Genetics of Chronic Lymphocytic Leukemia: Practical Aspects and Prognostic Significance

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1. Introduction

B-cell chronic lymphocytic leukemia (CLL) is a mature B-cell neoplasm. Affecting mainly the elderly, CLL represents the most common hematological malignancy in Western countries, and 6-7% of non Hodgkin’s lymphomas.

The disease course is heterogenous. Clinical staging systems (i.e. Rai and Binet) are used for estimating the tumor burden and prognosis and for making therapeutic decisions in individual patients. However, the evolution, even in the early stages, remains highly variable with at least 50% of cases showing early or late progression. Since the large majority of newly diagnosed cases present with early or intermediate stage, it is important to assess the risk profile within this group.

Several biological variables have been proposed for the prognostic stratification of early stage CLL, including chromosomal abnormalities [as assessed by karyotyping or fluorescent in situ hybridization (FISH)], expression of CD38, the proportion of ZAP-70-positive cells, somatic hypermutation of the variable part of the B-cell receptor gene (IGVH) and VH 3-21 usage. In addition, acquisition of particular chromosomal aberrations could be relevant, i.e. a 17p deletion appearing during the disease course confers resistance to alkylating agents and purine analogs, underscoring the need for defining the genetic patterns of disease evolution.

Here, chromosomal aberrations in CLL will be reviewed. First, the different techniques to detect abnormalities will be described. Second, the CLL-associated (cyto)genetic abnormalities and their relevance for clinical practice will be discussed, with a focus on the role of these aberrations in disease onset, progression, and on their prognostic significance.

2. Cytogenetic techniques

Numerous studies have shown that the presence, number, and type of chromosomal aberrations represent an independent predictor of prognosis in CLL (Döhner et al, 2000; Juliusson et al, 1990; Mayr et al, 2006; Van Den Neste et al, 2007). Therefore, cytogenetic analysis is now routinely performed in this disease. Different techniques are available to detect chromosomal abnormalities. Conventional cytogenetic analysis (CCA) can be performed, but is hampered by the poor mitotic index of CLL lymphocytes in vitro.
Although several mitogens have been used to overcome this problem, alternative approaches allowing analysis of nondividing cells are available, i.e. interphase FISH is widely used and has become the standard technique. In addition multiplex ligation-dependent probe amplification (MLPA) (Coll-Mulet et al, 2008; Fabris et al, 2011) and more recently analysis by means of different array-platforms (Gunn et al, 2008; Hagenkord et al, 2010) have been investigated in research and routine setting.

2.1 Conventional cytogenetic analysis

CCA or chromosome banding analysis (CBA) examines the patient’s chromosomes in a sample of cells. Counting the number of chromosomes and evaluating their structural aberrations (banding patterns) results in the construction of a karyogram and karyotype. The resolution is determined by the number of bands seen in a haploid set of chromosomes (300-850 bands, each band contains approximately 5-10 megabase of DNA) (Shaffer et al, 2009). The work-flow of the technical procedure is shown in Fig 1. Peripheral blood is the preferred tissue for CCA in CLL, but bone marrow, lymph node, spleen or effusions can be analyzed as well.

Since CLL is a malignancy of mature B-cells, these cells are often arrested at the G₀-G₁ phase of the cell cycle and do not divide spontaneously. They accumulate primarily as a result of lack of apoptosis, rather than by accelerated cell division (Chiorazzi, 2007). As a consequence, CLL lymphocytes have a poor mitotic index in vitro. Therefore longer culture duration has been introduced, i.e. 72 hours instead of 24-48 hours, and several stimulating agents have been added to the culture medium. Mitogens and agents such as 12-O-tetradecanoylphorbol-13-acetate (TPA), the lectine phytohemagglutinin (PHA), lipopolysaccharide (LPS) and pokeweed mitogen (PWM), the cytokine interleukin-2 (IL-2) and Epstein-Barr virus, have been used to improve the yield of aberrant metaphases. However, abnormal karyotypes were revealed in only 40–50% of cases (Juliusson et al, 1990). These low abnormality detection rates can be attributed to a lack of aberrant metaphases, i.e. proliferation disadvantage of the aberrant B-cell clone, and to the presence of cryptic deletions escaping the low resolution of CCA. Recently, improved culture methods have been introduced, i.e. CD40 ligand (CD40L)-induced cell cycle stimulation, and the immunostimulatory CpG oligonucleotide (DSP30) (Dicker et al, 2006; Haferlach et al, 2007; Mayr et al, 2006; Put et al, 2009a; Struski et al, 2009).

2.1.1 TPA

Before the introduction of DSP30, the phorbol ester TPA was considered to be the stimulating agent of choice to improve the mitotic index of CLL cells. TPA stimulates slowly proliferating immature B-cells by activating protein kinase C. This results in phosphorylation of downstream proteins, maturation of these cells towards a plasmacytoid phenotype and inhibition of apoptosis (Barragan et al, 2002). However, the induction of cells in G₂ and metaphase is weak (2-10%) (Carlsson et al, 1988; Stephenson et al, 1991).

In addition, TPA has been shown to induce the IL-2 receptor and CLL colony formation. The addition of the cytokine IL-2 to TPA stimulated CLL cell cultures was reported to directly stimulate CLL proliferation, even in absence of T-lymphocytes (Touw and Lowenberg, 1985). Although the latter findings provide evidence for the addition of IL-2 to TPA cultures, it is not mandatory for successful CCA (Put et al, 2009a; Struski et al, 2009).
Fig. 1. Conventional cytogenetic analysis: summarized procedure

PB, peripheral blood; BM, bone marrow; CLL, chronic lymphocytic leukemia

Patient Material
Fresh PB is preferred

Medium
L-glutamine

MITOGEN

Antibiotic(s)
Antimycotic(s)

Foetal Calf Serum

Cells are incubated at 37°C for 72h

Colcemid arrests cell cycle in metaphase by interference with microtubules

Colcemid

Further computer processing of microscopic images to establish a karyotype

Microscopy of stained slides
Visualization of metaphases

Denaturing (therm) and staining

Giemsa (G)-
Quinacrine (Q)-
OR

MITOGEN Colcemid

Medium
L-glutamine
Antibiotic(s)
Antimycotic(s)
Foetal Calf Serum
2.1.2 CD40-ligand (CD40L)

As metaphase induction by TPA is weak and aberration detection is inferior compared with FISH, efforts were made to improve culture methods. In contrast to the environment of lymph node proliferation centers, in vitro cultures do not protect the lymphocytes from apoptotic and cytotoxic triggers. The addition of CD40 was able to induce an antiapoptotic profile in CLL cells (Hallaert et al., 2008) and therefore it could improve the generation of metaphases. CD40 is an antigen expressed on the surface of normal and malignant B-cells and induces cell cycle progression after activation by its ligand (Buhmann et al., 2002). CD40L-induced cell cycle stimulation resulted in a threefold increase in generation of metaphases compared with stimulation with B-cell mitogens such as TPA, LPS and PWM. In addition, the success rate of CCA and aberration detection rate were higher in the CD40L cultures (93% vs. 78% and 89% vs. 22%, respectively) (Buhmann et al., 2002). However, this technique is labor-intensive and expensive, and therefore not applicable for routine analysis.

