FISH on a conventional 4-probe panel. This set of data also showed that, in CLL patients with "normal" FISH, conventional cytogenetic analysis identifies a subset of cases with adverse clinical and prognostic features to be considered for the design of risk-adapted treatment strategies.

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Contributions

GMR, GP, RF, PG and AC created and designed the study; GMR, FC, FC, MC, IN, AD, FM, NT and FZ provided study materials or patients; ET and AB performed the cytogenetic analysis; ES, LR and MN performed the molecular studies; GMR, FC, SM, LF, FC, IN, NT and FZ collected and assembled data; all authors assisted in the analysis and/or interpretation of the data; all authors critically revised the manuscript; and all authors gave final approval of the manuscript.

Conflict of interest disclosure

The authors declare no competing financial interests.

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Table 1. Learning and validation cohorts: clinical correlations according to karyotype and analysis of factors affecting TFT.

CLINICAL CORRELATIONS		Learning cohort			Validation cohort		
Parameter		Normal karyotype (51 cases)	Abnormal karyotype (14 cases)	p	Normal karyotype (54 cases)	Abnormal karyotype (30 cases)	p
Age							
Mean	(range)	64 (31-84)	69 (50-87)	ns	63 (41-90)	63 (32-84)	ns
Sex							
M/F	n. of pts	31/20	8/6	ns	34/20	23/7	ns
Matutes score							
3/4/5	n. of pts	3/25/23	4/3/7	0.030	4/24/25	7/5/12	0.035
Binet Stage							
A/B/C	n. of pts	47/3/1	10/3/1	0.089	45/9/0	20/7/3	0.040
CD38							
pos/neg	n. of pts	21/29	8/6	ns	11/41	12/18	0.079
ZAP-70							
pos/neg	n. of pts	15/6	5/4	ns	16/31	15/11	0.083
IGVH							
Unmutated/mutated	n. of pts	nd	nd		11/38	10/17	0.191
Therapy							
yes/no	n. of pts	16/35	11/3	0.002	6/48	19/11	<0.0001

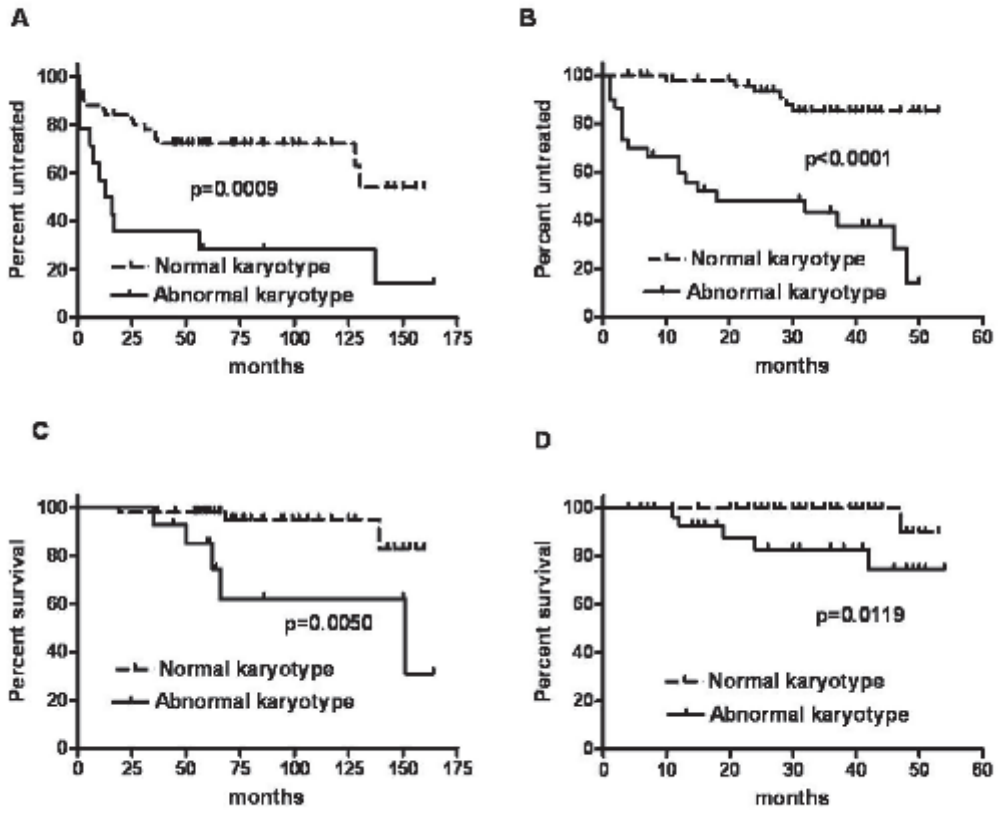
TFT ANALYSIS		Learning cohort			Validation cohort		
Parameter		N. of cases	HR (95% CI)	p	N. of cases	HR (95% CI)	p
Karyotype							
Abnormal vs Normal		14 vs 51	3.33 (2.03-15.66)	0.0009	34 vs 50	7.80 (4.36-25.12)	<0.0001
Binet stage							
B-C vs A		8 vs 57	4.56 (4.43-89.97)	<0.0001	19 vs 65	3.66 (2.47-26.83)	0.0006
CD38							
pos vs neg		29 vs 35	3.26 (1.60-7.78)	0.0018	23 vs 59	2.00 (0.91-5.73)	0.0786
ZAP70							
pos vs neg		10 vs 20	2.31 (0.81-5.78)	ns	32 vs 41	1.52 (0.70-3.41)	ns
IGHV							
Unmutated vs Mutated		nd	nd	nd	21 vs 55	2.40 (1.11-7.64)	0.0297

