Molecular cytogenetic investigations in chronic B-cell lymphoproliferative disorders: novel mitosis stimulators and clonal evolution

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I. Introduction & Literature Review

1.1. Cytogenetics: Historical Background:

Cytogenetic analysis has become an integral part of the diagnosis and management of many malignancies. Theodor Boveri was the first to suggest that malignant tumours could be due to an abnormal chromosome constitution (Sandberg, 1979). He also postulated the existence of enhancing or suppressing chromosomes, suggesting that malignant growth would result from loss of suppressing chromosomes or the predominance of enhancing chromosomes. The term chromosome was first coined by Waldeyer in 1888. The birth of cytogenetics is generally dated from Tjio and Levan’s identification of the true chromosome complement in human cells, as it is from this time that abnormalities of chromosome number and subsequently chromosome structure were reported (Tjio & Levan, 1956). Their discovery was made possible by a number of advances. In his delightful book, Hsu divided the study of human cytogenetics into four periods: the pre-hypotonic period (or Dark Ages), the period from 1952 to 1959 that included the discovery of hypotonic solution pretreatment for cytological preparations, the third period (1959–1969) during which time chromosome abnormalities were linked to clinical syndromes, and the post-banding (modern) period (Hsu, 1979). During the pre-hypotonic era, chromosomes were studied in mouse and rat cancers and camera lucida drawings of metaphases suggested the presence of many more chromosomes than normal and of structural abnormalities within these chromosomes.

The drawings were taken from squash preparations; a technique that was used to flatten metaphase spreads of chromosomes into a two-dimensional configuration, but still resulted in crowded overlapping aggregations of chromosomes that were very difficult to count. Colchicine, an extract of the autumn crocus, was used to arrest the cells in the metaphase stage of the cell cycle and increase the number of mitoses available for analysis. The use of a hypotonic pre-treatment method was an enormous step forward in the production of analysable chromosome preparations. The chromosomes could now be separated and viewed individually. Counting chromosomes was simplified and gross structural abnormalities could be discerned. Hsu describes the discovery of the utility of a hypotonic pre-treatment as a laboratory accident, the perpetrator of which never owned up to the error; thus, a major discovery in the history of cytogenetics was apparently made by an unknown technician. It was in this era, in 1956, that Tjio and Levan finally answered the question that had been plaguing investigators for more than 30 years, when they reported that there were 46
chromosomes in the human cell rather than 48. Subsequently, a number of researchers were able to identify chromosome abnormalities that appeared specific for clinical syndromes. Lejeune and his colleagues published the chromosomal nature of Down syndrome in 1959 (Lejeune et al., 1959). Their observation of an extra G group chromosome in patients with a specific congenital malformation syndrome showed for the first time that cytogenetic analysis could be used to diagnose a human condition. The study of the constitutional karyotype was aided by the discovery that phytohemagglutinin (PHA) could induce peripheral blood lymphocytes to divide (Nowell, 1960). This method was adopted by Moorhead et al. for the study of human chromosomes and remains one of the mainstays of modern cytogenetic analysis (Moorhead et al., 1960).

The confirmation of the correct chromosome number in human cells led in the late 1950s and early 1960s to a flood of publications describing numerical and structural abnormalities of chromosomes. The resulting confusion in the literature made clear that there was a need for a common nomenclature to describe these rearrangements in a manner that was intelligible to other workers in the field. Thus, a small group met in Denver, CO, to establish a system of describing chromosome abnormalities. They published the results of their deliberations in a report entitled “A Proposed Standard System of Nomenclature of Human Mitotic Chromosomes,” also known as the Denver Conference (1960), and this report has formed the basis for all subsequent nomenclature reports, now published as An International System for Human Cytogenetic Nomenclature (ISCN) (Shaffer et al., 2009).

The advent of banding enabled chromosomes to be individually identified and the normal homologues paired. Initially, banding patterns along the length of each chromosome were induced by preparations stained with quinacrine mustard and visualized via a fluorescence microscope (Caspersson et al., 1968) or depended upon a method whereby slides were incubated in warm saline or buffer solutions prior to staining by Giemsa. The initial Giemsa staining method required 3 days for completion. Seabright’s rapid banding technique was therefore embraced as the whole procedure could be carried out at room temperature using air-dried slides and producing G-banded chromosomes ready for observation within 10 minutes (Seabright, 1971). Once banding became available, there were numerous publications describing recurrent chromosome abnormalities that appeared to be found in specific tumour types.
1.2. Overview of Cancer Cytogenetics:
The history of cancer cytogenetics is not a long one but it has been eventful and much knowledge has been accumulated in the 50 years since Peter Nowell and David Hungerford published their finding of a small marker chromosome in the chromosome complement of cells cultured from seven patients with chronic myeloid leukaemia (CML) (Nowell & Hungerford, 1960). Nowell and Hungerford made their landmark discovery in 1960, only just beating another group from Edinburgh who had also noted the same marker chromosome in their CML patients (Baikie et al, 1960). Although the hypothesis that malignant cells were derived from normal tissue cells that had acquired abnormal chromatin content was first proposed by Boveri, it was not until Nowell and Hungerford’s description of the Philadelphia chromosome that a revolution in our understanding of the processes underlying the development of malignancy began. Nowell and Hungerford called the marker chromosome the Philadelphia chromosome 1, Ph1, after the city in which they worked and the number 1 superscript signalled that they fully expected that there were many more cytogenetic markers of cancer to be discovered. This was an exciting finding and an exciting time that effectively launched the field of cancer cytogenetics but the following years were frustrating as abnormalities were observed in various cancers but the inability to identify specific chromosomes by any method other than their basic shape limited researchers’ abilities to link abnormalities with different morphological subtypes of haematological or solid tumours. All this changed with the advent of banding techniques. Banding allowed the chromosomes to be clearly distinguished from one another and, most importantly, revealed the nature of structural abnormalities: balanced translocations of material between chromosomes, deletion of part of a chromosome, duplication of another segment, or an inversion of a chromosome segment. In 1973, Janet Rowley reported that a reciprocal translocation between chromosomes 9 and 22 resulted in the Philadelphia chromosome (Rowley, 1973). Since then, hundreds of rearrangements have been identified including not only translocations but also deletions and additions of part or all of chromosomes and also inversions of genetic material within chromosomes. From the descriptions of chromosome rearrangements, long before the Human Genome Project shone a light on the location of genes strung along our chromosomes, the molecular biologists were able to discover critical genes at the breakpoints of translocations. Banding continues to allow us to identify new chromosome abnormalities in both haematological and solid tumours and these abnormalities provide the sign posts to the critical genetic changes that underlie the transformation of normal cells into cancer cells. The first cytogenetic abnormality to have its genetic secrets unlocked was the 8;14 translocation which
characterizes Burkitt lymphoma/leukaemia. Researchers identified that the translocation caused two genes, MYC on 8q24 and the immunoglobulin heavy chain gene, IGH on 14q32, to come together (Dalla-Favera et al, 1982, Taub et al, 1982). It is now known that two classes of translocations are found in malignancies. The first type is epitomized by the t(8;14) in Burkitt lymphoma, one gene which is already actively transcribed in the cell type, such as IGH in B lymphocytes, is juxtaposed to a gene such as MYC which is, by virtue of its resulting proximity to IGH, up-regulated. Other translocations such as the t(8;21) in acute myeloid leukaemia (AML), also described by Janet Rowley in 1973 (Rowley et al, 1973), and the t(9;22) in CML, form fusion genes with a “new” gene product which incorporates part of the normal genes broken at the sites of translocation.

It took time for the medical and scientific worlds to realize the importance of the cytogenetic discoveries of the 1960s and 1970s. It was necessary to convince clinicians that the chromosome changes being described in the marrow and peripheral blood of their patients with a variety of malignancies could provide valuable information about the type and prognosis of these disorders. Many important clinical correlations were either identified or confirmed in the International Workshops on Chromosomes in Leukaemia. These constituted gatherings of physicians and scientists from around the world who brought together case studies of chromosome analyses together with clinical and laboratory data relating to each case. The first of these was held in Helsinki, Finland in August 1977 (Rowley & de la Chapelle, 1978). Laboratories participated from Belgium, Finland, Sweden, England, Germany, and the USA and the participants reviewed the data of 223 patients with Ph1-positive CML and 279 patients with acute non-lymphocytic leukaemia. A number of further workshops were held and information regarding the incidence and prognostic significance of rearrangements in CML, AML, acute lymphoblastic leukaemia (ALL), and myelodysplastic syndromes (MDS) provided by these workshops formed the basis for all future studies. Subsequently, national and multinational clinical trial groups have incorporated cytogenetic studies into their prospective trials and provided a wealth of data to show that cytogenetic analysis is of diagnostic and prognostic importance in most haematological malignancies and a number of solid tumours. It has only been by the careful observation of chromosome abnormalities and their correlations with clinical features that true insights have been obtained as to the underlying genetic basis of malignancy.

However, although the basic cytogenetic methods used in laboratories around the world today are very similar to those first described in the 1960s and 1970s, there are areas where improvements have been made. Mitogens were introduced into cultures to induce chronic
lymphoid malignancy cells to divide in the late 1970s (Gahrton et al, 1980) but further refinements and combinations of mitogens are still being discovered.

1.3. Genetic analysis of B-CLL:

1.3.1. Chromosome banding analysis (metaphase cytogenetics):

In the 1960s and 1970s most cytogenetic studies of B-CLL were performed on blood lymphocytes stimulated with phytohemagglutinin and the vast majority of tumors exhibited a normal karyotype. A review by Mitelman & Levan (Mitelman & Levan, 1978) on chromosome aberrations in human neoplasms associated no specific aberration with B-CLL. Specific chromosome aberrations in B-CLL were not identified before the late 1970s when B-cell mitogens were shown to induce leukemic cells from B-CLL tumors to proliferate in culture (Robèrt et al, 1978; Autio et al, 1979; Hurley et al, 1980; Gahrton et al, 1980a; Gahrton et al, 1980b). Among the mitogens that have been used are tetradecanoyl-0-phorbol-13-acetate, Epstein-Barr virus, lipopolysaccharide, pokeweed mitogen, cytochalasin B, anti-human IgM, B-cell growth factor, calcium ionophore (Ca2+), and an anti-CD40 antibody (Döhner et al, 1993; Crawford & Catovsky, 1993; Oscier et al, 1994). Despite the use of these mitogens, conventional chromosome banding analysis has remained difficult in CLL even with improved culture techniques clonal genomic abnormalities can be detected in only 40–50% of CLL cases (Juliusson et al, 1990; Juliusson et al, 1991). In the cases without clonal abnormalities mitotic cells often stem from nonleukemic T-lymphocytes, as shown by the study of (Autio et al, 1986) using the technique of sequential immunophenotyping and karyotype analysis.

Specific chromosomal abnormalities have been shown to affect the overall survival of patients with acute leukemia. Juliusson et al assessed this possibility; blood mononuclear cells from 433 patients with B-cell CLL in five European centers were cultured with B-cell mitogens (Epstein–Barr virus, lipopolysaccharide from Escherichia coli, 12-O-tetradecanoylphorbol-13-acetate, cytochalasin B, pokeweed mitogen, and phytohemagglutinin) according to the protocols of the individual institutions and banded metaphases were studied. They evaluated 391 patients cytogenetically, and 218 had clonal chromosomal changes. The most common abnormalities were trisomy 12 (n = 67) and structural abnormalities of chromosome 13 (n = 51; most involving the site of the retinoblastoma gene) and of chromosome 14 (n = 41). Patients with a normal karyotype had a median overall survival of more than 15 years, in contrast to 7.7 years for patients with clonal changes. Patients with single abnormalities (n = 113) did better than those with complex karyotypes (P<0.001).
Patients with abnormalities involving chromosome 14q had poorer survival than those with aberrations of chromosome 13q (P<0.05). Among patients with single abnormalities, those with trisomy 12 alone had poorer survival than patients with single aberrations of chromosome 13q (P = 0.01); the latter had the same survival as those with a normal karyotype. A high percentage of cells in metaphase with chromosomal abnormalities, indicating highly proliferative leukemic cells, was associated with poor survival (P<0.001). Cox proportional-hazards analysis identified age, sex, the percentage of cells in metaphase with chromosomal abnormalities, and the clinical stage of the disease (Binet classification system) as independent prognostic variables (Juliusson et al, 1990).

1.3.2. Fluorescence in situ hybridization (FISH):

FISH is a process by which specific fragments of DNA are labeled with a fluorochrome (a dye that emits visible light when irradiated with ultraviolet light) and are then allowed to attach to a particular location on a chromosome. The FISH technique is now well established and generally robust and reliable. However, the correct interpretation of the results is not always obvious, and there are some factors that need to be considered.

The chromosomes obtained in studies of malignancy are often of poor morphology, and tend to be involved in complex and subtle rearrangements; in such cases, it is often impossible to define the entire karyotype by simply using G-banding. There is a variety of other newer genetic techniques that are available. These include polymerase chain reaction (PCR) assays that can detect specific gene rearrangements (including translocations) but not gains or losses of chromosome material, and array comparative genomic hybridization (CGH) that can detect minute gains and losses but not balanced rearrangements. FISH has some of the attributes of both assays and forms a very powerful combination with karyotype studies. In particular, FISH can compensate for one of main restrictions of karyotype studies, the need for metaphase divisions: clinically useful information can often be obtained from FISH studies of interphase nuclei (Tkachuk et al, 1990). The impression is sometimes given that FISH studies are simply a matter of buying a kit with the right DNA probe, following the supplier’s instructions, and reading a simple positive or negative result. In practice, getting a reliable result from a FISH study requires experience, time spent in testing to determine the precise local conditions needed for optimum hybridization, and time spent in assessing and scoring positive and negative controls to determine local baseline levels.

The development of FISH in the late 1980s and early 1990s provided a very powerful tool for the detection of chromosome aberrations in tumors, Lichter (Lichter et al, 1990), especially in
CLL. Delineation of specific DNA sequences in the cells by the technique of in situ hybridization is the basis for this molecular cytogenetic approach, by which the genomic abnormalities can be detected on the single cell level in interphase nuclei or metaphase spreads. Therefore, this approach is also referred to as ‘interphase cytogenetics’ (Cremer et al, 1986). Numerical and structural chromosome aberrations that involve changes in the copy number in tumor cells are identified by aberrant signal numbers per cell, while translocation breakpoints are detected by the pattern of spatial distribution of the fluorescence signals. The sensitivity of detection is determined by the probe and the target size. While chromosome banding analysis detects only gross aberrations, i.e., rearranged or deleted subregions several megabasepairs in size, FISH identifies aberrant regions as small as the sequences targeted by the DNA probe(s). To achieve a sufficient hybridization efficiency the cloned DNA fragment should be at least 30–40 kb in size, i.e., DNA fragments cloned in cosmid vectors (Lichter et al, 1995). Routine diagnosis of heterogeneous tumor material using interphase cytogenetics generally require the DNA probes to be of even higher complexity, i.e., DNA fragments of 80 kb to several hundred kilobases cloned in P1, PAC, BAC, and YAC vectors. The diagnostic potential of these molecular cytogenetic techniques is not restricted to the study of metaphase chromosomes but, most importantly, includes the analysis of interphase nuclei, referred to as interphase cytogenetics (Joseph et al, 1984; Cremer et al, 1986).