2.1.3 DSP30

At the present time, the best CCA results in CLL are obtained with the addition of CpG oligonucleotides (ODN) and IL-2 to the culture medium. ODN containing a CpG motif, such as DSP30, stimulate cells of the immune system via the Toll-like receptor 9 (TLR9). In humans, the only cell types known to express TLR9 are B-cells and plasmacytoid dendritic cells (Hornung et al., 2002). It has been established that CpG stimulates a broad spectrum of B-cell malignancies, i.e. CLL (Jahrsdorfer et al., 2005). CpG induces proliferation in normal B-cells; however, proliferation is weaker and followed by increased apoptosis in CLL cells (Jahrsdorfer et al., 2005). The lower proliferative response to CpG-ODN in CLL cells compared with normal B-cells can be overcome by addition of IL-2. Indeed, compared with normal B-cells, CpG causes a stronger induction of the IL-2 receptor α chain (CD25) in CLL, resulting in higher numbers of IL-2 receptors with a stronger affinity. Costimulation with CpG and IL-2 might alter IL-2 signaling in CLL cells in addition to increase cytokine production and surface molecule expression (Decker et al., 2000a).

The use of CpG/IL-2 improves proliferation capacity of CLL cells, and therefore it enables karyotyping in more cases (79-98%). Moreover, the technique yields detection rates of aberrations comparable with interphase FISH (81-83%) (Dicker et al., 2006; Haferlach et al., 2007). Other groups confirmed an improvement of the aberration detection rate in CpG/IL-2 (i.e. an increase of 9-13% of cases with aberrations) compared with TPA stimulated cultures (Put et al., 2009a; Struski et al., 2009). Moreover, the detection of translocations and del(13q) in particular, has been found to be superior after CpG/IL-2 stimulation compared with TPA (Put et al., 2009a).

The influence of CpG/IL-2 on quality of banding and metaphase generation is not clear (Put et al., 2009a; Struski et al., 2009).

Another question to address is whether abnormalities found after CpG/IL-2 stimulation might be related to activation-induced cytidine deaminase (AID). CpG stimulation of CLL and normal B-cells induces expression of AID, an enzyme that is linked to the development of genetic abnormalities (Capolunghi et al., 2008). However, culturing B-cells of healthy blood donors with CpG/IL-2 did not induce clonal abnormalities, thus validating CpG/IL-2 as a tool for the cytogenetic analysis of CLL (Dicker et al., 2006; Put et al., 2009a; Wu et al., 2008).
In conclusion, CpG/IL-2 should be preferred for routine CCA of CLL. However, as neither conventional cytogenetics nor CLL-specific FISH can detect all aberrations, both techniques should be complementarily applied.

2.2 FISH

FISH uses labeled DNA probes directed to selected targets and has a higher resolution than standard cytogenetics (approximately 40 Kb - 1 Mb, depending on the size of the FISH-probes vs. 10 Mb, respectively). Moreover, it can be used on metaphases and on nondividing cells. Sample types that may be used for FISH include in most cases peripheral blood or bone marrow, but also lymph node, spleen or effusions. Either uncultured fresh or frozen cells, cultured fixed cells, or paraffin-embedded tissue sections can be investigated.

The procedure is summarized in Fig 2. Interphase FISH yields high rates of detection of abnormalities, i.e. 80% (Döhner et al., 2000). However, this technique provides only partial information confined to the chromosomal loci examined, whereas CCA gives an overview of all microscopically visible aberrations.

Although FISH is a very sensitive technique, one should consider certain shortcomings. As already mentioned, a limited number of probes is applied. For this reason FISH can underestimate genomic complexity. False-positive and false-negative interpretations occur in 5% of FISH assays (Smoley et al., 2010). Wrong results may be due to i.e. inadequate cut-offs, co-hybridization or poor hybridization of probes, background signals, difficulties in visualizing probe signals in different planes of the nucleus, inadequate probes [in case of microdeletions or microduplications, i.e. ATM or miR-15a/16-1, in which the probe may be too large or not covering the deletion]. Lack of proliferation of the aberrant clone can occur when FISH is performed on cultured material. Furthermore, complex and cryptic translocations may generate special patterns of FISH signals that do not match the normal, expected signal pattern.

In clinical practice, FISH is performed for the regions 17p13 (TP53), 11q13 (ATM), chromosome 12 and 13q14 (RB1 and miR15a/16.1). The panel can be extended with probes for the regions 6q21 and 14q32 (IGH). Of interest, particular aberrations detected by FISH (discussed in section 3.1), e.g. loss of 17p13, were identified as major prognostic markers in CLL.

Hence abnormalities detected by FISH may guide patient monitoring and therapeutic decisions. Moreover FISH analysis is recommended for pretreatment evaluation and before subsequent, second- or third-line treatment (Hallek et al., 2008).