nd: not done
ns: not significant
HR: hazard ratio

Legend to figure 1

Figure 1
TFT and OS respectively in the learning (A, C) and the validation (B, D) cohorts.

Figure 1


A
Percent untreated
p=0.0009
Normal karyotype
Abnormal karyotype
months
B
Percent untreated
p<0.0001
Normal karyotype
Abnormal karyotype
months
C
Percent survival
p=0.0050
Normal karyotype
Abnormal karyotype
months
D
Percent survival
p=0.0119
Normal karyotype
Abnormal karyotype
months

APPENDIX V

Gene mutation analysis of a panel of 20 genes in untreated CLL patients: clinical and biological correlations

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ABSTRACT

Background: chronic lymphocytic leukemia (CLL) is a hematological malignancy with clinicobiologic heterogeneity. CLL exhibits variable clinical presentation and evolution. The outcome of CLL can be predicted based upon presence or absence of somatic mutations of the variable region of the immunoglobulin heavy chain gene (IGHV) or CD38 and ZAP70 positivity; chromosomal aberrations, including deletions at 17p and 11q have also been linked to poor prognosis. Whole genome sequencing has provided new insights into the mutational status of the disease, by revealing genetic lesions affecting TP53 and several genes previously not involved in CLL, such as NOTCH1, SF3B1, MYD88 and BIRC3.
Objectives: to study a panel of 20 genes (ATM, BIRC3, BRAF, CDKN2A, CTNNB1, DDX3X, FBXW7, KIT, KLHL6, KRAS, MAPK1, MYD88, NOTCH1, NRAS, PIK3CA, POT1, SF3B1, TP53, XPO1, ZMYM3) potentially affected by mutations in untreated CLLs and to correlate mutational status with clinicobiologic parameters.
Methods: Agilent HaloPlex Target Enrichment kit was used to produce libraries of exonic regions from the above 20 genes. Sequencing was performed using the Ion Torrent PGM platform. Sequencing data were aligned to the human reference genome (GRCh37).
Results: 28 consecutive untreated CLL patients were included in this study. The frequencies of mutant reads ranged from 6.4 to 76.5%. Somatic mutations were identified in 12 cases. Mutations were identified in the following genes: TP53 (3 with >20% mutant reads, 2 with 5-19% mutant reads), SF3B1 (1), POT1 (3), ATM (1), MYD88 (1), FBXW7 (1), MAPK1 (1), DDX3X (1), KLHL6 (1), KRAS (1). Eight cases presented with one mutant gene, two mutant genes were detected in 4 cases. The presence of mutations correlated with high risk FISH (11q- and/or 17p-) ($p=0.0198$) and unfavourable cytogenetic (11q-, 17p- or complex karyotype) ($p=0.011$) findings. No correlations were instead observed with sex, age, Binet stage, CD38 and IgVH. Patients carrying CLLs with gene mutations showed a significant ($p<0.001$) shorter median time to first treatment in comparison to those without mutations (20 months vs not reached at 76 months).
Conclusions: The frequency of mutations in the 20 investigated genes is in line with published data in the literature using whole exome sequencing. This study shows that the simultaneous sequencing of a panel of genes implicated in CLL is feasible. More importantly, the semiconductor sequencing technology represents a relatively economical important tool for the characterization of CLL genetic heterogeneity and clinical-prognostic outcome.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most frequent leukemia in adult in Western countries [1]. Despite a homogeneous immunophenotype, it may exhibit variable clinical presentation and evolution. Some patients present an indolent disease, whereas others are characterized by aggressive disease and short survival times. The outcome of CLL can be predicted based upon stage at diagnosis [2, 3], presence or absence of somatic mutations of the variable region of the immunoglobulin heavy chain gene (IGHV) [4] or