At the molecular level, many critical genomic regions have only very recently been characterized in CLL and in contrast to PCR-based methods no sequence information of the region under investigation is required. Another major advantage of FISH compared to conventional banding analysis (CBA) or comparative genomic hybridization (CGH) is a higher spatial resolution for the detection of genomic aberrations resulting in a higher sensitivity. Therefore, the different findings regarding the spectrum and frequency of genomic abnormalities seen in various FISH studies compared to banding studies are not surprising (Do’hner et al, 1999). Using the molecular cytogenetic FISH approach with a comprehensive probe set, today genomic aberrations are detected in approximately 80% of CLL cases (Do’hner et al, 2000).

To assess the frequency and clinical relevance of genomic aberrations in CLL, Dohner et al. designed a comprehensive set of DNA probes for evaluating genomic changes in CLL by interphase cytogenetics. They evaluated 325 cases over a period of eight years. Of these cases, 268 (82 percent) exhibited abnormalities. In 175 patients there was one aberration, 67 patients had two aberrations, and 26 patients had more than two aberrations. Among the 178 patients with 13q deletion, the deletion was the sole abnormality in 117 (66 percent). In the remaining
61 patients (34 percent), 13q deletion was accompanied by 11q deletion (28 patients), 12q trisomy (13 patients), 11q deletion and 12q trisomy (1 patient), 17p deletion (8 patients), or other abnormalities (11 patients). An 11q deletion occurred as the sole aberration in 19 of 58 patients (33 percent), 12q trisomy in 22 of 53 patients (42 percent), 17p deletion in 4 of 23 patients (17 percent), and 6q deletion in 6 of 21 patients (29 percent). All deletions were monoallelic except for the 13q14 region: in 43 of the 178 patients with 13q deletions (24 percent), there were biallelic or concomitant monoallelic and biallelic deletions. In all cases, biallelic deletion affected the D13S25 locus, and in 2 of the 43 patients there was also biallelic RB1 deletion. Of the 12 patients with the translocation t(14q32), 7 had t(14;18), and the rest had t(14q32) with an unidentified partner (Dohner et al, 2000).

They reported that the most frequent abnormality was a deletion involving chromosome band 13q14, which occurred in 55 percent of cases. The second-most-frequent change was a deletion in 11q (found in 18 percent of patients). The third most frequent aberration was 12q trisomy, which was long considered the most frequent chromosomal abnormality in chronic lymphocytic leukemia.

These aberrations are among the most important factors in predicting survival. Patients with 17p deletions had by far the worst prognosis, followed by patients with 11q deletions, those with 12q trisomy, and those with normal karyotypes, whereas patients with 13q deletions as the sole abnormality had the longest estimated survival times. These observations parallel the more frequent finding of advanced disease at enrollment in patients with 17p or 11q deletions.

In a smaller series of patients, extensive lymphadenopathy was particularly striking in patients with an 11q deletion (Döhner et al, 1997). In the multivariate analysis, both 17p deletion and 11q deletion provided statistically significant prognostic information, with 17p deletion being the strongest predictor of poor survival. The poor prognosis of patients with 17p deletion or p53 mutation has been reported in only a few studies (El Rouby et al, 1993; Döhner et al, 1995; Geisler et al, 1997). El Rouby et al. found that mutation of p53 was the strongest independent prognostic factor (El Rouby et al, 1993). In a prospective study using chromosome banding, abnormality of chromosome 17 was associated with poor survival, and it was the only cytogenetic finding with independent prognostic value (Geisler et al, 1997). Neilson et al. found that 11q deletions were associated with rapid disease progression and shorter survival times (Neilson et al, 1997). The prognostic effect of 12q trisomy has been controversial (Juliusson et al, 1990; Oscier et al, 1990; Escudier et al, 1993; Juliusson & Merup, 1998). Döhner et al. study revealed that patients with 12q trisomy have shorter survival than those who have a 13q deletion as the sole aberration (Döhner et al, 2000).
1.3.3. Comparative genomic hybridization analysis:

In contrast to FISH, the novel technique of CGH (Kallioniemi et al, 1992) allows comprehensive screening for the presence of chromosomal imbalances in a tumor genome and does not depend on the knowledge of candidate regions that are altered in a specific tumor (Kallioniemi et al, 1992; Du Manoir et al, 1993; Joos et al, 1993). For CGH the whole genomic DNA of the tumor of interest is hybridized as probe to well-defined (normal) metaphase cells under suppression conditions (reverse in situ hybridization, reverse painting). Hybridization of genomic DNA results in a more or less homogeneous staining of all chromosomes. Chromosome regions that are overrepresented (e.g., trisomies or DNA amplifications) or underrepresented (e.g., monosomies or deletions) in the test genome can be detected by a stronger or a weaker staining of the respective target regions in the metaphases. Because signal inhomogeneities can also be caused by experimental parameters, an internal standard is introduced by cohybridization of normal genomic DNA. Signal inhomogeneities of diagnostic relevance are identified by comparison of the differentially visualized signal intensities of the test and control DNAs along the chromosomes. Dohner’s (Dohner et al., 1999) study on CGH of 28 patients with chronic B-cell leukemia shows the potential of this method for diagnosis of genetic alterations in B-CLL (Bentz et al, 1995). Whereas many of the aberrations detected by CGH were already well known to occur in this disease, a gain of material on chromosome arm 8q that was identified in 3 of 28 patients had not been described before. Comparison of the CGH data with banding results revealed a high proportion of cases (13 of 28) in which additional imbalances were detected by CGH. CGH has proven to be a sensitive method for the detection of high-level DNA amplifications, and high incidences of such amplifications have recently been reported in various subtypes of lymphoproliferative disorders (Houldsworth et al, 1996; Joos et al, 1996; Monni et al, 1996; Bentz et al, 1996a; Werner et al, 1997). In addition to identifying amplified sequences, CGH also provides information for localization within the genome. The involvement of candidate proto-oncogenes mapping to the respective bands has been tested in the two B-CLL tumors by Southern blot and interphase FISH analyses. These studies demonstrated amplification of the proto-oncogene MYC (8q24) and the cell cycle gene encoding cyclin D2 (CCND2; 12p13) (Bentz et al, 1995; Werner et al, 1997). Delineation of critical genomic regions by CGH has provided important information for selecting locus-specific DNA probes to be used for rapidly screening large numbers of B-CLL cases by interphase cytogenetics.
1.3.4. Molecular genetic techniques:

Deletion screening detecting loss of heterozygosity (LOH) by quantitative Southern blot or microsatellite analyses and mutation analyses of genes by single strand conformational polymorphism (SSCP) or DNA sequence analysis have long been limited in CLL due to the lack of candidate genes. Recently, the elucidations of the pathogenic role of p53 and ATM (in a subset of CLL patients) have made molecular genetic screening possible.

The identification and sequencing of clonal VDJ rearrangements will be of growing importance to further evaluate the prognostic impact of the VH gene mutational status as biological risk factor in CLL. Chronic lymphocytic leukemia is a monoclonal expansion of antigen-stimulated mature CD5+ B lymphocytes, expressing functional, rearranged immunoglobulin genes. During normal B-cell maturation in primary lymphoid organs, rearrangements of immunoglobulin heavy (IGH) variable (IGHV) – diversity (IGHD) – junction (IGHJ) genes and immunoglobulin kappa (IGK)/lambda (IGL) V-J genes provide the basis for the structure of the B-cell receptor (BCR). Upon antigen engagement, at least two maturation pathways can be followed by BCR-expressing antigen-inexperienced B cells in the context of secondary lymphoid tissues: (i) a T cell-dependent antigenic stimulation, usually occurring in germinal centres (GC) of secondary lymphoid organs, which needs a close cooperation with GC T cells, and is always associated with the induction of somatic mutations in B cell immunoglobulin variable genes; this process, known as somatic hyper-mutation (SHM), is finalized to a better antigen recognition by BCR; (ii) a T cell-independent antigenic stimulation, usually occurring outside GC, which does not need a cooperation with T cells, and may or may not induce somatic mutations in B cell immunoglobulin variable genes (Dal-Bo et al, 2011). Different antigens have been reported to be responsible for a T cell-dependent or T cell-independent antigenic stimulation of mature B cells. In the case of CLL, tumour cells from more than half of the cases have a significant number of point mutations in IGHV genes, whilst IGHV genes from the remaining cases present minimal or no somatic mutations (Dal-Bo et al, 2011). This observation has been demonstrated to harbour clinical relevance, as shown by two seminal studies from the late 1990s, concordantly demonstrating the role of IGHV mutational status as an independent prognosticator in CLL (Damle et al, 1999; Hamblin et al, 1999). In particular, unmutated (UM) CLL, i.e. bearing a BCR with less than 2% somatic mutations in IGHV genes, had a more aggressive clinical course compared to CLL characterized by a BCR with mutated (M) IGHV genes. Despite this clinical correlation, studies of gene expression profiles and extensive immunophenotypes comparing CLL cells expressing UM or M IGHV genes with subpopulations of normal B cells concordantly
demonstrate a CLL profile more related to memory, i.e. antigen-experienced, than naïve, i.e. antigen-inexperienced, B cells, regardless of the *IGHV* gene mutational status (Dal-Bo et al, 2011). Thus, CLL can be considered as a disease derived from antigen-experienced B lymphocytes differing in the immunoglobulin variable gene mutational load (Dal-Bo et al, 2011).

Because of the prognostic relevance of *IGHV* mutational status in patients with CLL, the diagnostic procedure for the detection of mutated VH genes has to become less labor- and cost-intensive. Currently a set-up consisting of a multiplex PCR from genomic DNA with a mixture of family specific unlabeled 3′-JH primers and fluorochrome-labeled 5′-VH primers can be used.

The PCR product is subsequently subjected to a genescan analysis through which the VH gene family involved in the clonal VDJ rearrangement can be identified. The product of the initial multiplex PCR is then directly sequenced with the unlabeled primer corresponding to the VH family involved in the clonal VDJ rearrangement of the respective case (Kroëber, et al, 2002).

1.4. Novel mitogenic stimulators in CLL:

Similar to other hematological malignancies, cytogenetic alterations are prognostically highly relevant in B-CLL. In contrast to acute leukemias or myelodysplastic syndromes, only a few studies have investigated chromosome banding analysis in CLL due to the low in vitro proliferative activity even in the presence of B-cell mitogens. Either the generation of metaphases was completely hampered or clonal aberrations were detectable in 40–70% of cases only – due to the poor quality of the metaphases or as the normal hematopoietic cells showed in vitro proliferation while the CLL cells did not (Juliusson et al, 1990; Oscier et al, 2002). Therefore, other methods were necessary to determine these recurrent abnormalities in CLL. FISH and CGH on interphase nuclei do not require proliferating cells. Interphase FISH analysis has become the standard technique for diagnosis in CLL. Mostly, probes are used for the detection of trisomy 12 and deletions of 6q21, 11q22.3, 13q14, and 17p13. This allows the detection of aberrations in ~80% of CLL samples. However, both CGH and FISH have shortcomings. CGH detects genomic imbalances but misses balanced translocations. Interphase FISH is restricted to the genes/loci for which probes were selected. Thus, new approaches were needed to identify specific cultivation techniques capable of generating sufficient metaphases in CLL. Stimulation of CLL cells with CD40 ligand (a B-cell mitogen) or a combination of CpG-oligodeoxynucleotides and IL-2 was demonstrated to overcome the
problem of established cultivation techniques in this disorder (Buhmann et al, 2002; Mayr et al, 2006).

1.4.1. Application of CD40:
In contrast to CBA, which gives an overview of all microscopically visible aberrations without previous knowledge of the affected regions, interphase FISH is able to detect limited patterns of aberrations only. Thus, efforts continue to improve the karyotyping technique in CLL. Conventional chromosome banding techniques used the B-cell mitogen 12-O-tetradecanoyl-phorbol-13-acetate (TPA). With this approach, the number and quality of metaphases were low in CLL cases. In lymph node proliferation centers in CLL, the environment protects the lymphatic cells from apoptotic and cytotoxic triggers. This environment is missing in in vitro culture. Prolonged in vitro CD40 stimulation of B-CLL cells was shown to result in upregulation of antiapoptotic Bcl-xL, A1/Bfl-1, and Mcl-1 proteins, and to mediate resistance to various classes of drugs, e.g., fludarabine or bortezomib. Thus, addition of CD40 was able to induce an antiapoptotic profile in CLL which was similar to the effects of BCR-ABL1 in chronic myeloid leukemia (Hallaert et al, 2008). Using these antiapoptotic effects of CD40 application, generation of metaphases is markedly improved in CLL.

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 (Buhmann et al, 2002; Mayr et al, 2006). CD40L stimulation induced metaphases in 93% of cases, versus 78% with conventional methods. Even more important, CD40L stimulation resulted in the detection of aberrations in 89% of cases versus only 22% by conventional methods (Buhmann et al, 2002) and confirmed all aberrations detected by FISH (Buhmann et al, 2002). 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.

More recently, the combination of an immunostimulatory CpG oligonucleotide combined with interleukin-2 (IL-2) has been introduced (Dicker et al, 2006; Mayr et al, 2006; Haferlach et al, 2007).

1.4.2. Application of Oligonucleotides and IL-2:
B-CLL cells are arrested in G0/early G1 phase of the cell cycle and are characterized by a marked hyporesponsiveness toward a variety of polyclonal B-cell activators. Regulation of
early cell cycle progression differs between B-CLL cells and normal B-cells. Costimulation with CpG-oligonucleotides and IL-2 can overcome this proliferative defect (Decker et al, 2002). Thus, another approach to induce in vitro stimulation of B-CLL cells is combination of the CpG-oligonucleotide (DSP30) and interleukin-2 (IL-2). Briefly, for metaphase induction, peripheral blood mononuclear cells are cultured in RPMI 1640 medium with 20% fetal calf serum (FCS) in the presence of the immunostimulatory CpG oligonucleotide DSP30 and interleukin 2 (IL-2). After 48 h, colcemid is added for another 24 h before chromosome preparation. The above procedure results, in most cases, in a resolution of 200–300 bands per haploid karyotype (Dicker et al, 2006). This method has now become the standard technique in several laboratories and is very robust in a routine setting.