2.3 MLPA

Since FISH is a quite laborious, time-consuming and expensive technique, MLPA has been developed as an alternative tool. This technique relies on the comparative quantitation of specifically bound probes that are amplified by polymerase chain reaction (PCR) with universal primers. The latter allows simultaneous processing of multiple samples and has proven to be accurate and reliable for identifying deletions, duplications, and amplifications (Coll-Mulet et al., 2008). The procedure is summarized in Fig 3. (Schouten et al., 2002) and an example of MLPA results is shown in Fig 4. In a study comparing FISH and MLPA on 100 samples of untreated early stage (Binet A) CLL patients, a high degree of concordance between both techniques was observed (95%). Seven aberrations were not detected by
Fig. 2. Fluorescent in situ hybridization: summarized procedure

- Patient ID: PB/BM
- PB, peripheral blood; BM, bone marrow; PBS, phosphate buffered saline; SSC, saline-sodium citrate (SSC) buffer; DAPI, 4',6-diamidino-2-phenylindole

**Patient Material:** Fresh PB/BM; cultured or frozen cells

**Fresh PB is preferred.**

**Cell suspensions are dropped on glass slides.**

**Cells are treated with pepsin (digestion) and formaldehyde (fixation), washed in PBS and dehydrated in ethanol.**

**Fluorescent probes are applied, covered with glass and sealed with rubber cement.**

**Further computer processing of microscopic images.**

**Microscopy of slides: visualization of fluorescent signals on metaphases and interphase nuclei.**

**DAPI staining of DNA.**
Fig. 3. Multiplex ligation-dependent probe amplification (MLPA): summarized procedure

DNA extracted from patient PB or BM is diluted and denatured at 98°C. After heating to 95°C, a probe mix is added.

Further software analysis of MLPA results reflects copy number status. Relative amounts of amplification products are separated by electrophoresis. Overnight hybridization at 60°C is followed by PCR amplification of the ligated Y and X sequences.
MLPA, probably due to the low percentage of leukemic cells (<30%) carrying the aberration (Fabris et al., 2011). The sensitivity may even be lower if no B-cell pre-enrichment is performed (i.e. aberrations not detected when the percentage of leukemic cells <50%). Moreover MLPA fails to detect concomitant mono- and biallelic losses at 13q (Fabris et al., 2011). However, the availability of multiple probes in MLPA allows the identification of genetic aberrations which are not incorporated in the standard FISH probe panel. In conclusion, MLPA can be used alone or in association with FISH to detect both recurrent and less frequent lesions in CLL.

2.4 Comparative genomic hybridization and single nucleotide polymorphism arrays

Very recently (2000s), comparative genomic hybridization arrays (aCGH) and single nucleotide polymorphism (SNP)-arrays have been validated as reliable tools to investigate global genetic abnormalities in CLL with a higher resolution (i.e. 200 basepairs – 10 kilobases), compared with FISH and conventional cytogenetics. Therefore, it allows to detect new, cytogenetically cryptic, recurrent chromosomal changes, such as microdeletions.

However, aCGH has shortcomings as it detects genomic imbalances, but not balanced aberrations. In contrast with aCGH, SNP-arrays have the additional advantage of detecting copy number neutral loss of heterozygosity (cnLOH) or uniparental disomy (UPD). LOH results from the loss of normal function of one allele of a gene in which the other allele has already been inactivated, whereas UPD is a cnLOH in which all copies of an allele are derived from one parent and no copies from the other parent are present. Until now, the application of aCGH and SNP-arrays is restricted to research setting, but may possibly be implemented in routine analysis of CLL in the near future. As many platforms from different companies are available and each platform has its own technical specifications, Fig 5. gives only a brief and general overview of the technique. In the next paragraphs, we will focus in detail on the main results.
Fig. 5. Array-technology: summarized procedure.
PB, peripheral blood; BM, bone marrow; BAC, bacterial artificial chromosome

2.5 Next generation sequencing

Next-generation sequencing (NGS, also known as massively parallel sequencing) technologies have a higher throughput than traditional sequencing methods. It allows
millions of sequencing reactions to happen in parallel, using different approaches, either by creating micro-reactors and/or attaching DNA molecules to solid surfaces or beads. Unlike previous methods NGS generates millions of short reads (21-400 base pairs) and does not require amplification as sequencing can be performed from a single DNA molecule. The short reads can be quantified, allowing accurate copy number assessment. Moreover, with approaches that sequence both ends of a DNA molecule (paired end massively parallel sequencing), it has become possible to detect balanced and unbalanced somatic rearrangements (i.e. fusion genes) in a genome-wide fashion. Since each type of NGS has specific artefacts, one should be aware of this phenomenon and new findings should be interpreted with caution (Reis-Filho, 2009). In addition, the high cost of the technique limits its use in (routine) practice.

3. Cytogenetic and molecular abnormalities in CLL prognosis

3.1 Five prognostically important FISH-categories

A landmark interphase FISH-study of 325 mainly untreated CLL patients identified five prognostically important hierarchical categories: 17p deletion (with or without concomitant lesions), 11q deletion (with no concomitant 17p deletion), 12 trisomy (with neither concomitant 17p- nor 11q deletion), none of these aberrations, and 13q deletion as the sole abnormality (Fig 6. and Fig 7.). Median survival times for patients in these five groups were 32, 79, 114, 111, and 133 months, respectively and the treatment-free survival was 9, 13, 33, 49 and 92 months, respectively (Döhner et al, 2000).

Fig. 6. Idiogram of G-banded chromosomes involved in prognostically important aberrations, at 550-band level.
Commonly deleted regions are indicated in red (caveat: deletions may be larger or smaller). Del(13q) type Ib can vary in length, as indicated by the dashed line.
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(A) 

1. Chromosomes 17
2. Chromosomes 11
3. Chromosomes 12
4. Chromosomes 13
5. Chromosomes

(B) 

(C) 

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Fig. 7. Examples of the four prognostically important aberrations in CLL, namely del(17p) (1), del(11q) (2), trisomy 12 (3) and del(13q) (4, 5), as observed by CCA (A), interphase FISH (B), metaphase FISH (C) and the Affymetrix 2.7M array platform (D). Applied FISH-probes are specific for TP53 (red) and centromere 17 (CEP17) (green) (1), for ATM (red) and centromere 12 (green) (2+3), for RB1 (green) and D13S319 (red) (4+5). Note a monoallelic loss of TP53 (B1, C1, D1 red arrow), monoallelic loss of ATM (B2, C2, D2 red arrow), trisomy 12 (B3, C3, D3 blue arrow), a monoallelic (B4, C4, D4 cases A+B+C, single red arrows) and biallelic del(13q) in (B5, C5, D4 case C, double red arrow).