CD38 [5] and ZAP70 positivity [6]. Chromosomal aberrations, including deletions at 17p and 11q, and TP53 mutations have also been linked to poor prognosis [7].
Next-generation sequencing (NGS) techniques have provided a better knowledge of the genetic complexity and heterogeneity of CLL [8-10]. Whole genome sequencing has provided new insights into the mutational status of the disease, by revealing novel genetic lesions affecting several genes previously not involved in CLL, such as NOTCH1, SF3B1, MYD88 and BIRC3 [10]. Alterations of these genes occur in approximately 5-10% of CLL patients at diagnosis and have shown significant correlations with survival [11-13].
Massive parallel sequencing technology provides a means of systematically discovering the genetic alterations that underlie disease and identifying new therapeutic targets and clinically predictive biomarkers [14, 15]. Since the launch of first NGS platform, a number of technological advances have been made, such as improved sequencing chemistry and novel detection methods. Here, we tested a panel of 20 genes that were found being mutated in CLL using small NGS system Ion Torrent Personal Genome Machine (PGM), a NGS platform that uses semiconductor sequencing technology [16], thus resulting in higher speed of analysis and lower cost. We sequenced DNA samples from 28 untreated patients affected by CLL and we correlated mutational status with clinic-biologic parameters.

METHODS

Patients

Twenty-eight newly diagnosed patients with CLL seen at our institution between 2006 and 2008 were included in the present study. Diagnosis was made according to standard NCI criteria [1]. Indications for treatment included: increased WBC count with <6 month lymphocyte doubling time, anemia or thrombocytopenia due to bone marrow infiltration or autoimmune phenomena not responding to steroids, disease progression in the Binet staging system. Fludarabine-containing regimens were used as first-line treatment; chlorambucil was used in some elderly and unfit patients.
Immunophenotyping with a standard diagnostic panel including anti CD38 monoclonal antibodies as well as FISH studies were performed as previously reported [17, 18]. Conventional karyotyping was performed using ODN and IL2 as previously reported [19].

Immunophenotypic analysis

At diagnosis, immunophenotypic analysis was performed according to NCI criteria [1]. The Matutes immunophenotypic score [20] was calculated giving 1 point each to CD5 positivity, CD23 positivity, CD22 weak positivity, sIg weak positivity and FMC7 negativity. Only patients with a score ≥3 (i.e. typical CLL) were included. The expression of CD38 were tested, as described [19], on fresh peripheral blood (PB) cells with a 20% cut-off for positivity.

IGHV mutational analysis

IGHV genes were amplified from genomic DNA and sequenced according to standard methods and the cut-off of 98% homology to the germline sequence was chosen to

discriminate between mutated (<98% homology) and unmutated (≥98% homology) cases, as reported previously [18].

IonTorrent Personal Genome Machine (PGM) analysis

Agilent HaloPlex Target Enrichment kit (Agilent Technologies, Santa Clara, CA, USA) was used to produce libraries of exonic regions from 20 genes (ATM, BIRC3, BRAF, CDKN2A, CTNNB1, DDX3X, FBXW7, KIT, KLHL6, KRAS, MAPK1, MYD88, NOTCH1, NRAS, PIK3CA, POT1, SF3B1, TP53, XPO1, ZMYM3) starting from genomic DNA from peripheral blood samples, according to HaloPlex Target Enrichment System (Agilent Technologies, Santa Clara, CA, USA) (see **Supplementary Table 1, Figure 1A**). Diluted libraries were linked to Ion Sphere Particles, clonally amplified in an emulsion PCR and enriched using Ion OneTouch emulsion PCR System (Life technologies, Foster City, CA, USA). Exon-enriched DNA was precipitated with magnetic beads coated with streptavidin. Enriched, template-positive Ion Sphere Particles were loaded in one Ion chip and sequenced using Ion Torrent PGM (Life technologies, Foster City, CA, USA). Sequencing data were aligned to the human reference genome (GRCh37). Data analysis and variants identification were performed using Torrent Suite 3.4 and Variant Caller plugin 3.4.4 (Life technologies, Foster City, CA, USA).