With combination of CpG-oligonucleotide DSP30 and IL-2 stimulation of metaphases was successful in ~95% of cases (Haferlack & Ulrick, 2011). Chromosomal aberration rates were detected in >80% of cases. This corresponded to an almost twofold increase when compared with earlier chromosomal banding studies and was comparable to the aberration rate detected by FISH analyses (Haferlach et al, 2007; Dicker et al, 2009). Also, cytogenetic alterations detected in ~30% of cases with an apparently normal karyotype as assessed by FISH (Haferlach & Ulrick, 2011). Only the use of CD40-ligand as a B-cell stimulus produced comparable results with respect to metaphase generation and chromosomal aberrations (Buhmann et al, 2002; Mayr et al, 2006). Importantly, three studies demonstrated that CpG/IL-2 does not induce clonal cytogenetic changes. The detected abnormalities hence represent true CLL-associated aberrations. Wu et al. cultured blood of healthy donors and did not observe clonal chromosome aberrations (Wu et al, 2008). Dicker et al. also showed that normal karyotypes were obtained after CpG/IL-2 stimulation of samples of some CLL patients and of healthy individuals and that sequential analysis yielded the same aberrations in two tested cases (Dicker et al, 2006). Accordingly, Put et al. did not detect clonal aberrations in samples from five healthy donors, and most abnormalities in patient samples were found in both CpG/IL-2 and TPA cultures (Put et al, 2009). Moreover, nonclonal aberrations were not more prevalent after CpG/IL-2 cultures compared with TPA cultures.

Until now, only two studies reported comparative data on stimulation with TPA and CpG/IL-2, showing an increase in detection rate of 9% (Struski et al, 2009 ) versus 13% ( Put , et al, 2009).

Interestingly, Put et al. detected most cases of CLL aberrations by CpG/ IL-2 (n =33) not TPA (n = 5). They reported that CpG/IL-2 cultures resulted in a higher detection rate of translocations (P = 0.027) and del (13q) (P = 0.0059) (Put et al., 2009). Most of the
aberrations that were undetected at the karyotypic level but detected by FISH were small deletions involving 13q14 (n = 71/80), which often are cytogenetically cryptic. While del(13q) is easily detected by FISH, translocations which represent an indicator of poor prognosis in CLL (Mayr et al, 2006; Van Den Neste et al, 2007) can escape FISH detection. This illustrates the importance of performing conventional cytogenetics in addition to FISH. Stimulation with CpG/IL-2 resulted in better quality of banding and a higher proliferation capacity; however, these results were not significant (Struski et al, 2009; Put et al, 2009). Indeed, detection rate of clonal chromosomal abnormalities, especially of translocations and del (13q), is superior after CpG/IL-2 stimulation compared with TPA. Chromosome banding analysis might contribute to prognostic predictions in CLL, and thus may lead to an improved understanding of the biological background of this heterogeneous disorder: one example was the description of reciprocal rearrangements in 34% of CLL patients by Mayr et al. Breakpoints clustered in the regions previously described as being deleted in CLL, e.g., 13q14, 11q21–1125, or 14q32. The occurrence of reciprocal translocations was an unfavourable prognostic parameter (Mayr et al, 2006). Previous studies demonstrated that increasing numbers of clonal cytogenetic abnormalities were associated with shorter survival (Juliusson et al, 1990; Mayr et al, 2006). Complex aberrant karyotype is defined by the combined occurrence of at least three clonal aberrations in accordance with the definition in other hematological malignancies and the cited studies in CLL (Juliusson et al, 1990; Schoch et al, 2005). In contrast to FISH data, which were able to identify complex aberrant karyotypes in only ~3% of cases, complex aberrant karyotypes were detected in ~20% of CLL cases with the above culture techniques. Similar to acute myeloid leukemia, there was an association between TP53 deletion and the number of chromosome aberrations (Dicker et al, 2009). Further, complex aberrant karyotypes were associated with an unmutated IGHV status. Therefore, FISH analysis seems to underestimate the complexity and heterogeneity of cytogenetic aberrations in CLL when compared to modern chromosomal banding techniques (Dicker et al, 2006, Haferlach et al, 2007). As an additional aspect, chromosome banding can reveal new subgroups within defined cytogenetic aberrations. In cases with a 13q deletion (according to interphase FISH), chromosomal banding can differentiate between interstitial deletions and reciprocal translocations. Also, additional cytogenetic abnormalities were identified with chromosomal banding in >30% of cases which seemed to have a sole del(13q) with interphase FISH (Haferlach et al, 2007). Furthermore, clonal evolution can easily be followed by chromosome analysis. Finally, the use of automated procedures, such as pipetting or relocation of
metaphases, allows improving the technical quality of chromosomal banding analysis, e.g., due to higher numbers of metaphases which can be screened for further evaluation. These novel options of automation in cytogenetics are able to contribute as well to the success of chromosomal banding in CLL cases. In conclusion, stimulation with the oligonucleotide DSP30 and IL-2 is an easy and efficient stimulus for metaphase generation in CLL. Two recently published studies confirmed that this novel cultivation technique leads to an optimization of chromosome analysis in CLL (Struski et al, 2009; Put et al, 2009). This results in a more comprehensive genetic characterization of CLL and improved insights in the whole genome. As demonstrated by the subgroup of complex aberrant karyotype in CLL, new clinically relevant subgroups can be identified in CLL with chromosome banding. A field of active interest is therefore the prognostic value of chromosome banding analysis in direct comparison to other clinically relevant parameters, e.g., interphase FISH or the IGHV mutational status (Rigolin et al, 2012). Finally, it remains to be clarified which combination of techniques is necessary to optimize prognostic predictions in CLL.

1.5. CLL Cytogenetics:
Chronic lymphocytic leukemia has a highly heterogeneous clinical course, with time to progression varying from months to many years. The classical clinical staging systems for CLL that were introduced 3 decades ago by Rai (Rai et al, 1975) and Binet (Binet et al, 1981) have been extremely useful in guiding disease management and treatment decisions. However, these staging systems have failed to predict the clinical course for individual patients at early-stage disease and to identify patients with poor prognosis. More recently, the analysis of chromosomal aberrations has provided significant prognostic information (Juliusson et al, 1985; Juliusson et al, 1990). The use of metaphase cytogenetics, however, has turned out to be problematic, due to the low mitotic index of most CLL cells even in the presence of B-cell mitogens (Gahrton et al, 1980). But even when metaphases could be generated, the quality was often poor and aberrations escaped detection. Thus, clonal aberrations were detected in only 40% to 50% of cases (Juliusson et al, 1990). Regardless of these problems, metaphase cytogenetics and, later, CGH (Bentz et al, 1995) could define the most common cytogenetic aberrations in CLL. Based on these results, the analysis of aberrant chromosomal regions with specific DNA probes by FISH, which can be applied to interphase cells, resulted in the detection of clonal aberrations in more than 80% of CLL patients (Do’hner et al, 2000).
1.5.1. Incidence of genomic aberrations in CLL:

In conventional chromosome banding studies, trisomy 12 was the first recurrent chromosome aberration described in the late 1970s and early 1980s (Robe`rt et al, 1978; Autio et al, 1979; Hurley et al, 1980; Gahrton et al, 1980). Several investigators confirmed trisomy 12 as frequent aberration in CLL in the following years (Morita et al, 1981; Han et al, 1984; Pittman & Catovsky, 1984; Juliusson et al, 1985; Nowell et al, 1986; Ross & Stockdill, 1987; Han et al, 1988; Bird et al, 1989; Oscier et al, 1990). Deletions and less frequently translocations involving chromosome band 13q14 were another recurrent aberration identified by several different groups in the late 1980s (Ross & Stockdill, 1987; Fitchett et al, 1987; Zech & Mellstedt, 1988; Peterson et al, 1992). Further genomic aberrations detected in varying frequencies by conventional cytogenetics included deletions of chromosome bands 11q (Callen DF, Ford et al, 1983; Pittman & Catovsky, 1984; Juliusson et al, 1985; Ross & Stockdill, 1987; Zech & Mellstedt, 1988), 6q14 (Pittman & Catovsky, 1984; Juliusson et al, 1985; Bird et al, 1989; Oscier et al, 1990) and 17p,34 partial or total trisomy 3q (Pittman & Catovsky, 1984; Ross & Stockdill, 1987) and translocations involving band 14q32 (Autio et al, 1979; Van den Berghe et al, 1979; Gahrton et al, 1980; Morita et al, 1981; Bloomfield et al, 1983; Pittman & Catovsky, 1984; Ueshima et al, 1985; Nowell et al, 1986; Bird et al, 1989; Oscier et al, 1990). This breakpoint was most frequently the result of a t(11;14)(q13;q32), today considered a hallmark of mantle cell lymphoma (MCL). (Raffeld & Jaffe et al, 1991). Therefore, many of these cases most likely represented leukemic MCL variants, rather than bona fide CLL cases. In 1990 and 1991 the largest CLL banding series were reported by the First and Second International Working Party on Chromosomes in CLL (IWCCLL) (Juliusson et al, 1990; Juliusson et al, 1991). Of 662 cases compiled in the Second IWCCLL, 604 were cytogenetically evaluable. Clonal genomic aberrations could be identified in 311 of these CLL cases, with trisomy 12 (19%) being the most frequent abnormality followed by aberrations of chromosome 13 (10%), 14 (8%), 11 (8%), 6 (6%), and 17 (4%) (Juliusson et al, 1991). However, in 351 cases no clonal abnormality was found. Due to the methodological problems of conventional chromosome banding studies, it became necessary to reassess the incidence of genomic aberrations in CLL with the aid of novel molecular cytogenetic techniques. Based on conventional cytogenetic analyses and CGH data, a comprehensive DNA probe set was developed allowing the evaluation of the incidence and prognostic significance of the most important CLL associated genomic aberrations. Dohner et al analyzed 325 CLL cases by FISH for deletions in the chromosome regions 6q21, 11q22-q23, 13q14, 17p13, for trisomies of bands 3q26, 8q24, 12q13 and for translocations involving
the immunoglobulin heavy chain locus on band 14q32. The prevalence of specific genomic aberrations in this large cohort was 82% (268 of 325 cases) and therefore almost twice as high as assumed from chromosome banding studies. The most common aberration was deletion 13q14 (55%), followed by deletion 11q22-q23 (18%), trisomy 12q13 (16%) deletion 17p13 (7%) and deletion 6q21 (7%) (Döhner et al, 2000). In multivariate analysis, the 17p and 11q deletions gave significant prognostic information showing that genomic aberrations are important independent predictors of disease progression and survival in CLL (Döhner et al, 2000).

1.5.2. Cytogenetic profile of CLL:
13q-
Structural aberrations involving the long arm of chromosome 13 were initially reported in smaller chromosome banding studies in the late 1980s (Ross & Stockdill, 1987; Fitchett et al, 1987; Zech & Mellstedt, 1988; Oscier et al, 1990; Peterson et al, 1992). While in the beginning trisomy 12 was often identified at a higher frequency, in more recent banding series deletions involving 13q turned out to be the most common abnormalities. The clinical significance of 13q14 aberrations was first shown by the multicenter studies of the First and Second IWCC, where patients with structural abnormalities of chromosome 13 seemed to have a more favorable prognosis exhibiting survival probabilities similar to those with a normal karyotype (Juliusson et al, 1990; Juliusson et al, 1991). These findings were supported by the results of the unicentric interphase FISH study by (Döhner et al, 2000). This deletion occurs in the hemizygous state in approximately 75-80% of the cases and in the homozygous state in the remaining 20-25% (Migliazza, et al, 2001). Patients with homozygous deletion of chromosome 13q14 normally show higher lymphocyte growth kinetics than patients with hemizygous deletions. Calin and colleagues identified a minimal deleted region of 29 Kb on 13q14, between the exon 2 and 5 of the LEU2 gene containing two micro RNA genes, miR15A and miR16-1 (Calin et al, 2002), the expression of which is significantly deregulated in a fraction of CLLs. The deletion of these micro-RNA genes was recently confirmed by other investigators using a high resolution CGH array (Grubor et al, 2009). The application of high resolution techniques in the study of CLL patients revealed multiple, discrete genomic alterations in 13q region including other genes such as RB and NUDT1 (Stilgenbauer et al, 1995; Calin et al, 2002) the role of which in the transformation process has not been defined yet.
Trisomy 12 was reported as the first recurrent chromosome aberration in CLL and was the most common chromosome aberration in many chromosome banding studies (Gahrton et al, 1980; Morita et al, 1981; Han et al, 1984; Pittman & Catovsky, 1984; Juliusson et al, 1985; Nowell et al, 1986; Ross & Stockdill, 1987; Han et al, 1988; Bird et al, 1989; Oscier et al, 1990). The frequency ranged from 7% to more than 25% according to the different investigations (Juliusson et al, 1990). Some CLL cases carrying a partial trisomy of chromosome 12 were reported (Einhorn et al, 1989). The duplicated segment extended between chromosomal bands 12q13 and 12q21.2, suggesting that this region may harbor genes important in the pathogenesis of CLL, although still unidentified. Among the considered hypothesis, the MDM2 gene, mapping at 12q14.3- q15 may be upregulated in CLL bearing trisomy 12 (Watanabe et al, 1994). MDM2 is a gene whose product acts as a major regulator of the tumor suppressor gene p53 (de Oca Luna et al, 1996) and its overexpression may correspond to a functional deletion of the TP53 product.

Trisomy 12 was assessed by interphase FISH by numerous groups (Perez et al, 1991; Anastasi et al, 1992; Raghoebier et al, 1992; Escudier et al, 1993; Que et al, 1993; Do’chner et al, 1993; Criel et al, 1994; Arif et al, 1995; Matutes et al, 1996; Hjalmar et al, 1998) determining the frequency of this aberration between 10% and 20%. The clinical significance of trisomy 12 was shown in the First and Second IWCCLL, where patients with this aberration had the shortest survival times among patients with single chromosomal abnormalities (Juliusson et al, 1990; Juliusson et al, 1991). Interphase cytogenetics showed an association between trisomy 12 and an increased percentage of atypical lymphocytes or prolymphocytes within the leukemic cell population, as well as an atypical immunophenotype (Que et al, 1993; Criel et al 1994; Matutes et al, 1996, Hjalmar et al, 1998). A sequential FISH analysis of trisomy 12 in CLL showed over a 4-year period similar requirement for treatment and similar overall survival for patients with and without FISH defined trisomy 12 (Auer et al, 1999). A long-term follow-up of a FISH study that initially reported increased need of therapy and reduced survival in patients with trisomy 12 found no statistical significant difference in survival between patients with and without chromosome 12 aberrations after a median observation time of 87 months (Hjalmar et al, 2001).