3.1.1 17p deletions

Patients with a deletion of 17p have worst outcome. The del(17p) is found in 3-8% of previously untreated patients, although higher incidences up to 45% have been reported in patients with relapsed or refractory CLL, as a consequence of clonal selection (Cramer and Hallek, 2011; Zenz et al, 2011). Del(17p) usually encompasses the TP53 locus at 17p13. A gene-dosage effect of TP53 has been reported. About 80-90% of the cases harbor a biallelic inactivation of TP53 (i.e. deletion of one copy and mutation of the remaining copy), but also the monoallelic inactivation of TP53 is an adverse prognostic marker (Cramer and Hallek, 2011; Zenz et al, 2011). The tumor suppressor p53 plays an essential role in inducing apoptosis or cell cycle arrest after DNA damage. Since therapy with purine nucleoside analogues (e.g. fludarabine) and alkylating agents (e.g. chlorambucil) is based on p53-dependent mechanisms, CLL patients with deletion 17p or inactivating mutations of TP53 are refractory to such chemotherapy (Van Bockstaele et al, 2008) and have impaired survival. A threshold of > 20-25% interphase nuclei harboring the del(17p) has been reported to correlate with adverse survival (Catovsky et al, 2007; Tam et al, 2009). Because of the very poor prognosis, risk-adapted treatment for this subgroup has been developed. Current treatment approaches (in clinical trials) use agents acting independently of p53 (e.g. alemtuzumab, high dose steroids) or allogeneic stem cell transplantation for fit patients (Zenz et al, 2011). In the future, optimization of the therapeutic strategies hopefully may improve outcome for this poor prognostic subgroup.
3.1.2 11q deletions

Deletions of 11q have been associated with adverse outcome. It is found in about 20% of the patients with CLL (Van Bockstaele et al, 2008; Zenz et al, 2011). The minimally deleted region (MDR) at 11q22.3-q23.1 harbors the ATM (ataxia telangiectasia mutated) gene. ATM is a protein that acts upstream of p53 in the DNA damage response pathway. Mutations of ATM have been reported in 12% of patients with CLL and in 30% of patients with del(11q) (Zenz et al, 2011). As not all patients with del(11q) have an ATM mutation (and vice versa), haplo-insufficiency of ATM or the presence of other tumor suppressor genes in the MDR can be suspected. In the patients with del(11q), the biallelic inactivation of ATM leads to a worse clinical outcome (Cramer and Hallek, 2011). Of note, rarely the del(11q) does not encompass ATM, but affects the telomerically located FDX locus (Heim and Mitelman, 2009). Patients with del(11q) are generally younger, have more B-symptoms and more advanced clinical stages. Furthermore, the del(11q) is typically associated with extensive lymphadenopathy (Cramer and Hallek, 2011; Van Bockstaele et al, 2008).

3.1.3 Trisomy 12

An intermediate outcome has been described for patients with trisomy 12. While progression free survival (PFS) may be shorter (PFS rate at 3 years of 48-83%), overall survival (OS) is rather favorable (OS rates at 3 years of 86-96%). Trisomy 12 has been associated with atypical morphology or immunophenotype (i.e. stronger surface immunoglobulin and FMC7 expression) (Zenz et al, 2011). The aberration is observed in 10-30% of patients (Van Bockstaele et al, 2008; Zenz et al, 2011). This variation probably reflects differences in patient selection. Partial trisomy 12q was reported in 10-20% of the cases and a minimal common gained region has been confined to 12q13 (Heim and Mitelman, 2009). The critical genes involved in the trisomy 12 are yet unknown. Small duplications of 12q have been reported and in particular the murine double minute 2 gene (MDM2) located at 12q15 has been found amplified in CLL (Merup et al, 1997). Overexpression of the MDM2 protein was also observed in CLL and this was significantly more frequent in the advanced rather than the earlier stages (Watanabe et al, 1996). The MDM2 SNP309 in B-CLL has been suggested to be an unfavorable prognostic marker; however the results of several recent publications are conflicting (Willander et al, 2010). The CLL upregulated gene 1 (CLLU1) located at 12q22 was overexpressed exclusively in CLL and its expression was shown to have a strong prognostic significance in patients younger than 70 years, namely higher expression was associated with shorter overall survival (Josefsson et al, 2007). However overexpression of CLLU1 occurs irrespectively of trisomy 12 or other large chromosomal rearrangements (Buhl et al, 2006).

Recurrent association of trisomy 12 with IG-aberrations, such as t(14;19)(q32;q13), t(14;18)(q32;q21) and del(14)(q24q32), and with trisomy 18 and/or trisomy 19, has been observed in a subset of cases (Heim and Mitelman, 2009). Trisomy 12 with concomitant TP53 mutations is rare.

3.1.4 13q deletions

Although deletions of 13q are often cytogenetically cryptic, they represent the most frequently observed FISH-aberration in CLL, with a prevalence of 40-60% (Van Bockstaele et al, 2008). Only when present as a solitary aberration (by FISH), the del(13q) implies a favorable prognosis. Higher percentages (that is > 65% or > 80%) of interphase FISH nuclei showing the
del(13q) have been associated with shorter overall survival and time to first treatment (Hernandez et al, 2009; Van Dyke et al, 2010). The MDR located at 13q14 contains miR-15a and miR-16-1. These microRNAs are small non-coding RNA genes that regulate gene expression. The miR-15a/16-1 cluster seems to negatively regulate the expression of multiple genes involved in proliferation and apoptosis (Klein and Dalla-Favera, 2010). Deletion of the MDR-region in mice models suggested that this lesion is sufficient for lymphomagenesis. In some CLL cases without del(13q), downregulation of miR-15a and miR-16-1 has been described, suggesting an epigenetic mechanism suppressing the miR-cluster (Klein and Dalla-Favera, 2010). Mutations in the miR-cluster appear to be very rare (Zenz et al, 2011). The del(13q) is most frequently heterozygous (monoallelic, 76% of cases), but can be homozygous (biallelic, 24% of cases). While the former is suggested to be an early event, the latter probably occurs at a later stage. A gene dosage-effect of miR-15a/16-1 has been reported (Zenz et al, 2011). In addition, SNP-arrays showed that the extent of the deletion (Fig 6) is associated with disease characteristics, for example del(13q) type II (long, involving RB1, related with disease progression) and del(13q) type I (short, not involving RB1, related with disease progression only when associated with other aberrations) (Malek et al, 2010; Zenz et al, 2011).