Statistical analysis

Quantitative variables were reported as mean values with standard deviations (SDs) and were compared using the Mann-Whitney test. The Fisher exact test was used for assessing the association of gene mutations with clinico-biological variables. The time to first treatment (TTFT) was calculated as the interval between diagnosis and the start of first-line treatment. Survival curves were compared by using the log-rank test. A P value <.05 was used as a criterion for statistical significance. Statistical analyses were performed using Stata software release 8.0 (Stata Corporation, College Station, TX, USA) and Prism 4.00 for Windows, GraphPad Software (San Diego California USA).

RESULTS

Patients

Twenty-eight consecutive untreated cases of CLL seen at our institution between 2006 and 2008 were included in this study. The main clinical and molecular characteristics of the CLL cases are reported in **Table 1**. The male/female ratio was 1.8 and mean age at diagnosis was 62 years (median = 62; range = 49–75). Binet stage A ($n = 22$), B ($n = 4$) or C ($n = 2$). The patients were classified in 3 cytogenetic risk categories: 17p-, 11q- or complex karyotype defined the high risk group, +12 or 1-2 cytogenetic aberrations defined the intermediate risk group, isolated 13q- or absence of detectable aberrations defined the low risk group.

Cytogenetic analysis by FISH and conventional karyotype analysis

Results of FISH and cytogenetic analysis are reported in **Table 2**. In 14/28 cases, FISH studies revealed genetic lesions. 17p deletion was seen in 2 cases, 11q deletion in 1 case, trisomy 12 in 4 cases and 13q deletion in 9 cases. In two cases, the 13q14 deletion was

associated respectively to the deletion of chromosome 11 and chromosome 17. 19/28 cases showed an abnormal karyotype by conventional cytogenetic analysis, including 5/15 cases without detectable lesions by FISH (see **Table 2**). A complex karyotype (≥ 3 aberrations) was found in 4 cases.

Mutational analysis by Next Generation Sequencing (NGS)

Parallel sequencing of exonic regions from 20 genes (ATM, BIRC3, BRAF, CDKN2A, CTNNB1, DDX3X, FBXW7, KIT, KLHL6, KRAS, MAPK1, MYD88, NOTCH1, NRAS, PIK3CA, POT1, SF3B1, TP53, XPO1, ZMYM3) (**Figure 1A**)was carried out in 28 CLL cases using the semiconductor technology of the Ion Torrent platform. Somatic mutations were identified in 12 (42.8%) cases, as reported in **Table 2**. Each segment had a number of reads > 500. Mutations were detected with a frequency ranging from 6.4 to 76.5% of the reads. Five cases showed mutations in the TP53 gene (range 6.4%-46.8% of reads), 3 cases presented POT1 mutations, while mutations of MYD88, FBXW7, MAPK1, DDX3X, KLHL6, SF3B1, KRAS and ATM were detected in 1 case each. Eight cases presented one mutated gene, while and 4 cases carried 2 mutated genes (TP53 in association with POT1 or DDX3X, ATM in association with KLHL6 and KRAS with POT1). Gene mutations found were validated using capillary Sanger sequencing (**Figure 1**)

Correlation between mutational status and clinico-biological parameters

The correlations between mutational status and clinic-biological parameters are reported in **Table 3**. The presence of somatic mutation did not correlate with sex, age, Binet stage, CD38 or IgVH status. A positive mutational status correlated with the presence of cytogenetic abnormalities detected by FISH and the relative risk status (favourable, intermediate and high) (p=0.0198) and unfavourable karyotype findings (p=0.0322). Mutated patients showed a significant (p=0.0002) shorter median time to first treatment in comparison to those without mutations (20 months vs not reached at 76 months) (**Figure 2**)

DISCUSSION

The development of massive parallel sequencing, also referred to as next-generation sequencing (NGS), allowed the possibility to carry out whole-genome sequencing in various diseases and define the transmissible or somatically acquired genetic alterations [21]. NGS studies applied to CLL identified novel oncogenes and tumor suppressor genes potentially implicated in pathogenesis. Wu and colleagues showed 25 significantly recurrent genetic alterations affecting 7 core signaling pathways [22]. Whole genome/exome sequencing of numerous CLL cases has disclosed the gentic landscape of CLL, providing comprehensive catalogs of somatic mutations and new insights into the genes that contribute to cellular transformation [8-12]. CLL shows a highly variable disease course, partly explained by the diverse combinations of somatic mutations uncovered by sequencing studies [9, 10].