11q-

The commonly deleted segment includes the ataxia teleangiectasia mutated (ATM) gene which is involved in the signal transduction pathway activated in response to DNA breaks
The remaining ATM allele is mutated in up to 36% of CLLs with 11q- and those patients with homozygous ATM defects may show a more aggressive disease than patients with 11q only (Austen, et al, 2007). The Second IWCCLL reported abnormalities of the long arm of chromosome 11 in 49 of 604 (8%) cytogenetically evaluable cases (Juliusson et al, 1991). Most of these resulted from the translocation t(11;14) (q13;q32) and aberrations involving 11q other than translocations at 11q13 were found in less than 5% of CLL cases. Evidence for the significance of 11q22-q23 aberrations came from smaller chromosome banding studies (Callen & Ford, et al, 1983; Pittman S, Catovsky, 1984; Juliusson et al, 1985; Ross & Stockdill, 1987; Zech & Mellstedt, 1988; Fegan et al, 1995). First evidence for a prognostic role of 11q aberrations in CLL came from two banding studies showing a correlation between 11q deletions and progressive disease with reduced survival times (Fegan et al, 1995; Neilson et al, 1997). Patients with 11q deletion exhibit extensive lymphadenopathy and have a more rapid disease progression as shown by a shorter treatment-free interval and reduced overall survival (Do¨hner et al, 1997).

11q- represented an independent adverse prognostic factor predicting for a shorter progression free survival (PFS) in several clinical trials. Anyway, patients with 11q- treated upfront with fludarabine and cyclophosphamide plus rituximab (FCR) showed a 100% overall response rate (complete remission rate (CR) 88%) with 77% relapse free survival at 3 years (Tsimberidou et al, 2009). Likewise, a good overall response rate (87% CR + Partial Remission (PR) ) was described using the monoclonal antibody anti CD52 alemtuzumab upfront (Hillmen et al, 2007).

17p-
Strucural aberrations of chromosome 17 most commonly resulting from loss of the short arm were only observed in 4% of CLL cases by the multicenter study of the Second IWCCLL (Do¨hner et al, 2000). The 17p- anomaly is frequently associated with additional aberrations and a complex karyotype by conventional banding analysis (Haferlach et al, 2007). Over 70% of CLLs with del 17p13 may carry mutations in the remaining TP53 allele. TP53 gene mutations may occur in patients with inferior prognosis in the absence of 17p deletion (Oscier et al, 2002). 17p-/TP53 mutation confer genetic instability, this gene being involved in the maintenance of effective DNA damage checkpoints, in cooperation with the DNA double-strand break sensitive ATM gene (Pettitt et al, 2001). The clinical outcome in 17p- is severe (Dohnerr et al, 2000; Zenz et al, 2002). Only 2 out of 8 patients (25%) with 17p- achieved CR using the FCR regimen upfront, as compared with a 72% CR rate in the remaining patients.
Alemtuzumab as single agent could induce responses in 7 out of 11 (64%) patients treated upfront (Hillmen et al, 2007). Flavopiridol was able to induce a partial response in 7/18 relapsed/refractory patients in a recent study (Phelps et al, 2008). Non myeloablative allogeneic transplantation may induce prolonged disease free survival in this subset of CLL (Caballero et al, 2005).

6q-
The Second IWCLL found structural aberrations of chromosome 6 in 6% of the evaluable tumors and described chromosome bands 6q15 and 6q23 as most commonly involved (Juliusson et al, 1991). In small lymphocytic lymphoma (SLL), the lymphomatous counterpart of CLL, especially deletions of 6q21-q23 were identified (Offit et al, 1994). Recently, in CLL the proximal location of the minimally deleted region was confirmed by Merup et al, who identified a critical deletion region spanning markers D6S283 to D6S270 on chromosome band 6q21 in 6% of CLL cases (Merup et al, 1998). The 6q- chromosome may be frequently associated with other chromosome changes. A subset of patients, however, may carry 6q- as single or early chromosome abnormality (Cuneo et al, 2004). The incidence of 6q- was found to be higher in patients requiring treatment or in relapsed/refractory patients than in patients with indolent disease. Patients with 6q- show distinct hematological features, consisting of high WBC count at presentation, atypical morphology, CD38+, unmutated IGHV gene in 60% of the cases, shorter treatment free interval and survival as compared with CLL with favorable cytogenetic aberrations (i.e. 13q- normal).

14q32 translocations
The Second IWCLL reported aberrations of chromosome 14 clustering in band 14q32, the locus of the immunoglobulin heavy chain (IgH) gene, in 8% of evaluable cases (Juliusson et al, 1991). Recurrent partner chromosomes include 18q21/BCL2 and 19q13/BCL3; other partners occasionally identified were 2p12/BCL11A; 2p13; 4p16; 4q31; 5q31; 6p21/CCND3; 7q21/CDK6, 8q11; 9q34; 17p11. The classical t(11;14) (q13;q32), indistinguishable from the translocation associated with mantle cell lymphoma, was documented in CLL by several groups: these cases are likely to represent an atypical form of CLL sharing some features with leukemic mantle cell lymphoma (Cuneo et al, 1997). The clinical outcome of CLL with 14q32 translocations may be worse as compared with CLL with favorable karyotype (Nelson et al, 2007; Cavazzini et al, 2008).
**Other translocations (balanced and unbalanced)**

Following the introduction of effective stimulation methods, a previously undescribed 34% incidence of balanced or unbalanced chromosome translocations was documented in CLL (Mayr et al, 2006). Some recurrent breakpoints were observed at 1p32, 1q21, 2p11, 6p11, 13q14, 14q32 and 18q21 and chromosome translocations were frequently found in the context of complex karyotype. A recurrent translocation t(1;6) (p35.3;p25.2) was documented in 8 patients in association with other chromosome lesions (i.e. 11q-, 17p-) and with unmutated IGHV and unfavorable outcome (Michaux et al, 2005). Chromosome translocations represented the strongest predictor of an inferior clinical outcome in a study (Mayr et al, 2006).

**Novel aberrations**

Up to 21% of CLL investigated by automated array-based genomic profiling (matrix CGH) could be shown to harbor genomic imbalances in regions not covered by conventional FISH probe-set (Gunn et al, 2008), including recurrent submicroscopic deletions of chromosome 22q11 in 15% of the cases (Gunn et al, 2008). Trisomy of a small segment at 2p24 with upregulation of the MYCN gene mapping at this region was documented (Schwaene et al, 2004). Novel regions of deletion were documented in >10% of the patients at 9q, 10q, 15q, 22q (Novak et al, 2002). Imbalances reflecting gain of chromosome material and/or amplification occurring in >10% of the cases involved 1p, 2q, 4q, 6q, 8q, 10p, 11p, 16q, 17q, 18q, 19q, 21q, 22q. Four distinct regions on chromosome 12 showed the highest rate of allelic imbalance and, interestingly, a gene (CLLU1) upregulated in CLL with aggressive clinical course was identified at 12q22 (Buhl et al, 2006; Josefsson et al, 2007). High density single nucleotide polymorphism (SNP) arrays were employed to study LOH and allelic loss without loss of gene dosage, also referred to as uniparental disomy (UPD) (Tsimberidou et al, 2009). In 4 out of 70 cases with unmutated IGHV a 3.5 Mb gain at 2p16 including BCL11A and REL was detected. In 3/11 patients with homozygous 13q14 deletion, UPD of a large segment on 13q, distal to the 13q14 deleted region was identified. Using a highly sensitive technique (Lucito et al, 2003), the presence of additional allelic losses or gains in the CD38+ lymphocytes vs. CD38− lymphocytes was observed in 3 cases, suggesting that genetically unstable subclones spend most of the time in the activated CD38+ status.
1.6. Clonal Evolution in CLL:

Clonal evolution of genomic aberrations has been documented in CLL. In a conventional cytogenetic analysis carried out by Oscier et al, karyotypic evolution was seen in 18 out of 112 patients (16%), but there was no correlation between the incidence of clonal evolution and disease progression (Oscier et al, 1991). In two other chromosomes banding studies a significant association between the presence of ongoing karyotype changes and disease progression was seen (Fegan et al, 1995; Finn et al, 1998). In these investigations 6q and 11q deletions were the most commonly acquired secondary chromosome aberrations associated with a shorter progression-free survival. Using a molecular cytogenetic approach Leupolt et al performed a sequential interphase cytogenetic study applying FISH on 55 CLL patients over a median observation time period of 42 months (Leupolt et al, 2001). Clonal evolution was seen in nine out of 55 patients (16%) with 17p deletion (four cases), 6q deletion (three cases), 11q deletion (one case) and evolution from mono- to biallelic 13q deletion (three cases) being the acquired aberrations. Stilgenbauer et al found a significant association between the presence of clonal evolution and progressive disease. Only 20% of the patients with a stable karyotype have died compared to two-thirds of those exhibiting clonal evolution. In consideration of these studies the sensitive detection of genomic aberrations by interphase FISH provides a basis for a more accurate correlation of genomic aberrations with clinical features in CLL. Interphase FISH and molecular genetic techniques represent excellent tools for a better characterization of the critical genomic regions and have allowed the identification of candidate genes involved in pathogenesis or disease progression of CLL (Stilgenbauer et al, 2002).

Recently the clonal stability of malignant cells in CLL has been focused for intensive investigation. Several studies suggest that some patients with no detectable chromosomal abnormalities on initial analysis can acquire new abnormalities that may correlate with more aggressive disease behavior (Finn et al, 1998; Fegan et al, 2003). This raises a question of whether retrospective studies on the prognostic utility of FISH relate to cytogenetic abnormalities present at diagnosis or acquired during the course of the disease. Early studies using conventional cytogenetic analysis suggested such clonal evolution was rare (Nowell et al, 1988; Oscier et al, 1991), but subsequent studies suggested it may be more common than originally believed (Finn et al, 1988; Fegan et al, 2003).

Sequential chromosome banding analyses indicated the acquisition of genomic aberrations over time (clonal evolution) as an infrequent phenomenon in CLL (Han et al, 1986, Nowell et al, 1988, Juliusson et al, 1990, Oscier et al, 1991). However, only limited data are available
from FISH studies of interphase cells, a more sensitive method for the detection of genomic aberrations in CLL (Raghoebier et al, 1992; Escudier et al, 1993; Cuneo et al, 1994; Auer et al, 1999; Hjalmar et al, 2001; Chevallier et al, 2002; Byrd et al, 2006; Shanafelt et al, 2006; Grever et al, 2007). Auer et al. and Hjalmar et al. found the acquisition of trisomy 12 in none of 41 and 2 of 77 CLL cases, respectively (Auer et al, 1999; Hjalmar et al, 2001). Chevalier et al. observed additional genomic aberrations with an extended probe set in 13/31 (42%) CLL cases after a median time of 83 months (Chevallier et al, 2002). There was no association between clonal evolution and CD38 expression or disease progression but the acquisition of del(17p13) was associated with death in 7/11 cases.

More recent studies on CE detected by FISH in CLL and its relation to other prognostic markers such as CD38, ZAP-70 and IGHV gene mutation status have inconsistent results. Shanafelt et al. reported the increasing occurrence of CE after more than 5 years of observation (27 incidence, n= 63) and concluded that ZAP-70 positive patients may be more likely to experience CE (n=159; CE in 13/31 ZAP-70 positive v 3/29 ZAP-70 negative, p=0.008; statistically relevant association with CD38 positivity or unmutated IGHV gene not found) (Shanafelt et al, 2006). Stilgenbauer et al. observed CE only in CLL patients with unmutated IGHV genes (n=64, p=0.002; ZAP-70 and CD38 had not been examined) and presented CE as an independent adverse prognostic factor (Stilgenbauer et al, 2007).

In a study done by Cavazzini et al.; immunophenotypic studies, FISH and conventional karyotyping were used to define the clinicobiologic significance of 14q32 translocations involving the immunoglobulin gene locus (14q32/IGH) in 252 chronic lymphocytic leukaemia (CLL) patients. The panel of probes included: 13q14, centromere 12, 6q21; 11q22/ATM; 17p13/TP53, 14q32/IGH. Patients were classified as group 1 (favourable, i.e. 13q-single or normal), group 2 (intermediate risk, i.e. +12, 6q-, 1–2 anomalies), group 3 (unfavourable, i.e. 17p-, 11q-, complex karyotype), or group 4 (14q32/IGH translocation). They found that one hundred and twenty patients had a clonal chromosome aberration in the karyotype; 31 had a chromosome translocation, of which 15 had either complex karyotype and/or 11q- or 17p-. Half of the patients (n = 110) were allocated to the favourable risk group (group 1); 99 to the intermediate risk group (group 2); 25 to the unfavourable risk group (group 3) and 18 to the 14q32/IGH translocation group (group 4). Two patients with 14q32/IGH rearrangements were included in group 3 because they had 3 or more chromosome changes in the karyotype. The presence of an additional 17p- was noted at relapse after first line treatment in one patient included in group 4. 14q32 rearrangement did not occur as a secondary change in their series. IGH probe splitting occurred in 20–99% of
the cells (median 70%) in 18 patients. The IGH translocation was the sole anomaly in eight patients; of the remaining 10 patients a concurrent 13q14 deletion was detected in nine cases and +12 in one case. Chromosome 14q32 translocation partners were identified in eight patients: in five cases BCL2/IGH rearrangement was detected; whereas fusions of IGH with BCL11A at 2p12 (63% of the cells), with CCND3 at 6p21 (71% of the cells) and CDK6 at 7q21 (30% of the cells) were seen in one case each.
Clonal evolution in CLL

Additional aberrations: 11 (17%) patients after 42 (23–73) months

Cytogenetic risk in CLL

- **TP53 mutations** by DHPLC + I-FISH)
- Conventional karyotyping with odn + IL2
- Evaluate before therapy and at relapse

- Feasibility — diagnostic networks —
- Research in clinical trials
II. Rationale

Numerous studies have shown that the presence, number, and type of chromosomal aberrations represent an independent predictor of prognosis in B-CLL (Juliusson et al, 1990; Döhner et al, 2000; Mayr et al, 2006; Van Den Neste et al, 2007). Therefore, cytogenetic analysis is routinely performed in this disease entity. However, CLL lymphocytes have a poor mitotic index in vitro. Several mitogens, such as 12-O-tetradecanoylphorbol- 13-acetate (TPA), lipopolysaccharide (LPS), pokeweed mitogen (PWM), and Epstein- Barr virus, have been added to the cultures to improve the yield of aberrant metaphases, revealing abnormal karyotypes in 40–50% of cases (Juliusson et al, 1990). The rate of detection can be increased to 80% by interphase FISH (Döhner et al, 2000). The variable incidence of specific lesions in different phases of the disease reflects their correlation with biologic and clinical features. However, FISH technique provides only partial information confined to the chromosomal regions examined. For this reason, there has been great interest in improving culture methods to detect the clonal aberrations in chronic lymphocytic leukemia and other B.cell neoplasia. The introduction of stimulation by CpG oligodeoxynucleotide (ODN-DSP30) combined with IL2 allowed for the identification of more cases with clonal aberrations than in previous analyses using other mitogens and showed that a fraction of cases with apparently normal FISH results may carry chromosome lesions in regions not covered by conventional probe panels (Dicker et al, 2006; Haferlach et al, 2007). Until now, few studies compared the results obtained by this new procedure (CpG/IL-2) with TPA for B-cell chronic lymphoproliferative disorders (Struski et al, 2009; Put et al, 2009). Thus, one of this study’s aims was to analyze the impact of the innovative combination of immunostimulatory ODN + IL2 in SMZL cell culture compared to traditional mitosis inductors: TPA and LPS.