3.2 Other cytogenetic aberrations

Several other recurrent genomic aberrations have been described in CLL, such as del(6q), del(14)(q24.1q32.33) involving IGH (Pospisilova et al, 2007), t(1;6)(p35;p25) involving MUM1/IRF4 (Michaux et al, 2005), total or partial trisomy 3, trisomy 8, trisomy 18 and 19 and changes leading to gains of 2p24-25, 3q26-27, and 8q24. These aberrations are rare in CLL (prevalence < 5-10%). Most of the genes involved are not yet identified and their prognostic relevance remains to be investigated (Heim and Mitelman, 2009; Van Bockstaele et al, 2008).

3.3 Translocations

Translocations have been reported in up to 34-42% of patients with CLL (Mayr et al, 2006; Van Den Neste et al, 2007). Balanced translocations are relatively rare, but unbalanced non-reciprocal aberrations are frequent and are often observed within complex karyotypes. Although translocations are heterogenous in CLL, many breakpoints are located in regions showing recurrent loss, like 13q14 and 17p13 (Heim and Mitelman, 2009). Chromosomal translocations in general may have a negative impact on response to therapy and survival, especially when unbalanced (Mayr et al, 2006; Van Den Neste et al, 2007). Balanced translocations, especially those involving immunoglobulin (IG) genes, are recurrent, but uncommon (i.e. 5%) (Haferlach et al, 2007). Recurrent partners include BCL2, BCL3, BCL11A and MYC (Table 2). In published reports (Cavazzini et al, 2008; Nowakowski et al, 2007), at least part of the cases have unknown partner genes. In most studies, CLL cases with translocations involving IG are analyzed as a single group (Cavazzini et al, 2008; Juliusson et al, 1990). However, the partner gene that becomes overexpressed as a result of the translocation, may be relevant for the outcome. The best described is the BCL3 gene involved in the t(14;19), often associated with atypical morphology, unmutated IGVH genes and inferior prognosis (Cavazzini et al, 2008; Chapiro et al, 2008; Martin-Subero et al, 2007; Nowakowski et al, 2007). Similarly, translocations involving MYC have been associated with loss (i.e. monosomy) of 17, del(11q) complex karyotype, additional unbalanced translocations and poor prognosis (Put et al, 2011). In contrast, translocations involving BCL2 are associated with mutated IGVH genes, trisomy 12, absence of del(11q) and more favorable outcome (Put et al, 2009b).
Translocation\textsuperscript{a,b} & Partner Gene & Morphology & IGH & Associated changes & Prognosis \\
\hline
\textbf{t(2;14)(p16;q32)} & \textit{BCL11a} & Atypical & U > M & Trisomy 12 & Uncertain \\
\textbf{t(8;14)(q24;q32)} & \textit{MYC} & PL / PT & U \approx M & Monosomy 17p & Poor \\
& & & & Del(11q) & \\
& & & & Complex karyotype & \\
& & & & Unbalanced translocations & \\
\textbf{t(14;18)(q32;q21)} & \textit{BCL2} & Typical & M > U & Trisomy 12 & Favorable \\
& & & & Absence of del(11q) & \\
\textbf{t(14;19)(q32;q21)} & \textit{BCL3} & Atypical & U > M & Trisomy 12 & Poor \\
\textsuperscript{a}IG-translocations involve most frequently \textit{IGH} located on 14q32. Variant translocations involve either \textit{IGK} on 2p12 or \textit{IGL} on 22q11. \\
\textsuperscript{b}To date, most cases with t(11;14)(q13;q32), involving \textit{CCND1}, are diagnosed as mantle cell lymphoma; however, rare cases of t(11;14)-positive CLL might exist.

Table 1. Overview of translocations involving immunoglobulin (\textit{IG})-genes in CLL

### 3.4 Genomic complexity

Cytogenetic complexity is defined as the presence of three or more clonal chromosomal aberrations. CCA was found to be superior in the detection of complexity, compared with FISH (Haferlach et al., 2007), probably due to the limited number of investigated loci in the latter approach. Complexity is found in a minority of the cases with CLL (10-30%) (Haferlach et al., 2007; Kujawski et al., 2008). A highly significant association was observed between complex aberrant karyotypes and 17p deletions, unmutated \textit{IGVH} and expression of CD38 (Haferlach et al., 2007). In addition, particular aberrations (i.e. translocations involving \textit{MYC}) have also been associated with a complex aberrant karyotype (Put et al., unpublished data). Prognostically, patients with complex genomic changes appear to have more aggressive disease. Similarly, genomic complexity detected by SNP-arrays ($\geq$ 3 genetic lesions) has been associated with poor outcome (Kujawski et al., 2008). An impaired apoptotic DNA double-strand break response and multiple genomic deletions, including del(17p), del(11q), and del(13q) type II were identified as independent strong predictors of genomic complexity in CLL. Moreover, a strong independent effect of aberrant p53 function on genomic complexity and a modest effect of decreased ATM function have been observed (Ouillette et al., 2010). Such multiple independent gene defects in CLL may contribute to genomic instability. In addition, telomere dysfunction as a consequence of telomere erosion may also drive genomic instability during the progression of CLL (Lin et al., 2010). Indeed, short telomeres have been associated with a high risk of genomic aberrations and genetic complexity (Roos et al., 2008).

### 3.5 Clonal evolution

Clonal evolution (CE) represents the acquisition of new or additional cytogenetic aberrations during disease course. As a consequence, CCA or FISH should not only be used for initial prognostication of patients with CLL, but also at the time of disease progression or before therapy initiation [FISH is mandatory in this setting for detection of del(17p)]. Initially, CE as evaluated by sequential CCA, was considered infrequent, i.e. in 16% of CLL patients (Oscier et al., 1991). Later studies reported higher frequencies of 25-43% (Fegan et al., 1995; Finn et al., 1998; Haferlach et al., 2007). Interphase FISH studies (Table 2) revealed CE in 27% and 17% after a median follow-up of more than 5 years and 42.3 months, respectively (Shanafelt et al., 2006;
Interestingly, CE occurred more frequently among cases with unmutated IGVH status (Shanafelt et al., 2006; Stilgenbauer et al., 2007). However, another study did not find a correlation between CE and unmutated IGVH, expression of CD38 and ZAP70 on one hand, but the combination of all three prognostic factors correlated highly significantly with CE and with a shift from lower to higher FISH risk category (Berkova et al., 2009). Patients with CE showed progression to more advanced stages, greater need for therapy and a higher hazard ratio for death. Moreover, CE was identified as an independent factor for survival (Stilgenbauer et al., 2007). As a consequence, CCA or FISH should not only be used for initial prognostication of patients with CLL, but also at the time of disease progression or before therapy initiation [FISH is mandatory in this setting for detection of del(17p)]