In this report, we describe the development a panel of 20 genes implicated in CLL, that can be all simultaneously sequenced in parallel using the Ion Torrent platform. This approach was tested in a group of 28 CLL patients. The panel was designed to cover the coding sequences of all the above mentioned genes. We found somatic mutations in 12

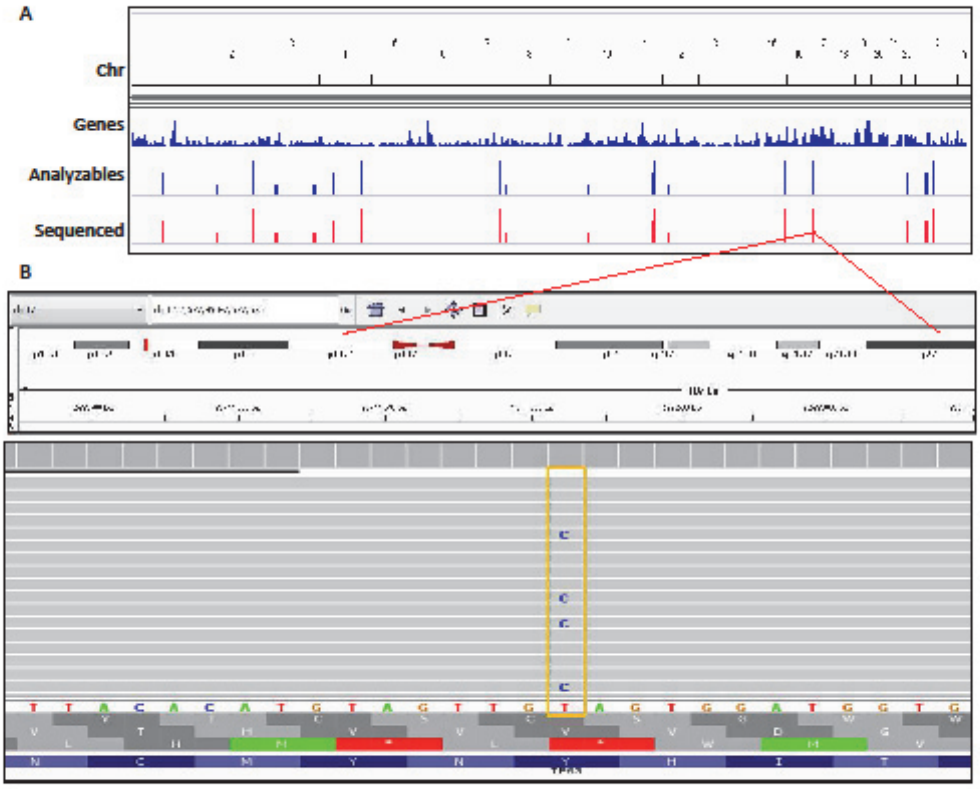
cases. TP53 was most frequently mutated (5 cases) and the frequency of mutations involving the other genes investigated was in line with data published in literature using whole exome sequencing [8, 10]. The percentage of reads showing mutation ranged from 6.4 to 76.5%. From the variability in the reads where mutation was found, we deduce that both clonal and subclonal mutations were present. The mutational profile of CLL can be characterized in two main group: clonal mutations, which are present in all tumor cells and represent early events in the leukemogenesis, and subclonal mutations, usually present in a small fraction of leukemic cells and possibly may represent progression events. This finding may explain the basis of the great heterogeneity within the CLL population [22]. ATM mutation present in the 76.5% of the reads analyzed is probably a predominantly clonal, whereas mutation with rates of 6-20% are probably subclonal mutations, that could expand over the time and lead to disease progression.

Among our 19 cases with genetic lesions by FISH and/or cytogenetic analysis NGS showed gene mutations in 9 cases, documenting that cytogenetic, FISH and sequencing may represent complementary methods that allow to detect genetic heterogeneity within CLL. NGS could also provide information in a significant proportion of CLL patients with favorable prognostic features. Our approach allow for the identification of genetic lesions in 25% of patients with non-informative cytogenetic analysis (FISH and karyotype) and 50% of FISH negative cases. In the three cases without cytogenetic and FISH abnormalities, we found mutated FBXW7, MAPK1 and TP53, respectively (see **Table 2**). Data are not surprising, in fact TP53 mutations in the absence of 17p13 deletion are detected in 30% of CLL at diagnosis [23].

We then correlated mutational status with clinicobiological parameters. Interestingly, the presence of mutations correlated with high risk FISH (11q- and/or 17p-) and unfavourable cytogenetic (11q-, 17p- or complex karyotype) findings. No correlations were instead observed with other parameters like sex, age, Binet stage, CD38 and IgVH. The mutational status also correlated at univariate analysis with time to first treatment, that was significantly shorter in patients harboring somatic mutations. At multivariate analysis, no variable independently predictive of shorter TFT was statistically significant. This data is probably due to small number of patients analysed. Definitely a future goal will be to expand the series of patients.