At diagnosis, up to 80% of CLL can be shown to harbour clonal chromosome aberrations (Döhner et al, 2000; Haferlach et al, 2007), some of which may have important clinical implications (Cuneo et al, 1998; Döhner et al, 2000; Haferlach et al, 2007). Despite previous claims that CLL is a genetically stable disease (Nowell et al, 1988), clonal evolution (CE) was more recently reported in 15-42% of CLL using conventional karyotyping (Juliusson et al, 1985) or FISH technique (Cuneo et al, 2002; Chevallier et al, 2002; Shanafelt et al, 2006; Stilgenbauer et al, 2007). The incidence of this phenomenon is variable, depending on the length of follow-up (Shanafelt et al, 2006) and on the number of probes used for interphase FISH analysis (Chevallier et al, 2002). In previous studies, CE was defined by the late
appearance of aberrations of chromosomes 17p, 11q, 6q, and 12 (Hjalmar et al, 2001; Stilgenbauer et al, 2007) and was associated with markers of active disease, i.e. ZAP70 positivity and unmutated IGHV gene (Shanafelt et al, 2006, Stilgenbauer et al, 2007).

Besides the classical CLL-associated aberrations, i.e. 13q- (40% of the cases), +12 and 11q- (10-15% of the cases), 17p- (2-5% of the cases) (Döhner et al, 2000; Haferlach et al, 2007), other recurring chromosome changes were described in CLL (Cuneo et al, 2000) and attention was recently devoted to 14q32 translocation involving the IGH gene. This aberration was found in up 6-19% of CLL patients at diagnosis (Haferlach et al, 2007, Qiu et al, 2008) and was associated with therapy demanding disease and inferior outcome (Cavazzini et al, 2008; Haferlach et al, 2010).

The incidence of this aberration at clonal evolution is presently unknown. To better define the incidence and significance of CE, including the late appearance of 14q32/IGH translocations, we analyzed 105 CLL patients sequentially over many years with a panel of probes including an IGH break-a-part probe.
III. Objectives

3.1. General objective:
- To test the efficiency of novel stimulators in SMZL.
- To analyze CE in CLL.

3.2. Specific objective:
- To compare the efficiency of CpG oligodeoxynucleotide + IL-2 with TPA and LPS for SMZL karyotypic cell culture.
- To detect the cytogenetic profile of SMZL.
- To detect the CE during the course of CLL.
- To correlate between CE and hematologic/clinical parameters.
IV. Material and Methods

4.1. Study area:
The present study was carried out in Haematology section- department of Bio-Medical Sciences and Advanced Therapies, University of Ferrara, Italy.

4.2. Study design:
A descriptive hospital based study was conducted.

4.3. Patients and Samples:

SMZL Patients: Eighteen cases of SMZL diagnosed between 2008 and June 2010 were included (Table 1). All the patients were studied by cytogenetic analysis as part of diagnostic workup. Samples from 13 patients were obtained at disease presentation, whereas 5 patients were sent for cytogenetic analysis 5–12 months after initial presentation.

CLL Patients: One-hundred five CLL patients diagnosed at the department between 1995 and 2004 were included. All enrolled patients were submitted to FISH analysis as part of routine diagnostic workup. FISH was repeated at least on 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.

The patients fulfilled the following criteria: a) Diagnosis of “bona fide” CLL based on morphology and immunophenotyping (score according to Matutes et al ≥3 (Matutes et al, 1994). Those cases with t(11;14)(q13;q32)/BCL1-IGH were excluded from this study. b) Successful FISH analysis at diagnosis and during follow-up. c) Clinical records available for review.

4.4. Methods:

4.4.1. Clinicobiologic investigations:

SMZL: Diagnosis was made according to the WHO (Swerdlow et al, 2008) histopathologic criteria in one patient in whom splenectomy was performed for diagnostic purposes; in the remaining 17 patients, diagnosis was based on the combination of presentation features, morphologic and immunologic features, and bone marrow findings (Mollejo et al, 1995). Minimal requirements were represented by (a) peripheral and bone marrow lymphocytosis (i.e., >5 × 10^9/L B-lymphocytes in the PB and/or >40% lymphocytes in the BM aspiration or
lymphoid infiltrate on biopsy sections), with or without splenomegaly and minimal adenopathy, (b) morphology consistent with SMZL (i.e., small-to-medium-sized lymphocytes, with or without villous lymphocytes and/or plasmacytoid features, and (c) immunophenotype consistent with chronic B-cell proliferation with a Matutes score ≤3 (Matutes et al, 2008).

**CLL:** Diagnosis was based on the presence of persistent lymphocytosis (>5 × 10^9/L), on the examination of the peripheral blood (PB) smear and on the results of immunophenotyping.

The following markers were tested in all cases by flowcytometric analysis as previously described (Cuneo et al, 2004), 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 the 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. ZAP-70 was assessed using the ZAP-70 Alexa Fluor 488 (Caltag/ Valter Occhiena, Torino, Italy) monoclonal antibody (Rigolin et al, 2010). All patients underwent physical examination, chest X-ray film and abdomen ultrasonography as part of the diagnostic workup. 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 NCI criteria (Cheson et al, 1996), included a rise of the WBC count with <6 month lymphocyte doubling time (LTD), the development of anaemia, neutropenia or thrombocytopenia due to BM infiltration or autoimmune phenomena non responsive to steroid drugs and disease progression in the Rai staging system (Rai et al, 1975). The patients were treated according to guidelines in use at the department 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).

### 4.4.2. Cytogenetic analysis:

**Conventional Cytogenetic Analysis of SMZL samples:** Conventional cytogenetic analysis was performed on cells obtained from peripheral blood (PB) in 14 cases, from BM aspirate in 3 cases, and from a spleen sample in 1 case (Table 2). Methods for cytogenetic analysis used in the department were previously published (Castoldi et al, 1987). Spleen samples were
minced with a scalpel to obtain a single cell suspension. After separation by centrifugation over Ficoll-Hypaque, PB, BM, and splenic cells were cultured for 72 h in 10 ml RPMI 1640 (Gibco-Invitrogen) supplemented with 20% fetal calf serum (FCS-Gibco-Invitro-gen), 2mmol/L GlutaMAX (Gibco-Invitro-gen), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco-Invitrogen). Three separate cell cultures were setup in all patients, using the 3 different mitogens: (i) TPA; 50 ng/mL– Sigma-Aldrich), (ii) LPS; 40 µg/mL– Sigma-Aldrich), and (iii) immunostimulatory CpG-oligonucleotide DSP30 plus IL2 (2 µmol/L GpC-ODN TCGTGCCTGTCTCCGCTTCTTTTGCC) (TibMolBiol, Berlin, Germany/IL2 100 U/mL Stem Cell Technologies Inc) according to the method described by Dicker et al. (Dicker et al, 2006). Whenever possible, an additional 72 h unstimulated control culture was setup (6 cases). All cultures were setup with a cell concentration of 2 × 10^6/mL and incubated at 37°C in a 5% CO2 fully humidified atmosphere under standard conditions which have remained unchanged at the department during the study period. Colcemid (Kario Max Colcemid Solution 0.05 µg/mL Gibco, Invitrogen) was added for four hours before harvest. Harvesting and slide preparation were performed using hypotonic treatment (20 minutes incubation in 0.075mol/L potassium chloride); a classical 3:1 methanol/acetic acid solution was used as fixative. Slides were prepared using a predetermined volume (i.e., 20 µl) of fixed cell suspension, and metaphases were G-banded with Wright’s stain (Rooney et al, 2001). Whenever possible, 20 or more metaphases were analyzed from each culture, and karyotypes were described according to the International System for Human Cytogenetics Nomenclature (ISCN 2005) (Karger et al, 2005). Complex karyotype was defined by the presence of 3 or more cytogenetic aberrations in the same clone. To compare the efficiency of the 3 different mitogens, the following cytogenetic features were assessed in the different culture types by visualization at the microscope of the metaphases present on one slide.

(a) Proliferation. Based on the number of mitotic figures, the following score was adopted.
Score 1: failure, defined by the presence of 0-1 mitotic figures.
Score 2: poor proliferation, defined by the presence of 2–10 mitotic figures.
Score 3: moderate proliferation, defined by the presence of 11–19 mitotic figures.
Score 4: good proliferation, defined by the presence of ≥20 mitotic figures.

(b) Quality of Banding. The number of chromosomal bands per haploid set of chromosomes was counted referring to the ideograms of banding patterns present in the guidelines of SCN 2005 (Karger et al, 2005). The following score was adopted.
Score 1: insufficient quality for karyotyping (<100 visible chromosome bands).
Score 2: poor quality (<200 visible chromosome bands).
Score 3: sufficient quality (200–300 visible chromosome bands).
Score 4: good quality (>300 visible chromosome bands).

(c) Stimulation Efficiency. The number of metaphases with clonal abnormalities in each culture system was evaluated, and the karyotypes were divided in three groups:
Score 1: failure, less than 10 analyzable metaphases.
Score 2: normal, absence of clonal abnormalities.
Score 3: abnormal, karyotype with clonal chromosomal abnormalities, that is, the same structural rearrangement or chromosome gain in at least two mitotic figures, or chromosome loss in at least three mitoses.

Cytogenetic and FISH studies of CLL samples: 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, as well as for the detection of trisomy 12 and translocations at 14q32/IGH. Methods were detailed elsewhere, 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 (Cavazzini et al, 2008). All the 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 suspect Richter’s syndrome). These samples were referred to the cytogenetic laboratories and processed for FISH studies. Those patients with 11q- and/or 17p- were considered as “high cytogenetic risk”, 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 (Cavazzini et al, 2008): 11q14/BCL1, 18q21/BCL2, 3q27/BCL6, 18q21/MALT1 and 8q24/MYC (Vysis/Abbott Co, Downers Grove IL, USA). Non-commercial bacterial artificial clones (BACs) were also used: RP11158I21 and 440P5 mapping at 2p12/BCL11A, RP11696P19/298J23/533O20/7K24 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 6 BACs (RP1146O23/137P5/773M18/44H14/811I15) was used for the evaluation of region 5p15.31-33 and BAC RP11145B1 was used as a centromeric control for sub region 5p15.2 (provided by Prof. M. Rocchi, University of Bari, Italy).
4.5. Statistical analysis:

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

5.1. Novel mitogenic stimulators for SMZL samples:

**Hematologic and Clinical Features:** All 18 patients had an unequivocal diagnosis of SMZL with PB and/or BM involvement by a clonal expansion of B-lymphocytes consistent with a marginal zone phenotype as assessed by immunophenotyping. The patients had 2.9–34.8 × 10⁹/L PB lymphocytes at time of sampling for cytogenetic analysis, and no patient had cytologic and/or histologic features suggestive of transformation into high-grade lymphoma. BM involvement with >40% lymphocytes was detected in 11/15 cases, splenomegaly was present in 11/18 cases. A minority of patients had anemia or thrombocytopenia. Demographics and hematologic datas are presented in Table 1.

**Outcome of Cytogenetic Investigations:** The karyotype could be defined in all 18 cases. No analyzable mitoses were obtained from 72 h unstimulated parallel culture in 6 cases. The outcome of cytogenetic investigations using different mitogens is shown in Figures 1–5.

**Proliferation:** Proliferation with at least 1 mitogen was assessable in all 18 cases. The number of cases with failure, low, moderate, and good proliferation is shown in Figure 1. More cases with score 3-4 (moderate-good) were seen in ODN+ IL2- stimulated cultures (17 cases = 94.4%) as compared with TPA and LPS (10 cases each; 55.5%) (P = .015). Seven/18 patients (38.8%) with TPA and 4/18 patients (22.2%) with LPS had score 1 (failure), whereas no failure was observed in ODN+ IL2-stimulated culture.

**Quality of Banding:** The quality of banding expressed as number of bands in mitotic figures from the different cell cultures is shown in Figure 2. A good quality of banding (score 4) was observed in 3/18 cases with ODN+ IL2 and in no case with TPA or LPS. Overall, 14/18 cases with ODN+ IL2 had score 3-4 (sufficient-good) (77.7%) as compared with 8/18 cases (44.4%) with TPA and LPS (P = .067). An example of the quality of chromosome banding is shown in Figure 3.

**Stimulation Efficiency:** The karyotypes are described in Table 2, along with outcome measures (i.e., quality of banding and proliferation score) using different mitogens. The karyotype could be defined from ODN+ IL2-stimulated cultures in all 18 patients, 14 of whom (77.7%) had a cytogenetic aberration. Clonal aberrations could be documented in 9 cases (50%) and in 3 cases (16.6%) by stimulation with LPS and TPA, respectively, whereas in the remaining cases, normal karyotype or failure was observed with these mitogens as shown in Figure 4. Five patients had a complex karyotype (pat. 3, 4, 5, 8, and 16 in Table 2),
with numerical gains and structural abnormalities. One case was in the near-tetraploid range (pat. 4). The most frequent abnormalities (see Figure 5) were represented by aberrations of chromosome 14q in 5 patients, 3 of whom had a 14q interstitial deletion (nos. 3, 11, and 15). In patient 8 a t(14; 19)(q32; q13) translocation was detected. Trisomy 12 and 7q deletion were observed in 4 cases each. Abnormalities involving 11q and 13q were observed in two cases each.

5.2. Clonal evolution in CLL:

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 3. Recurring aberrations at CE were 14q32/IGH translocation in 7 patients; 17p- in 4 patients, 11q- in 2 patients, biallelic 13q- in 4 cases, hemizygous 13q- in 1 case, and 14q32 deletion in 1 patient. A 17p deletion was associated with 14q32/IGH rearrangement in 3/7 patients (Nos 1, 2, 6), one of whom also developed a biallelic 13q14 deletion (no 6). In two cases with 14q32/IGH translocation at CE (Nos. 1, 7), 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 in the LN sample preceded its appearance in PB samples (table 3) by 13-58 months. The 14q32/IGH translocation persisted at subsequent analyses in both cases.

All the patients with 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 a IGH-BCL2 fusion signal consistent with a t(14;18)(q32;q21) translocation. In the remaining 4 cases it was not possible to identify the partner chromosome with our probe panel.