<table>
<thead>
<tr>
<th>Reference</th>
<th>CLL Patients</th>
<th>Follow-up (months)</th>
<th>CE: previously treated patients (n)</th>
<th>CE: abnormalities</th>
<th>Other findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shanafelt et al, 2006</td>
<td>108+</td>
<td>67 (23-136)</td>
<td>18 (11%)</td>
<td>del(13q) (72%) &gt; del(17p) (22%) &gt; del(11q) (6%)</td>
<td>CE not confined to unmutated IGVH (association ns)</td>
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<td>Correlation between ZAP70+ and CE</td>
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<td>CE more frequent after 50 months compared with before 24 months (27% vs. &lt;2%, respectively)</td>
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<td>Stilgenbauer et al, 2007</td>
<td>64</td>
<td>42 (23-73)</td>
<td>11 (17%)</td>
<td>del(17p) (36%) &gt; del(13q) = del(6q) (27%) &gt; del(11q) (18%) &gt; +8q24 (9%)</td>
<td>CE confined to unmutated IGVH</td>
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<td>CE correlates with progressive clinical stages, greater need for therapy, higher hazard ratio for death</td>
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<td></td>
<td>CE as independent factor for survival</td>
</tr>
<tr>
<td>Berkova et al, 2009</td>
<td>97</td>
<td>66 (22-304)</td>
<td>25 (26%)</td>
<td>del(13q) (64%) &gt; del(17p) = del(11q) (16%) &gt; trisomy 12 (4%)</td>
<td>Combination of unmutated IGVH, CD38+ and ZAP70+ correlates highly significantly with CE and with a shift from lower to higher FISH risk category</td>
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<tr>
<td>Loscertales et al, 2010</td>
<td>81</td>
<td>67 (16-111)</td>
<td>17 (21%)</td>
<td>del(17p) (53%) &gt; del(11q) (35%)</td>
<td>CE not confined to unmutated IGVH</td>
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<td>del(17p) observed in untreated patients</td>
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</tbody>
</table>

N, number; CE, clonal evolution; ns, not significant.
*Sequential samples were available in 108/159 patients.

Table 2. Overview of clonal evolution investigated by FISH

### 3.6 Molecular karyotyping

The introduction of aCGH and SNP-arrays enables to investigate CLL at a resolution, greatly surpassing this of conventional cytogenetics. Different array-platforms were validated as a powerful, cost-effective tool for clinical risk assessment in CLL (Table 3) (Gunn et al., 2008; Hagenkord et al., 2010; O'Malley et al., 2011). Of note, the sensitivity of these platforms varies and is related to i.e. the resolution of the array. For example, the Affymetrix SNP6.0 array was found to be superior to the 250K array in detecting small aberrations of uncertain significance and equivalent to the 250K array in detecting clinically relevant lesions. Since the cost of the 250K array is lower, it is preferred for routine use. In contrast, the 10K array is not suitable for routine clinical use due to its low resolution (Hagenkord et al., 2010).

New recurrent cytogenetic abnormalities were detected by aCGH and SNP-arrays. In Table 3 an overview of selected publications on array-applications in CLL is shown, describing known prognostically important lesions and new molecular cytogenetic findings (Grubor et al, 2009;
Genetics of Chronic Lymphocytic Leukemia: Practical Aspects and Prognostic Significance

Gunn et al, 2008; Gunnarsson et al, 2008; Guennarsson et al, 2010; Hagenkord et al, 2010; Kujawski et al, 2008; Lehmann et al, 2008; O’Malley et al, 2011; Ouillette et al, 2010; Ouillette et al, 2011; Pfeifer et al, 2007; Rinaldi et al, 2011). Other recent studies using array-platforms revealed new insights in the disease: i.e. the genome of CLL appeared to be quite stable over time (Brown et al, 2010); disease progression has been associated with large, but not small copy number alterations (Gunnarsson et al, 2010), genomic complexity, 13q deletion in the presence of other aberrations (Gunnarsson et al, 2011) and deletions involving RBL1 (Malik, 2010).

Table 3. Overview of selected publications on genomic array-applications in CLL

<table>
<thead>
<tr>
<th>Reference</th>
<th>Array</th>
<th>Patients (n)</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Other highlighted abnormalities</th>
<th>LOH/HC L/H</th>
<th>Scenario</th>
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<tbody>
<tr>
<td>Philbert et al, 2007</td>
<td>E. coli. Affymetrix</td>
<td>70</td>
<td>5%</td>
<td>25%</td>
<td>4%</td>
<td>0%</td>
<td>3%</td>
<td>Gain of 2p11.2 (n=6), 13q</td>
<td>11 patients with large CN aberrations, 10 patients with 13q deletions</td>
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<tr>
<td>Lehmann et al, 2008</td>
<td>50K Affymetrix</td>
<td>90</td>
<td>5%</td>
<td>10%</td>
<td>7%</td>
<td>4%</td>
<td>Loss of 3q26 (n=2)</td>
<td>10 patients with LOH and 7 patients with UDG</td>
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<tr>
<td>Ouillette et al, 2010</td>
<td>50K Affymetrix</td>
<td>131</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Gain of 12q13.3q15.1</td>
<td>Genomic aberrations of the genome including UDG and very common events in early stage CLL</td>
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<tr>
<td>Kujawski et al, 2010</td>
<td>44K Agilent</td>
<td>171</td>
<td>7%</td>
<td>15%</td>
<td>10%</td>
<td>9%</td>
<td>Gain of 3q (n=5)</td>
<td>17 patients with UDG</td>
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<tr>
<td>Ouillette et al, 2011</td>
<td>32K BAC, 13K Affymetrix, 50K Affymetrix, and 1.7K BAC</td>
<td>17</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Gain of 3q (n=2), gain of 7p (n=2), gain of 11q (n=3), gain of whole chr 11 (n=1) and chr 2</td>
<td>Genomic instability (e.g. 23% of translocated by BAC array) was observed in 27% of the cases</td>
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<tr>
<td>Ouillette et al, 2012</td>
<td>150K BAC, 150K Affymetrix, and 3.7K BAC</td>
<td>18</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Gain of 3q (n=2), gain of 7p (n=2), gain of 11q (n=3), gain of whole chr 11 (n=1) and chr 2</td>
<td>Genomic instability (e.g. 23% of translocated by BAC array) was observed in 27% of the cases</td>
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<tr>
<td>Conforti et al, 2009</td>
<td>45K and 36K single nucleotide polymorphisms (SNP)</td>
<td>12</td>
<td>3%</td>
<td>12%</td>
<td>20%</td>
<td>7%</td>
<td>Loss of 22q11.21 (n=2), 11q22.3 (n=2), 11p15.5 (n=2), 11q13.3 (n=2), 11q14.3 (n=2), 5p15.31 (n=2), 5q35 (n=2)</td>
<td>LOH/HC and UDG in 3/12 cases</td>
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Table 3 (continued).