In conclusion, NGS by Ion Torrent might represent an important tool for the characterization of CLL genetic heterogeneity and clinical prognostic outcome. Ion Torrent is surely feasible due to the low cost and the short time for reaction. This techniques, applied to clinics, could expand our knowledge on the molecular mechanisms involved in the pathogenesis of the disease and offer new perspectives for clinical management of the CLL patients.

Figure 1. Sanger validation of a TP53 mutation detected by Ion Torrent PGM.



Location	Gene	Chr	Position	Ref.	Var.	Zygosity	dbSNP ID	Mutation Type	Freq. %	Coverage	PP2 Index	Variant Details
exonic	TP53	chr17	7577580	T	C	het		nonsynonymous SNV	19,89	926	1	NM_000546 exon7:c.737A>G p.Y246C

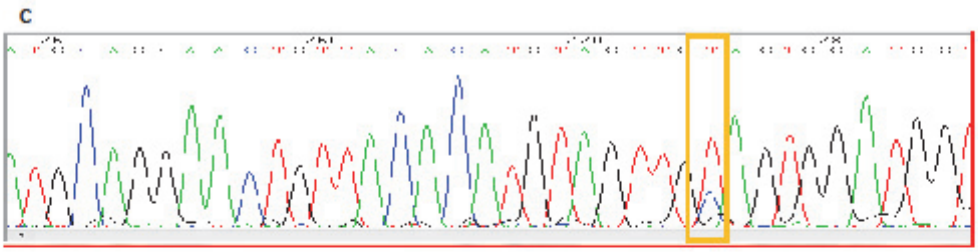


FIGURE 2. Time to first treatment in patients with (Mutated, n=12) and without somatic mutations (Unmutated, n=16).

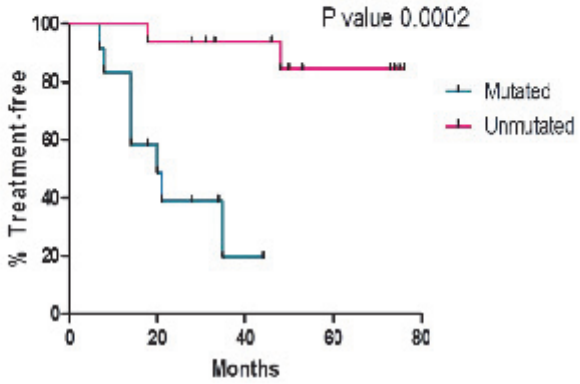


Table 1. Baseline characteristics and clinical data in 28 cases submitted to NGS sequencing.

CLINICAL AND BIOLOGICAL CHARACTERISTICS	
N of patients	28
Age mean yrs (range)	62 (49-75)
M/F	18/10
Stage (Binet) A/B/C	22/4/2
CD38 (>20%) neg/pos	20/7
IgVH Mutated/Unmutated	17/11
FISH Low /Intermediate/High	21/4/3
Karyotype Low /Intermediate/High	15/8/5

Table 2. Outcome of NGS sequencing, FISH investigations and conventional karyoptyping analysis.