5.3. Correlation between CE, 14q32/IGH translocations, hematologic and clinical parameters in CLL:

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, 7 of whom had a 14q32/IGH translocation, after 1-4 lines of treatment (median 3). To the contrary none of 47 untreated patients developed CE (p<0.0001), as shown in table 4, 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 4); 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 5. 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 with 90 patients without CE (p=0.0033, Fig. 6A). A total of 27 patients, including 11/15 with CE (6 out of 7 with 14q32/IGH translocation) became refractory to fludarabine after a median of 72 months from start of treatment and a significant association was noted between short TTCR and CE (table 5, Fig. 6B). 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 6), which maintained their predictivity at multivariate analysis (table 7).

Survival after the development of CE was 32 months (standard error 8, 5 months) in 15 patients (Fig. 6C) and 32 months (standard error 0, 9 months) in 7 patients with late appearing 14q32 translocation.
Table 1: Clinical features at presentation in 18 cases of SMZL.

<p>| | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Median age, y (range)</td>
<td>74 (56–85)</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>13/5</td>
</tr>
<tr>
<td>Splenomegaly yes/no</td>
<td>11/7</td>
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<tr>
<td>Lymphadenopathy (yes/no)</td>
<td>1/14</td>
</tr>
<tr>
<td>&gt;40% lymphs in the BM aspirate</td>
<td>11/4</td>
</tr>
<tr>
<td>Lymphocytosis ≥5 × 10^9/L yes/no</td>
<td>11/7</td>
</tr>
<tr>
<td>Absolute lymphocyte count (×10^9/L)</td>
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</tr>
<tr>
<td>Villous lymphocytes yes/no</td>
<td>6/9</td>
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<tr>
<td>Hb &lt; 12 g/dL yes/no</td>
<td>5/13 (8.6–15.2)</td>
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<tr>
<td>Platelet count ≤100 × 10^9/L yes/no</td>
<td>4/14 (52–239)</td>
</tr>
<tr>
<td>CD5 expression yes/no</td>
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</tr>
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Table 2: Efficiency of different mitogens and karyotypes in 18 patients with SMZL.

<table>
<thead>
<tr>
<th>Pts</th>
<th>Ages</th>
<th>Sample</th>
<th>TPA</th>
<th>LPS</th>
<th>ODN+IL-2</th>
<th>Karyotype</th>
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<tr>
<td></td>
<td></td>
<td></td>
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<td>prol</td>
<td>efficiency</td>
<td>qua</td>
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<tr>
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<td>78</td>
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<td>3</td>
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</tr>
<tr>
<td>7</td>
<td>55</td>
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<tr>
<td>10</td>
<td>63</td>
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<td>3</td>
<td>A</td>
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</tr>
<tr>
<td>18</td>
<td>82</td>
<td>PB</td>
<td>3</td>
<td>2</td>
<td>N</td>
<td>0</td>
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</table>

Absolute lymphocyte count × 10^6 at the time of cytogenetic investigation, or % BM infiltration, as appropriate. NA: not available. *See materials and methods for details; A: abnormal, N: normal, F: failure. PB: Peripheral Blood, BM: Bone Marrow, Spl: Spleen.
Figure 1: No. of patients with failure, poor, moderate, and good proliferation following stimulation by different mitogens.
Figure 2: Quality of banding: black-coloured column corresponds to insufficient chromosome quality, grey-coloured column corresponds to poor quality, light grey and white correspond to sufficient and good quality, respectively, in every stimulation procedure.
Figure 3: G-banding karyotypes showing some examples of poor quality (score2—(a), patient 14), sufficient quality (score3—(b), patient 4), and good quality (score4—(c), patient 16).
Figure 4: Stimulation efficiency: white colour column represents abnormal cells, and dark and grey colour columns represent failure and normal, respectively, in every stimulation procedure.
Figure 5: Total clonal chromosome abnormalities: distribution of recurrent abnormalities if they were found as single aberration or in association with other abnormalities.
Table 3: outcome of FISH investigations in 15 patients with clonal evolution

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

(*) detected in BM/LN sample before its appearance in the PB

IgH R: rearrangement of the IgH with split signal by FISH (14q32/IGH translocation).
Table 4: Baseline characteristics and clinical data in 105 CLL submitted to sequential FISH investigations

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All the pts (No of cases)</th>
<th>Without CE (90 pts)</th>
<th>With CE (15 pts)</th>
<th>Without 14q32 translocation at CE (98 pts)</th>
<th>With 14q32 translocation at CE (7 pts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age years (range)</td>
<td>63 (31-86)</td>
<td>63 (31-86)</td>
<td>63 (51-78)</td>
<td>63 (31-86)</td>
<td>60 (51-71)</td>
</tr>
<tr>
<td>M/F ratio</td>
<td>66/39</td>
<td>57/33</td>
<td>9/6</td>
<td>62/36</td>
<td>4/3</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;30X10⁹/L</td>
<td>89</td>
<td>78</td>
<td>11</td>
<td>84</td>
<td>5</td>
</tr>
<tr>
<td>&gt;30X10⁹/L</td>
<td>16</td>
<td>12</td>
<td>4</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Rai stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-1</td>
<td>84</td>
<td>74</td>
<td>10</td>
<td>80</td>
<td>4</td>
</tr>
<tr>
<td>2-4</td>
<td>21</td>
<td>16</td>
<td>5</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>CD38+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>neg</td>
<td>72</td>
<td>64</td>
<td>8</td>
<td>69</td>
<td>3</td>
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<tr>
<td>pos</td>
<td>30</td>
<td>23</td>
<td>7</td>
<td>26</td>
<td>4</td>
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<td>ZAP-70</td>
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<td></td>
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<td></td>
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<tr>
<td>neg</td>
<td>46</td>
<td>40</td>
<td>6</td>
<td>42</td>
<td>4</td>
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<tr>
<td>pos</td>
<td>25</td>
<td>17</td>
<td>8</td>
<td>22</td>
<td>3</td>
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<td>FISH aberrations</td>
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</tr>
<tr>
<td>Standard risk</td>
<td>92</td>
<td>81</td>
<td>11</td>
<td>87</td>
<td>5</td>
</tr>
<tr>
<td>High risk</td>
<td>13</td>
<td>9</td>
<td>4</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Treated before CE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>58</td>
<td>43</td>
<td>15(*)</td>
<td>51</td>
<td>7(**)</td>
</tr>
<tr>
<td>No</td>
<td>47</td>
<td>47</td>
<td>0</td>
<td>47</td>
<td>0</td>
</tr>
<tr>
<td>Relapsed/refractory</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>27</td>
<td>16</td>
<td>11(***)</td>
<td>21</td>
<td>6(****)</td>
</tr>
<tr>
<td>No</td>
<td>31</td>
<td>27</td>
<td>4</td>
<td>30</td>
<td>1</td>
</tr>
</tbody>
</table>

(*) p<0.0001, (**) p=0.014, (***) p=0.016, (****) p=0.02
Table 5: Correlation of outcome measures and development of CE

<table>
<thead>
<tr>
<th>Outcome measure</th>
<th>Months</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TTT (months)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All the patients (No. 105)</td>
<td>54 (st err 11.1)</td>
<td></td>
</tr>
<tr>
<td>With CE (15)</td>
<td>35 (st err 3.6)</td>
<td>0.0033</td>
</tr>
<tr>
<td>Without CE (90)</td>
<td>71 (st err 14.0)</td>
<td></td>
</tr>
<tr>
<td>With 14q32 translocation (7)</td>
<td>36 (0.40) (*)</td>
<td>0.067</td>
</tr>
<tr>
<td>Without 14q32 translocation (98)</td>
<td>63 (13.90)</td>
<td></td>
</tr>
<tr>
<td><strong>TTCR (months)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All the patients (58 patients)</td>
<td>72 (st err 8.7)</td>
<td></td>
</tr>
<tr>
<td>With CE (15)</td>
<td>34 (st err 5.2)</td>
<td>0.0046</td>
</tr>
<tr>
<td>Without CE (43)</td>
<td>86 (st err 12.1)</td>
<td></td>
</tr>
<tr>
<td>With 14q32 translocation (7)</td>
<td>27 (st err 0.2)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Without 14q32 translocation (51)</td>
<td>75 (st err 6.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Survival from diagnosis (months)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All the patients (105)</td>
<td>173 (st err 35.1)</td>
<td></td>
</tr>
<tr>
<td>With CE (15)</td>
<td>124 (st err 2.03)</td>
<td>Ns</td>
</tr>
<tr>
<td>Without CE (43)</td>
<td>173 (st err 18.80)</td>
<td></td>
</tr>
<tr>
<td>With 14q32 translocation (7)</td>
<td>125 (st err 2.0)</td>
<td>Ns</td>
</tr>
<tr>
<td>Without 14q32 translocation (51)</td>
<td>173 (st err 33.0)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CE: clonal evolution, TTT: Time to treatment, TTCR = Time to chemorefractoriness
Ns: Not significant
Table 6. Impact of baseline characteristics on TTCR in 58 treated patients

<table>
<thead>
<tr>
<th>Baseline Characteristics</th>
<th>Median TTCR (months)</th>
<th>Std err</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M (n=38)</td>
<td>72</td>
<td>7.8</td>
<td>0.95</td>
</tr>
<tr>
<td>F (n=20)</td>
<td>86</td>
<td>19.7</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-2 (n=47)</td>
<td>86</td>
<td>8.7</td>
<td>0.0004</td>
</tr>
<tr>
<td>3-4 (n=11)</td>
<td>27</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>CD38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>neg (n=34)</td>
<td>75</td>
<td>8.1</td>
<td>0.0857</td>
</tr>
<tr>
<td>pos (n=23)</td>
<td>48</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>ZAP70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>neg (n=28)</td>
<td>72</td>
<td>15.1</td>
<td>0.34</td>
</tr>
<tr>
<td>pos (n=21)</td>
<td>58</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>Cytogenetics at diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard N=46</td>
<td>86</td>
<td>10.7</td>
<td>0.0032</td>
</tr>
<tr>
<td>High n=12</td>
<td>36</td>
<td>4.8</td>
<td></td>
</tr>
</tbody>
</table>

Table 7: Cox analysis of factors prediction for TTCR

<table>
<thead>
<tr>
<th>Variable</th>
<th>Haz. Ratio</th>
<th>[95% Conf. Interval]</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytogenic Risk Group</td>
<td>2.53</td>
<td>1.08 – 3.92</td>
<td>0.033</td>
</tr>
<tr>
<td>Stage at 1st treatment</td>
<td>2.72</td>
<td>1.06 – 7.01</td>
<td>0.038</td>
</tr>
</tbody>
</table>
Figure 6:
A) Time to first Treatment in patient with (“yes”, n=15) and without CE (“no”, n=90); B) Time to Chemorefractoriness in patient with (“yes”, n=15) and without CE (“no”, n=43); C) survival from the development of CE in 15 patients with CE
VI. Discussion

a) Novel mitogenic stimulators in SMZL:
Cytogenetic analysis has an established role in the diagnostic workup (Hernandez et al, 1995) and risk assessment of chronic lymphoproliferative disorders (Salido et al, 2010; Gruszka-Westwood et al, 2001; Zenz et al, 2007). Because the mitotic index in these indolent disorders is low, stimulation with LPS and TPA was widely employed (Oscier et al, 1993; Juliusson et al, 1990). Evidence was recently provided that conventional karyotyping may allow for the detection of aberrations, especially translocations, not detectable by molecular cytogenetic methods (Haferlach et al, 2010) and that ODN+IL2 stimulation may disclose more cytogenetically abnormal cases than was previously thought in CLL (Dicker et al, 2006, Buhmann et al, 2002). In particular, in a CLL Research Consortium study, more clonal abnormalities were observed after culture of CLL cells with ODN than with the traditional pokeweed mitogen (PWM) plus TPA (Heerema et al, 2010). All clonal abnormalities in PWM+ TPA cultures were observed in ODN cultures, whereas ODN identified some clones not found by PWM+ TPA. These results were reproducible in five different laboratories, and all abnormalities were concordant with FISH.
In this study, we were able to show that improved mitotic stimulation can be obtained in SMZL, a low grade lymphoproliferative disorder, by using ODN+ IL2 in analogy with CLL. Indeed, a significantly greater number of mitotic figures could be observed in ODN+ IL2-stimulated cultures, which offered chromosomes of better quality with more clonal aberrations with respect to TPA/LPS-stimulated cultures. The karyotype could be defined in 100% of ODN+ IL2-stimulated cultures, 77.7% of which showed a clonal abnormality, as compared with 50% karyotypically abnormal cases obtained by a combination of results from LPS/TPA-stimulated cultures in the same patients. The percentage of cytogenetically abnormal cases in LPS/TPA stimulated cultures was in line with previous reports (Sol´e et al, 1997; Troussard et al, 1998; Salido et al, 2010). It is worth noting that at the time of sampling for cytogenetic analysis the majority of our patients were at an initial stage of the disease with moderate lymphocytosis and splenomegaly, no lymph node involvement, and absence of anemia or thrombocytopenia in the majority of them. Interestingly, the capability to detect abnormal clones was independent of the degree of lymphocytosis and BM involvement, since all cases with <5 × 10^9/L lymphocytes in the PB and with <40% BM lymphocytes could be shown to have an abnormal karyotype.
Thus, our data demonstrate that ODN+ IL2 is the method of choice to enhance cell divisions of good quality for karyotyping in SMZL, in keeping with a recent analysis documenting a 97% rate of aberrant karyotypes in 29 SMZL (Meloni-Ehrig et al, 2009). The cytogenetic profile in our patients confirms that 7q deletion, 3q abnormalities, +12, and 14q32 translocations are frequently encountered in this disease. Interestingly, a 14q22-24 deletion occurred as single abnormality in 2 cases and as additional aberration in 1, resulting in a 16.6% incidence for this aberration. Only 3% of the SMZL studies by Salido and coworkers (Salido et al, 2010) were found to carry 14q deletions, which showed a 1.5% overall incidence in a study of 3054 mature B-cell neoplasms (Reindl et al, 2010). The majority of cases with 14q deletion in the latter analysis were represented by atypical CLL cases having a therapy-demanding disease. Our 3 SMZLs with 14q deletion also had a therapy-demanding disease, requiring treatment after 1, 8, and 13 months from cytogenetic analysis.