<table>
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<tr>
<th>Key et al., 2010</th>
<th>11q Abnormal</th>
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<th>17p Abnormal</th>
<th>17q Abnormal</th>
<th>13q Abnormal</th>
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<td>Loss of 1p, 1q, 14q, 14p or 11q or 11p and aberrations present in 10%</td>
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<td>3 patients with UPD of 17p31.3 (2 cases) and IGH deletion (2 cases); 29 cases with IGH deletion (1 case); 2 cases with LOH at 11p15.5; 17 cases with IGH deletion (1 case); 1 case with LOH at 11p15.5; 1 case with LOH at 11q13.1</td>
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<td>Acquired loss of 10q (6 cases) including 1 case with 6q loss and 1 case with genetic gain of 2q (2 cases) and loss of 1p (5 cases); 2 cases with IGH deletion (1 case); 2 cases with IGH deletion (1 case); 2 cases with IGH deletion (1 case)</td>
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<td>Genomic complexity (10 cases with IGH deletion and 1 case with LOH at 11q13.1); 1 case with IGH deletion (1 case); 1 case with IGH deletion (1 case); 1 case with IGH deletion (1 case)</td>
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<td>Genomic complexity (10 cases with IGH deletion and 1 case with LOH at 11q13.1); 1 case with IGH deletion (1 case); 1 case with IGH deletion (1 case); 1 case with IGH deletion (1 case)</td>
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<td>Clonal evolution (CE)</td>
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<td>Untreated – treated patients, respectively</td>
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<td>At diagnosis + at follow-up, respectively</td>
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<td>Low frequency of (partial) gain of 12q</td>
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Chronic Lymphocytic Leukemia
3.7 Next generation sequencing

Whole genome sequencing of cases with CLL led to the discovery of several genes, previously unsuspected to be involved in this disease. For example, combining NGS and copy number analysis in 5 patients, < 20 clonal genomic alterations/case and recurrent mutations of NOTCH1, TGM7, BIRC3, and PLEKHG5 were observed (Fabbri et al, 2011). Lesions of MYD88, BIRC3, and PLEKHG5 are all linked to alteration of the NF-κB pathway. In a screening cohort of 48 CLL cases, NOTCH1 mutations were found in 8.3% of CLL cases at diagnosis and were associated with aggressive disease (i.e. higher frequency of NOTCH1 mutations were associated with Richter transformation and refractoriness to chemotherapy, in 31.0% and 20.8% of the cases, respectively). Moreover NOTCH1 mutation at diagnosis emerged as an independent risk factor for poor survival (Fabbri et al, 2011). Another NGS and exome sequencing study identified four genes that were recurrently mutated, namely NOTCH1, XPO1 predominantly in CLL with unmutated IGVH, and MYD88 and KLHL6 in CLL with mutated IGVH status (Puente et al, 2011). NOTCH1, XPO1 and MYD88 mutations are suspected to be oncogenic changes, contributing to disease progression, based on their patterns of mutation and functional analyses, (Puente et al, 2011). In conclusion, NGS appears to be a highly effective technique in identifying new genetic lesions and future studies are promising to contribute to an improved understanding of disease onset and evolution.

4. The origin of cytogenetic abnormalities

Genomic imbalances, such as gains and losses of chromosome segments or whole chromosomes (aneuploidy), are more frequently observed than translocations in CLL. However, in the following paragraphs we will focus mainly on the origin of translocations, in particular translocations involving IG loci, as the underlying mechanisms are quite specific for lymphoid malignancies, i.e. CLL.

4.1 The origin of aneuploidy and structural aberrations

Aneuploidy may arise due to defects in segregation of chromosomes during cell division, including multipolar spindles, but also abnormal kinetochore-spindle interactions, premature chromatid separation, centrosome amplification, and abnormal cytokinesis. Defects of centrosome function in particular have been suggested to be involved in a wide variety of human malignancies. Centrosomes have central role in organizing microtubuli and the mitotic spindle. An aberrant number, size, shape of the centrosome, as well as aberrant phosphorylation of centrosome proteins, may missegregate chromosomes, resulting in aneuploid cells. In addition, errors in the separation of sister chromatids could also be a cause of aneuploidy. Finally, checkpoint controls are expected to be abrogated in order to enable unequal chromosome segregation during cell cycle progression (Gollin, 2004; Schwab, 2001).

Structural chromosomal instability results from chromosome breakage and rearrangement due to defects in the cell cycle checkpoints, the DNA damage response and/or loss of telomere integrity (Gollin, 2004). When a chromatid break occurs, an unprotected chromosomal end will probably fuse with either another broken chromatid or its sister chromatid to produce a dicentric chromosome. During the anaphase, the two centromeres
are pulled to opposite poles, forming a bridge that breaks, resulting in more unprotected chromosomal ends, thus resulting in breakage-fusion-bridge cycles. Telomere mechanics, defects in DNA damage response and cell cycle checkpoint may play important roles in the development and maintenance of chromosomal instability (Gollin, 2004).