Case	Mutated genes	% mutation	FISH results	Conventional karyotyping results
Emato Unife-942	TP53	6,43	del(13q), del(17p)	46,XY,add(8)(p23) [19] / 46,XY [1]
	TP53	18,69		
Emato Unife-121	Nessuna		neg	46,XY,t(14;18)(q32;q21) [5] / 46,XY , [12] / 46,XY,del(1)(q32),add(9)(q34),-14,+mar [4] CNC
Emato Unife-662	MYD88	10,83	neg	46,XY,t(4;11)(q21;q24),add(22)(p13) [8] / 46,XY,dic(15;15)(p11;p11) [3] / 46,XY [9]
Emato Unife-83	Nessuna		neg	47,X,der(X)(p11),+del(3)(p11p14) [4] / 46,XX [16]
Emato Unife-884	FBXW7	32,94	neg	46,XY [20]
Emato Unife-658	MAPK1	18,84	neg	46,XY [20]
Emato Unife-874	Nessuna		neg	46,XX [20]
Emato Unife-84	Nessuna		neg	46,XY[20]
Emato Unife-1572	TP53	23,1	neg	46,XY,del(7)(q32) [4] / 46,XY [21]
	POT1	22,28		
Emato Unife-288	POT1	19,92	trisomy 12	47,XX,+12 [16] / 46,XX[4]
Emato Unife-437	Nessuna		del(13q)	46,XY,del(13)(q14q22) [6] / 46,XY [14]
Emato Unife-455	TP53	39,37	trisomy 12	47,XY,+12 [7] / 47,XY,+12,add(19)(p13) [3] / 46,XY [10]
	DDX3X	66,81		
Emato Unife-666	Nessuna		trisomy 12	47,XX,+12 [17] / 46,XX[3]
Emato Unife-676	KLHL6	33,1	del(11q), del(13q)	46-47,XX,add(2)(p25), add(2)(q36), add(3)(p26), add(4)(p16), del(11)(q21q23), del(13)(q14q21), add(14)(p13), add(15)(p13), add(18)(p11), +mar [21] / 46,XX [1]
	ATM	76,51		
Emato Unife-974	Nessuna		neg	46,XY[20]
Emato Unife-978	Nessuna		del(13q)	46,XY,del(13)(q14q22) [11] / 46,XY [10]
Emato Unife-1184	Nessuna		del(13q)	46,XY,del(13)(q14q22)[11] / 46,XY [9]
Emato Unife-1214	Nessuna		del(13q)	46,XY,t(14;18)(q32;q21)[13]/46,XY,del(13)(q14q22),t(14;18)(q32;q21) [5] / 46,XY [2]
Emato Unife-194	TP53	16,96	del(17p)	45-46,XX,der(2)(p23),add(16)(p13),der(14)(q32),der(17)(p12),+19,-21 [cp10] / 46,XX [10]
Emato Unife-1343	Nessuna		del(13q)	46,XX,del(13)(q14q22) [3] / 46,XX [17]
Emato Unife-1353	Nessuna		del(13q)	46,XY,del(13)(q14q22)[4]/46,XY,del(10)(q22q24),del(13)(q14q22) [5] / 46,XY [11]
Emato Unife-1363	Nessuna		neg	46,XX [20]
Emato Unife-1451	POT1	29,3	trisomy 12	47,XY,+12 [20]
	KRAS	8,17		
Emato Unife-1456	TP53	46,81	neg	46,XY[20]
Emato Unife-1466	Nessuna		del(13q)	46,XX,del(13)(q14q22) [5] / 46,XX [15]
Emato Unife-1473	Nessuna		neg	46,XY[20]
Emato Unife-1486	SF3B1	29,21	neg	46,XX,der(7)t(7;?)(q32;?) [7] / 46,XX [13] aspetto 6q-CNC [3]
Emato Unife-1498	Nessuna		neg	46,XY[20]

Table 3. Clinical correlations according to presence or absence of somatic mutation by NGS sequencing.

CLINICAL CORRELATIONS				
Parameter		**Mutation positive (12 cases)**	**Mutation negative (16 cases)**	**P**
Age				
Mean	(range)	62 (49-70)	63 (51-75)	ns
Sex				
M/F	n. of pts	8/4	10/6	ns
Binet Stage				
A/B,C	n. of pts	8/4	14/2	ns
CD38				
Pos/Neg	n. of pts	4/7	3/13	ns
IgVH				
Mutated/Unmutated	n. of pts	6/6	11/5	ns
FISH				
Low/Intermediate/High	n. of pts	6/3/3	15/1/0	0.0198
Karyotype				
Low/Intermediate/High	n. of pts	3/5/4	12/3/1	0.0322
Therapy				
Yes/No	n. of pts	7/5	2/14	0.014
Time to First Treatment				
	months	20.0	Not reached	0.0002