One case in this study with a t(14;19)(q32;q13) adds to a previous report of 4/330 cases carrying this translocation (Salido et al, 2010). The 14;19 translocation involving IgH and BCL3 is a rare aberration usually associated with a heterogeneous group of B-cell malignancies (Martín-Subero et al, 2007), including an atypical form of CLL with aggressive clinical features (Huh et al, 2007). The identification of this subtle rearrangement may be difficult when banding quality is suboptimal, and it is possible that improved resolution obtained in ODN+ IL2-stimulated cultures made the detection of this subtle rearrangement easier. In line with previous studies (Michaux et al, 1997), our case had a rapidly progressive disease requiring treatment 3 months after diagnosis due to rapid lymphocyte doubling time.

b) CE in CLL:

CLL may undergo CE, with late appearance of 11q deletion (Cuneo et al, 2002) 17p deletion and 6q deletion in 16-17% of the cases (Shanafelt et al, 2006; Stilgenbauer et al, 2007; Deambrogi et al 2009). Although recent evidence was provided by several groups that the 14q32/IGH translocation may identify a cytogenetic group of CLL patients characterized by a therapy-demanding disease (Cavazzini et al, 2008; Haferlach et al, 2010; Haferlach et al, 2010; Matutes et al, 2010), scanty 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 7 of these 15 cases showed a 14q32/IGH translocation. This is the first report identifying 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
lymphoma characterized by relatively aggressive behaviour and therapy-demanding disease (Cuneo et al, 2001). The presence of 14q32/IGH translocation was previously documented to involve a minority of interphase cells in some CLL patients (Cavazzini et al, 2008) and one case was described harboring IGH translocations with different partners (Nowakowski et al, 2005). These findings argue in favor of a possible secondary nature of this translocation in some patients.

Interphase FISH studies on the 7 cases of our series with late appearance of 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 the t(14;18)(q32;q21)/IGH-BCL2 is the most frequently occurring translocation involving the IGH locus in CLL (Cavazzini et al, 2008, Nowakowski et al, 2005; Nguyen-Khac ete al, 2010).

Interestingly, in our series the appearance of a 14q32 translocation in BM and LN samples preceded its appearance in the PB in 2 cases. Recent studies indicated that CLL cells in lymph nodes and bone marrow may have an important role in tumor proliferation (Messmer et al, 2005; Van Gent et al, 2008; Herishanu et al, 2001) and, interestingly, a relatively high incidence of 14q32/IGH translocation (17-19%) was noted by Flanagan and coworkers who performed FISH studies on paraffin-embedded lymphoid tissue from CLL/SLL (Flanagan et al, 2008).

In 2 previous analyses 14q32/IGH translocation was not observed at CE (Shanafelt et al, 2006; Stilgenbauer et al, 2007), nor was this aberration detected at Richter's transformation (Deambrogi et al, 2009). 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 7 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 it was associated with a minor clone carrying 17p deletion in 3 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 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 coworkers (Shanafelt et al, 2006). Deletion affecting
chromosome 14q32 locus were previously described in CLL at a frequency of approximately 2-8% (Chesi et al, 2000; Pospisilova et al, 2007; Wlodarska et al, 2007; Reindl et al, 2010). 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 (Stilgenbauer et al, 2007). In our series the development of CE occurred only in previously treated patients. Among baseline hematologic parameters, ZAP70-positivity and the presence of 11q deletion and/or 17p 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 holds 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 TTCT 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 (Figure 6).
VII. Conclusions

In conclusion, we have shown that stimulation of mitosis with ODN+ IL2 offers more mitotic figures of better quality and results in an increased rate of clonal aberrations in SMZL, in analogy with CLL. The profile of chromosome lesions obtained with this mitogen was in line with previous data, confirming that cytogenetic findings may be useful for the diagnosis of this lymphoid neoplasia. The detection of subtle rearrangements, such as 14q deletion and 14;19 translocations, might have been facilitated by improved banding resolution. Although more sensitive molecular genetic techniques using array CGH technology (Robledo et al, 2009) may be of value for the detection of subtle unbalanced genetic lesions, this cytogenetic method may be ideal for prospective studied aiming at the definition of the prognostic impact of chromosome aberrations in SMZL and other lymphoproliferative disorders.

Furthermore, our data show that 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 in CLL occurs in pre-treated patients with short TTT and TTCR and survival after the development of CE with and without 14q32 translocation is relatively short.
References


Cremer T, Landegent J, Brückner A, et al. (1986) Detection of chromosome aberrations in the human interphase nucleus by visualization of specific target DNAs with radioactive and non-


Haferlach C, Dicker F, Schnittger S, et al. (2010) Translocation involving the IGH@ locus occur in 3.7% of Chronic Lymphocytic Leukemia and are associated with Unmutated IGHV status and shorter time to treatment: a study on 2,135 cases. 52nd ASH Annual Meeting Abstracts, Blood 116: 1480.


Martín-Subero JI, Ibbotson R, Klapper W, et al. (2007) A comprehensive genetic and histopathologic analysis identifies two subgroups of B-cell malignancies carrying a t(14;19)(q32;q13) or variant BCL3-translocation. Leukemia. 21(7): 1532–1544.


Appendix

1. Conventional cytogenetic using traditional mitogenic inductors (TPA+ LPS)

Procedure of Chromosomes preparation from peripheral blood in chronic lymphoproliferative syndrome:

1. Medium: RPMI 1640 +20% FCS + antibiotic (penicillin / streptomycin 100U/ml)
2. Cells: Precede to the separation of 20 cc of heparinized peripheral blood (from Na Heparin 15a70U / ml) 3 ml of Ficoll density gradient separation '1,077 centrifugation at 2000 rpm 20'. At the end of the ring is taken MNC (mononuclear Cells) and washed with saline by centrifugation at 1200/10 '. Ccounts in chamber- Burcker, 20x10^6 cells in 10 ml cultures of medium for 72 h with different mitogen.
3. Mitogens:
4. 1 cultured with LPS (40 µ g / ml) (400 µ l)
5. 1 cultured with TPA (50ng/ml TPA) (50 µ l)
6. 1 cultured with LPS + TPA + PWM (10 µ g / ml) (400 µ l1 +100 +50 µ l)
a. If the cells are few use culture with TPA / LPS / PWM together.
7. Times of culture: culture for 72 h
8. Colchicine block: 24h before the end of the incubation time add colchicine to arrest the cells in the metaphase stage

[use= 0.05 µ g / ml Kariomax Gibco10 µ g / ml] was added to each flask 50µ.
9. Centrifugation: At the end of incubation transfer the contents of the flask into the centrifuge tube and centrifuge at 1200 rpm * x 10 ’*
10. Suction: ** SN aspirate and resuspend the bottom **
11. Hypotonic solution: add 5 ml of 37 ° C -preheated hypotonic solution of 0.075 M KCI to the sediment slowly and incubate at 37 ° C for 20 ’
12. Repeat step 9 from * to * spin
13. Repeat step 10 ** from ** to aspirate and resuspend
14. Fixation: # in a vortex of continuous shaking add 5 ml of fixative methanol: glacial acetic acid 3: 1 #
15. Repeat step 9 from * to *
16. Repeat step 10 from ** to **
17. Repeat step 14 from # #
18. Repeat step 9 from * to *
19. Repeat step 10 from ** to **

20. The content is ready to be diluted appropriately for the preparation of glass
2. Novel stimulators (Oligodeoxynucleotide + Interleukin-2)

2.1 Materials

1. A fresh sample of peripheral blood or bone marrow is required. In the case of a peripheral blood leukocytosis, use 5–10 ml of heparinized peripheral blood (peripheral blood is preferred to bone marrow for karyotyping in CLL/ malignant lymphoma, as contaminating dividing cells of normal hematopoiesis are less frequent). Alternatively, heparinised bone marrow may be used.

2. Medium: RPMI 1640 medium (Gibco, Gaithersburg, MD) with l-glutamine – 400 mL.

3. FCS (20%): 100 ml of FCS is added to 400 ml of RPMI; together with antibiotics/antimycotics. Store at 4°C temperature.

4. CpG-oligonucleotide DSP30: Order 1 mmol of DSP30: sequence: 5¢-TsCsgsTsCsgsCsTsgsTsCsTsCsTsgsCsTsCsTsCsTsgsCsC, dilute 500 nmol in 5 mL distilled water to a concentration of 100 pmol/mL. Use 100 mL (=10 nmol) of DSP30 solution per 5 mL prepared medium.

5. Interleukin 2 (IL-2): Biological activity of IL-2: \(\frac{31 \times 107}{10 mg} = (105 U/10 mg)\). Add 10 mg IL-2 to 1,000 mL RPMI media 1640 (=100,000 U/1,000 mL = stock solution). Aliquot stock solution: 50 mL (=5,000 U) stock solution + 950 mL RPMI media 1640 into 20 Eppendorf tubes. Concentration of IL-2 for use: \(\approx 5,000 U/1,000 mL\). Add 100 mL (=500 U) IL-2 per 5 mL prepared medium (see Note 3).

6. TPA (Phorbol-12-myristate-13 acetate).

7. Colcemid (0.15 g/mL) (Sigma, Munich, Germany).

8. Potassium chloride (KCl): 5.592 g KCl added to 1,000 mL distilled water. Store at 4°C temperature.

9. Carnoy's fixative: acetic acid (100%) – 50 mL; methanol – 150 mL. Storage temperature: −20°C, stability: 24 h (maximum 48 h).

10. Phosphate buffer: Di-Na-hydrogen phosphate – 11.4 g; K-dihydrogen phosphate – 4.9 g; add 1,000 mL distilled water. Store at 4°C temperature.

11. 2× SSC for chromosome banding: NaCl – 7.65 g; tri-sodium citrate dehydrate – 44.10 g; add 2,000 mL distilled water. Store at room temperature.

12. Giemsa Staining solution: 60 mL of phosphate buffer with 600 mL distilled water; remove 37.5 mL and add 37.5 mL of Giemsa stain.
2.2. Methods

2.2.1. Preparation of Samples for Cultivation of Metaphases
1. Measure the white blood cell (WBC) count of patient sample (Sysmex® nucleocounter); required cell count: $1 \times 10^7$ leukocytes/5 mL medium.
2. Add sample volume to culture tube with 5 mL of prepared medium.
3. Prepare two culture tubes: Add 100 mL of IL-2 solution and 100 mL of DSP30 solution to one culture tube. Add 50 mL TPA to the other culture tube (see Table 1).

Table 1

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<th>Supplements</th>
<th>Volume (mL)</th>
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<tr>
<td>Prepared medium</td>
<td>5,000</td>
</tr>
<tr>
<td>TPA</td>
<td>50</td>
</tr>
<tr>
<td>DSP30</td>
<td>100</td>
</tr>
<tr>
<td>IL-2</td>
<td>100</td>
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2.2.2. Supplements for cultivation

2.2.3. Cultivation of Metaphases for Cases with CLL
1. Incubate culture tubes for 48 h (37°C).
2. Add 150 mL colcemid (1.5 mg) to DSP30 + IL2 culture and incubate for additional 24 h to generate a higher number of metaphases.
3. Incubate TPA culture for 72 h and then add colcemid for 2 h.

2.2.4. Harvesting of Cultures

1. Harvesting of cultures using Tecan® pipetting robot.
2. In case the Tecan robot is not available or cannot be used due to technical problems (e.g., clots in the culture due to autoantibodies), the following manual procedure is used.
3. After centrifugation, remove the supernatant.
4. Suspend the sediment in 10 mL KCl (5.592 g/L).
5. After centrifugation and removal of supernatant, resuspend sediment in methanol/acetic acid (−20°C), wait for 30 min (room temperature) before proceeding.
6. Repeat step 5 twice without waiting period.
7. After centrifugation and removal of supernatant, resuspend sediment.
8. Drop suspension on glass slides and allow slides to dry.

2.2.5. Banding and Staining
1. Banding is carried out by incubation of dried slides at 60°C in 2× SSC solution.
2. Staining is performed using Giemsa stain diluted in distilled water and phosphate buffer for 3 min.
2.2.6. Evaluation of Metaphases

1. Capture metaphases using the metaphase finder Metafer (Metasystems®).
2. A minimum of 20 metaphases should be analyzed in CLL cases to establish a normal karyotype.
3. An abnormal karyotype should be correlated with the interphase FISH results for reporting.
3. FISH

3.1. Materials and Equipment

Note that many of the reagents used are potentially harmful by contact, inhalation, and/or ingestion. It is important that principles of good laboratory practice are followed, and that appropriate health and safety precautions are taken. Material Safety Data Sheets can be obtained from the manufacturers, either supplied with the reagent or available online. Most of these reagents can be obtained from any good supplier such as Sigma or Gibco. Other suppliers are indicated where necessary.

3.1.1. Solutions

1. 20× SSC (SSC = sodium chloride and sodium citrate solution): dissolve 175.3 g of sodium chloride and 88.2 g of sodium citrate in 900 mL of distilled water (dH2O). Adjust the pH to 7.0 using sodium hydroxide or hydrochloric acid, then make up to 1 L with more dH2O. This can be stored at room temperature for up to 6 months. For other concentrations, either dilute this stock or else modify these amounts accordingly; for example, for 4× SSC, use 34.1 g sodium chloride and 17.6 g sodium citrate per liter of solution.

2. 2× SSC.

3. 0.4× SSC.

4. Hybridization buffer (supplied by the probe manufacturer).

5. Pepsin (SIGMA).

6. NP40 (Vysis) or Igepal (SIGMA) or Tween 20.

7. 2× SSC/0.1% NP40/Igepal: Add 100 mL of 20× SSC to a 1,000-mL cylinder. Add 1 mL of NP40/Igepal with a clean disposable plastic pipette. Add dH2O to the 1,000-mL mark. Mix well. Adjust pH if necessary to 6.8–7.5. Label and date the bottle. Store the stock solution in a fridge; the in-use solution can be kept on the bench at room temperature.

8. 0.4× SSC/0.3% NP40/Igepal: Add 20 mL of 20× SSC to a 1,000-mL cylinder. Add 3 mL of NP40/Igepal with a clean disposable plastic pipette. Add dH2O to 1,000 mL mark. Transfer to a 1 L clean bottle. Mix well. If necessary, adjust pH to 6.8–7.5. Label and date the bottle. Store the stock solution in a fridge; the in-use solution can be kept on the bench at room temperature.

9. Counterstain: The choice of counterstain is affected by the choice of fluorochrome being used. DAPI (4’, 6-Diamidino-2-phenylindole dihydrochloride) is better for red or green fluorochromes, such as SpectrumOrange and SpectrumGreen. An alternative to DAPI is propidium iodide, and this is better for yellow fluorochromes, such as fluorescein. The counterstain solutions and mountant can be bought separately and diluted as needed to final
concentrations of 0.3 mg/mL of PI or 0.1 mg/mL of DAPI. DAPI is used as a counterstain to highlight the nuclei and to contrast the fluorescence signals. It also produces faint G-banding of metaphase chromosomes, so that most of them can be identified. Suppliers include Vector Laboratories and Abbott (Vysis). This stain contains agents to reduce quenching (fading) of the fluorochromes, caused by oxidants and/or free radicals. DAPI stock is kept at 4°C in the fridge, ready to use.

10. Ethanol, used to dehydrate slides; a series of dilutions with water is prepared, 70, 85, and 100%, in containers such as Coplin jars.