4.2 The origin of translocations

Recurrent translocations in CLL often involve $\text{IG}$ loci. These translocations may follow DNA double strand breaks (DSBs) that are generated during V(D)J recombination (i.e. recombination of Variable, Diversity, and Joining segments of $\text{IG}$-genes) and somatic hypermutation (SHM) in developing B-cells and in the context of class switch recombination (CSR) in activated mature B-cells. DSBs in the partner loci may be generated by off-target VDJ recombination, CSR activities or may result from more general factors, such as oxidative metabolism or genotoxic agents. Misrepair of these DSBs can promote oncogenic translocations. When a translocation involves oncogenes or tumor suppressor genes, it can be positively selected in the context of neoplastic transformation. Selection likely plays the main role in the appearance of most clonal translocations in tumors (Gostissa et al., 2011).

4.2.1 VDJ recombination and RAG-mediated DSB

The complete VDJ recombination involves RAG-mediated cleavage, which generates DSBs, and the DSB repair pathway “classical nonhomologous DNA end-joining” (C-NHEJ). The latter promotes chromosomal integrity and suppresses the formation of translocations. In the absence of C-NHEJ, DSBs still can be joined by alternative end-joining (A-EJ), a process that contributes to oncogenic chromosomal translocations (Gostissa et al., 2011; Nussenzweig and Nussenzweig, 2010).

4.2.2 SHM, CSR and AID-mediated DSB

Although representing different processes, SHM and CSR are both initiated by AID (Gostissa et al., 2011; Perez-Duran et al., 2007). SHM generates point mutations, small deletions and insertions in variable region exons. This occurs in the germinal centers (GCs) and allows the selection of B-cells that express higher affinity B-cell receptors. CSR can also occur within the GC, as well as in extrafollicular regions (Gostissa et al., 2011).

AID initiates both SHM and CSR in B-cells by deaminating cytosines on the DNA of $\text{IG}$ genes. The generated lesion can be processed into a mutation (SHM) or a DSB followed by a recombination reaction (CSR) (Perez-Duran et al., 2007). CSR requires the generation of AID-initiated DSBs. In contrast, SHM generally does not require DSBs. The latter are only occasionally generated as by-products of AID activity (Gostissa et al., 2011). It has been suggested that AID may have a dual role; initiating chromosomal translocations on one hand and generating secondary hits by mutagenesis on the other (Perez-Duran et al., 2007). Aberrant SHM and involvement of AID were reported to be involved in mutations of $\text{TP53}$ (Malickova et al., 2008), $\text{MYC}$, $\text{PAX5}$ and $\text{RhoH}$ (Reiniger et al, 2006). Moreover, AID activity has been linked to the generation of DSBs involved in translocations in both $\text{IG}$ and non-$\text{IG}$ loci (Gostissa et al., 2011). While AID was shown to initiate the formation of translocations and mutations, ATM, p53 and ARF provide surveillance mechanisms to prevent these aberrations (Perez-Duran et al., 2007).
AID expression results from interaction with an activated microenvironment. In a study of CLL patients with unmutated IGVH, high AID expression was found exclusively in the small subset of cells with ongoing CSR (Palacios et al, 2010). In addition, in CLL and small lymphocytic lymphoma, AID expression has been associated with unfavorable clinical course and with adverse biological parameters, i.e. higher proliferation rate, deletion of ATM and TP53 (Leuenberger et al, 2010). AID expression has been considered to be predictive for CLL with unmutated IGVH status (Palacios et al, 2010). However, in other reports the association of AID expression and IGVH mutational status is considered controversial (Leuenberger et al, 2010).

4.2.3 Combined action of RAG and AID

In conclusion, RAG and AID can generate DSBs leading to translocations via VDJ recombination and CSR, respectively. RAG and AID are usually expressed in distinct B-cell developmental compartments. Activity of RAG has been observed in developing bone marrow B-cells, whereas AID activity has been found in peripheral mature B-cells. Breakpoint sequences can provide information regarding the developmental stage at which the translocation occurred (Gostissa et al, 2011; Nussenzweig and Nussenzweig, 2010). However, collaboration between RAG and AID in generating translocations has been reported. RAG induced DSBs can persist in the absence of ATM, an essential DNA damage checkpoint regulator, or in absence of the NHEJ factor XRCC4, leading to abnormal or delayed repair of RAG-mediated DSBs. In addition, AID may facilitate off-target DSB formation by RAG. As a consequence RAG and AID-mediated DSBs may coexist and become partners in translocation formation (Nussenzweig and Nussenzweig, 2010). Finally, not all DSBs that are precursors of translocations in lymphomas appear to be initiated by RAG or AID (Gostissa et al, 2011). The mechanism(s) involved herein remain largely unknown.

4.2.4 Oncogene activation

Most recurrent translocations activate oncogenes, either by generating oncogenic fusion proteins or by deregulating oncogene expression by linking it to strong transcriptional control elements. The IGH locus contains two known major transcriptional enhancer regions: the intronic enhancer (iEμ), which promotes optimal VDJ recombination in developing B-cells and the IGH 3′ regulatory region (IGH3′RR), which modulates CSR in mature B-cells by long-range (over 100 kb) activation of certain promoters. The IgH3′RR does not gain full enhancer activity until late in B-cell development. It was reported that iEμ has low oncogenic activity, suggesting that VDJ-mediated translocations that retain iEμ near the translocation breakpoint may arise in early B-cell developmental stages but remain oncogenically silent until the IgH3′RR becomes fully active at the mature B-cell stage. Alternatively, the development of mature B-cell tumors from cells carrying VDJ-mediated translocations might reflect the time required for the accumulation of secondary mutations necessary for transformation. Another explanation is that translocations may be generated directly in mature B-cells, either by persisting VDJ breaks arisen at the pro-B-cell stage or by RAG-mediated breaks in peripheral B-cells (Gostissa et al, 2011).
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Chronic Lymphocytic Leukemia


B-cell chronic lymphocytic leukemia (CLL) is considered a single disease with extremely variable course, and survival rates ranging from months to decades. It is clear that clinical heterogeneity reflects biologic diversity with at least two major subtypes in terms of cellular proliferation, clinical aggressiveness and prognosis. As CLL progresses, abnormal hematopoiesis results in pancytopenia and decreased immunoglobulin production, followed by nonspecific symptoms such as fatigue or malaise. A cure is usually not possible, and delayed treatment (until symptoms develop) is aimed at lengthening life and decreasing symptoms. Researchers are playing a lead role in investigating CLL’s cause and the role of genetics in the pathogenesis of this disorder. Research programs are dedicated towards understanding the basic mechanisms underlying CLL with the hope of improving treatment options.

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