Chromosome	Gene	Start genomic position	End genomic position	Analyzable region
chr2	XPO1	61719173	61719706	"NC_000002.11:61719173-61719706"
chr2	SF3B1	198267115	198267848	"NC_000002.11:198267115-198267848"
chr2	SF3B1	198266411	198267036	"NC_000002.11:198266411-198267036"
chr2	SF3B1	198265215	198265916	"NC_000002.11:198265215-198265916"
chr15	CHD2	93499516	93499959	"NC_000015.9:93499516-93499959"
chr15	CHD2	93510384	93511033	"NC_000015.9:93510384-93511033"
chr15	CHD2	93534475	93535000	"NC_000015.9:93534475-93535000"
chr15	CHD2	93540268	93540784	"NC_000015.9:93540268-93540784"
chr15	CHD2	93555303	93555804	"NC_000015.9:93555303-93555804"
chr15	CHD2	93552180	93552765	"NC_000015.9:93552180-93552765"
chrX	DDX3X	41196436	41196991	"NC_000023.10:41196436-41196991"
chrX	DDX3X	41204135	41205040	"NC_000023.10:41204135-41205040"
chrX	ZMYM3	70461788	70462292	"NC_000023.10:70461788-70462292"
chrX	ZMYM3	70460768	70461442	"NC_000023.10:70460768-70461442"
chrX	ZMYM3	70472711	70473246	"NC_000023.10:70472711-70473246"
chrX	ZMYM3	70469561	70470208	"NC_000023.10:70469561-70470208"
chr17	TP53	7576768	7577268	"NC_000017.10:7576768-7577268"
chr17	TP53	7577349	7577687	"NC_000017.10:7577349-7577687"
chr17	TP53	7577851	7578801	"NC_000017.10:7577851-7578801"
chr17	TP53	7579055	7579834	"NC_000017.10:7579055-7579834"
chr4	KIT	55595386	55595769	"NC_000004.11:55595386-55595769"
chr4	KIT	55602365	55602872	"NC_000004.11:55602365-55602872"
chr4	FBXW7	153249048	153249593	"NC_000004.11:153249048-153249593"
chr4	FBXW7	153245138	153245696	"NC_000004.11:153245138-153245696"
chr4	FBXW7	153246985	153247682	"NC_000004.11:153246985-153247682"
chr4	FBXW7	153258767	153259117	"NC_000004.11:153258767-153259117"
chr3	MYD88	38182079	38182867	"NC_000003.11:38182079-38182867"
chr3	CTNNB1	41265794	41266469	"NC_000003.11:41265794-41266469"
chr3	PIK3CA	178951808	178952299	"NC_000003.11:178951808-178952299"
chr3	KLHL6	183272884	183273632	"NC_000003.11:183272884-183273632"
chr22	MAPK1	22126949	22127493	"NC_000022.10:22126949-22127493"
chr22	MAPK1	22159919	22160421	"NC_000022.10:22159919-22160421"
chr9	NOTCH	139390287	139391047	"NC_000009.11:139390287-139391047"
chr1	NRAS	115256330	115256815	"NC_000001.10:115256330-115256815"
chr1	NRAS	115258445	115258986	"NC_000001.10:115258445-115258986"
chr7	POT1	124498740	124499390	"NC_000007.13:124498740-124499390"
chr7	POT1	124510738	124511207	"NC_000007.13:124510738-124511207"
chr7	POT1	124532174	124532633	"NC_000007.13:124532174-124532633"
chr7	POT1	124538031	124538662	"NC_000007.13:124538031-124538662"
chr7	POT1	124536926	124537435	"NC_000007.13:124536926-124537435"
chr7	BRAF	140452990	140453495	"NC_000007.13:140452990-140453495"
chr11	BIRC3	102201521	102202174	"NC_000011.9:102201521-102202174"
chr11	BIRC3	102207344	102207768	"NC_000011.9:102207344-102207768"
chr11	ATM	108119490	108120023	"NC_000011.9:108119490-108120023"
chr11	ATM	108117519	108118136	"NC_000011.9:108117519-108118136"
chr11	ATM	108123199	108123716	"NC_000011.9:108123199-108123716"
chr11	ATM	108137735	108138170	"NC_000011.9:108137735-108138170"
chr11	ATM	108154862	108155488	"NC_000011.9:108154862-108155488"
chr11	ATM	108173355	108173950	"NC_000011.9:108173355-108173950"
chr11	ATM	108170161	108170733	"NC_000011.9:108170161-108170733"
chr11	ATM	108172302	108172703	"NC_000011.9:108172302-108172703"
chr11	ATM	108180579	108181241	"NC_000011.9:108180579-108181241"
chr11	ATM	108200697	108201254	"NC_000011.9:108200697-108201254"
chr11	ATM	108205463	108206084	"NC_000011.9:108205463-108206084"
chr11	ATM	108206442	108206939	"NC_000011.9:108206442-108206939"
chr11	ATM	108204378	108204828	"NC_000011.9:108204378-108204828"
chr11	ATM	108217938	108218252	"NC_000011.9:108217938-108218252"
chr11	ATM	108225292	108225744	"NC_000011.9:108225292-108225744"
chr11	ATM	108235751	108236396	"NC_000011.9:108235751-108236396"
chr12	KRAS	25397964	25398541	"NC_000012.11:25397964-25398541"

Table 1, suppl. Genomic segments analysed with NGS by Ion Torrent.

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