3.1.2. Equipment

1. Pipettes: very small volumes of liquids need to be accurately dispensed; a Gilson pipette is suitable, to which sterile disposable pipette tips can be attached.
2. Microfuge tubes (e.g., Eppendorf) 1.5 mL.
3. Water bath, capable of maintaining a temperature of 75°C.
4. Vortex mixer.
5. Microfuge/microcentrifuge: This is used to spin down small amounts of DNA probe, hybridization buffer, and small quantities of fixed sample.
6. Incubator or oven set at 37°C.
7. PH Meter: this may need a special probe for testing the SSC wash solutions. These solutions have to be made fresh for every experiment and it is essential that the pH is between 6.8 and 7.5.
8. Rubber solution, sometimes called rubber cement, for sealing the edges of coverslips. If it cannot be obtained from your usual supplier, it can often be obtained from shops that sell or repair bicycles. It is convenient to have this in tubes with a fine opening, so that a fine line can be squeezed out. Alternatively, it can be drawn into a 5 mL syringe when needed and squeezed out through a blunt needle.
9. Coverslips: 22 mm × 22 mm (any thickness).
10. Coverslips: 22 mm × 50 mm, thickness grade 0.
11. Epifluorescence microscope: This microscope has an ultraviolet light source and a filter wheel; one that is electronically controlled is preferable.
12. Fluorescence filters (see Note 1).
13. A phase-contrast microscope is also very useful, for examining unstained slides to assess the quality of the spreading.
14. A programmable hotplate, such as a HybriteTM or a ThermobriteTM. This is not an essential item of equipment, but is recommended as it helps to semi-automate the
hybridization part of the FISH process. It can accommodate up to 12 slides. If this is not available, then the following two items will be needed:

15. Humidified container: This can simply be a plastic box containing a slide rack and damp towels or tissues, and which has a close-fitting lid.

16. Hotplate that can maintain a temperature of 75°C.

3.2. The Method

Always read the probe manufacturer’s protocols carefully. The method described here is robust and generally reliable, but some probes may need alterations to the procedure.

3.2.1. Slide Preparation

For FISH studies of metaphases, standard culturing, harvesting, and fixation procedures are used. If a rapid result is needed and can be obtained from interphase cells, it is not necessary to wait to collect cells in division: simply suspend the cells in hypotonic KCl for 10 min, fix using 3:1 absolute methanol:glacial acetic acid, and then change the fixative at least three times. Metaphase preparations can be made from both freshly fixed and archived fixed samples. A fixed cytogenetic material stored over 15 years can be used. Although there will be a degree of DNA degradation during this time, its quality is usually adequate for retrospective FISH studies. Resuspend the fixed cells in fresh fixative for a few hours, centrifuge, remove the supernatant, and then resuspend in fresh fixative for a few more minutes before spreading. Slides for FISH studies are spread in the same way as those for karyotype studies. For high-quality FISH preparations, it is essential not to spread cells too densely on the slides, as this can increase background signal levels. Adequate fixative changes are also necessary to reduce cell debris, which can adversely interfere with a FISH analysis. The presence of large numbers of dead cells can also lead to difficulties; this is most common in samples from high-count diagnostic studies, in samples that have been delayed reaching the laboratory, and also sometimes in samples collected in EDTA instead of heparin.

As the quality of the slides affects the formation of the metaphase spreads, it is necessary to use thoroughly clean washed slides. Slides can be bought precleaned or else washed in ethanol and then kept in a freezer at −20°C before being used.

1. Drop the fixed cell suspension (usually about 10–20 mL) on to a clean slide.

2. Add 3–4 drops of fresh fixative on to the spread region and then leave to air-dry. It is helpful later if the area to be hybridized (usually about 20 mm diameter) is defined by scoring underneath the slide with a diamond marker.

3. Check the slide using a phase-contrast microscope. If a study of metaphases is planned, ensure that the chromosomes are well spread, with good contrast, and that there is little
cytoplasm. The chromosomes should appear dark gray, not black and shiny, or pale. Some cytoplasm can be cleared, if necessary, by using a pretreatment with RNase prior to hybridization, as described below.

4. Slides should be left to age overnight before being treated for FISH. However, satisfactory results can be obtained from slides that are aged for just an hour in an incubator at 37°C. If slides are being prepared for FISH studies more than a few days later, they should be stored at −20°C.

3.2.2. Slide Pretreatment

Freshly prepared slides do not usually require any enzyme pretreatment. However, metaphase spreads can be treated to facilitate disruption of the cell membrane and to allow efficient hybridization of the probe mixture to the target DNA; this may help when using archived fixed material.

3.2.3. Probe Dilution

Perform this procedure in reduced light. Some manufacturers supply their probe in diluted form. If this is not the case, then use the following procedure to make 10 mL of probe solution for immediate use:

1. Remove the stock probe and hybridization buffer from the freezer and leave it in the dark for about 1 h at room temperature.
2. Flick with a finger to mix, and then briefly vortex the probe and the hybridization buffer to ensure thorough mixing.
4. Remove 7 mL hybridization buffer and add to a labelled microfuge tube.
5. Add 2 mL sterile water.
6. Remove 1 mL DNA probe and add to the microfuge tube.
7. Flick the microfuge tube with fingers and briefly vortex.
8. Pulse spin in the microfuge for 10 s at 140 g. The procedure can be adapted to dilute all of the probe as soon as it is received in the laboratory, instead of diluting small quantities as needed.

3.2.4. Co-denaturation and Hybridization

The target DNA on the slides is denatured, i.e., the DNA is rendered single-stranded to allow hybridization with the fluorescently labeled probe. Ordinarily, DNA needs prolonged exposure to temperatures of greater than 90°C to denature. However, using formamide, an organic solvent that is contained in the hybridization buffer, allows denaturation to take place at lower temperatures. It is usual to leave hybridization to take place overnight. If an urgent
result is needed, then the hybridization time can sometimes be reduced to as little as 2 h. Hybridization is most efficiently done automatically using a programmable hotplate, such as a Thermobrite™ or Hybrite™, and use of equipment such as this is recommended. Instrument specific usage instructions will be provided by the supplier and only a brief description is given below. If such equipment is not available, then hybridization can be done perfectly satisfactorily in a humidified chamber, as described below.

3.2.4.1. Adding the Probe: 1. Warm the slide(s) at 37°C for an hour in an incubator.
2. Take the slides through the alcohol series, i.e., 70, 85, and 100% ethanol for 2 min each.
3. 10 mL of probe is usually enough to cover the cells spread on the slide. Pipette the probe slowly onto the slide.
4. Immediately place a 22 mm × 22 mm coverslip gently over the area. Be careful to avoid the formation of air bubbles, as the probe will not hybridize uniformly around a bubble.
5. Seal around the edge of the coverslip with rubber solution. (The rubber solution can be squeezed directly from the tube onto the slide, or aspirated into a 5-mL syringe and squeezed out through a blunt needle).

3.2.4.2. Hybrite Method: At this stage the slides are placed in a Hybrite, if one is available. This machine typically has four hybridization programs available. For FISH studies of fixed cells from blood or bone marrow, it is usual to run the program that uses a melt temperature of 75°C for 2 min, whereas for FISH studies of a section, 85°C for 5 min is usually more appropriate. The machine then lowers the temperature to 37°C and the slides are left for as long as needed, usually overnight.

3.2.4.3. Hotplate Method: If a Hybrite is not available, heat up the hotplate and ensure that the temperature is 75°C. (Slightly higher is acceptable but do not exceed 80°C.) Warm the humidified chamber to 37°C in the incubator. Place the slide on the hotplate for 2 min, then transfer to the humidified chamber, and leave at 37°C for as long as needed, usually overnight.

3.2.5. Posthybridization Washes and Signal Detection
After hybridization is complete, unbound probe is removed by a series of washes. These washes are usually carried out in a slightly more stringent solution than the hybridization buffer, to denature and remove weakly bound probe. This should leave only the positively bound probe-target DNA. It is important that the slides are prevented from drying out. Please note that all the wash buffers should have pH between 6.8 and 7.5
1. Place a Coplin jar containing 0.4× SSC and 0.3% NP40/ Igepal in a water bath and bring the temperature to 75°C.
2. Prepare a Coplin jar containing 2× SSC and 0.1% NP40/Igepal at room temperature.
3. Carefully remove glue from around the coverslip using fine forceps.
4. Gently tap the slide on the side of a plastic beaker until the coverslip falls off. The coverslip must be removed gently from the slide to avoid damage to the cells. If the coverslip does not come off readily, check for residual glue which may be still holding it in place. If no traces of glue are found, then soak the slides in 1× SSC for 1–2 min at room temperature to gently lift the coverslip off the slide.
5. Place the slide(s) (no more than two at a time) into the Coplin jar containing 0.4× SSC and 0.3% NP40/Igepal at 75°C for 2 min.
6. Agitate for a few seconds.
7. Remove the slide and place it in a Coplin jar containing 2× SSC and 0.1% NP40/Igepal at room temperature for at least 1 min.
8. Remove the slide and leave to air-dry in the dark.

3.2.6. Counterstaining
1. Add 10–20 mL DAPI stain to a 22 × 50-mm cover slip.
2. Place cover slip (stain side down) on top of the FISH slide.
3. Carefully blot the slide to remove excess mountant solution.
4. It can be helpful to place the slide in a fridge for about 20 min before viewing under the microscope.
5. The slides may be stored for up to 6 months if kept at 4°C in the dark.

3.2.7. Assessing the Result
It can take a while for eyes to adjust to see fluorescence; the light emitted by fluorochromes is often very low, and it will be easier to see if the microscope is in a dark room or is surrounded by dark curtains. It is prudent to systematically examine several fields across the slide if the first field examined does not appear to have any signals. However, the FISH procedure is not infallible and sometimes it fails to give a result even when performed in an experienced laboratory. There are many factors can cause the failure. The fluorescence lasts for a brief time, after which it fades. The amount of light produced by fluorochromes is often very limited, especially if the probe is very small; sometimes it cannot be seen clearly by the human eye. Therefore, it is often necessary to photograph the cells with a digital camera, so that a record can be made before the fluorescence fades. Also, the digitized image can be enhanced to make the colors brighter. This is usually done using a computer program specifically designed for cytogenetic studies. Do not issue a report if there is any doubt about
the validity of the result. If the hybridization is poor and scoring is difficult, then do not hesitate to repeat the study using fresh slides.

3.2.8. Screening and Analysis
Visualization and analysis of FISH signals from larger probes, such as chromosome paints, alpha satellite probes, and yeast artificial chromosomes or bacteria artificial chromosomes, can be successfully effected using a simple epifluorescence microscope with appropriate filter sets. Such a microscope has an ultraviolet (UV) light source as well as white light. The pathway for each kind of light is different, with white light passing through the slide and UV light being projected onto it. The detection of smaller signals (e.g., from phages or cosmids) may require the help of a computer-based image analysis system: a video camera or a low light charge-coupled device (CCD) camera is used to create a digital image, which is enhanced by the computer using image-analysis software. Some systems are also able to control the filters, the camera, the exposure times, and the microscope focusing. The combination of CCD cameras and image analysis makes it possible to process very faint signals and produce images with remarkable clarity. Two of the major manufacturers are Applied Imaging International, and Metasystems, GmbH, Germany. The slide is screened at low power to locate suitable cells, i.e., those with low background and with clear signals. It is good practice to take several photographs of each study as a record. A single photograph is usually composed of several images: these are usually collected using a digital camera mounted on the microscope, each image taken through a different filter, with the computer controlling the exposure time for each filter. The computer analyzes these images, calculates the contribution made through each filter, and produces a pseudo-colored image based on the combined data. If the probe is large, the signals strong, and the hybridization efficient, then it is possible to screen a slide by eye and score the number of signals in each nucleus or metaphase. If the signals are small, or faint, or if the hybridization has been poor, then it can be difficult to see the signals by eye, and it may be necessary to capture (photograph) each nucleus or metaphase and then use a computer to enhance the image. A repeat study with careful attention to the process may be better than struggling with a poor preparation. The hybridization efficiency can vary across a slide, so choose an area with well-spaced cells, low background, and clear signals. Systematically work across the area and record the signal pattern for each nucleus. Enough cells should be scored to give a clear and unambiguous result. For example, if a patient with a possible diagnosis of chronic myeloid is being screened and the first 20 cells all show a BCR–ABL1 fusion, then it is not necessary to do more to confirm the diagnosis. Conversely, if the same patient is being studied after
treatment, when low levels of positivity would be expected, then several hundred cells may need to be screened. No report should be issued that is based on the analysis of just one person. Every case should be scored by two people, at least one of whom is an experienced analyst. The second scorer should not know the result obtained by the first. Afterward, the scores are compared: if the difference could be clinically significant, then a third person should be called on to undertake a further analysis. Records should be kept of the hybridization efficiency of all the probes kept in stock. A probe with reduced hybridization efficiency may fail to provide signals for all the genes in a cell, giving an underestimate of the true incidence. A control study will indicate the expected distribution of signals. Traditionally, the mean number of normal/abnormal control signals has been determined, and then a range of ±3 standard deviations has been calculated. However, this custom presupposes that loss follows a normal distribution, when in fact it is more likely to follow a Poisson distribution. Any test results should be outside this range before they can be accepted as being significant. This is particularly important when screening using a single-color probe. In reality, it can be impractical to determine a precise “normal” range for each stock of probe, scored on different tissues by different analysts. Scoring for loss of a single-color signal is particularly imprecise. If a cell appears to have just one signal, then the cell should be checked at different levels of focus through a single-band pass filter to ensure that the other signal is really missing. Note that two signals that happen to be on top of each other will be indistinguishable. A cell may also appear to have only one signal if there has been a failure of hybridization. It can also happen that a cell can lose a chromosome through random nondisjunction. For all of these reasons, it can be impossible to detect low levels of clonal loss, and many laboratories have decided that the level of loss has to be at least 10% before it can be regarded as being significant. It is safer to score loss using a break-apart probe, or a dual-color probe with both being located on the same chromosome. It is common practice, for example, to screen for monosomy 7 and deletion of the long arms of a chromosome 7 (7q-) by using a pair of probes, one located at the 7 centromere and one in the area commonly deleted. Scoring gain of signals is more reliable. Some cells in a bone marrow aspirate may be tetraploid, and so a cell with three signals might be a tetraploid cell in which two of the four expected signals are again superimposed. However, this coincidence is likely to occur at only a low frequency. Other examples of different kinds of signal patterns are exist.

3.2.9. Interpreting Results

It can be unsafe to report results in isolation; it is wise to be aware of the results of other laboratory tests. For example, a negative result can have two meanings: either the clonal cells
do not have the abnormality being tested, or else there may be no clonal cells present in the sample. Knowledge of any immunophenotyping and/or morphological studies on the same sample can be particularly helpful. It is not possible to identify all the different types of cells in a bone marrow aspirate using only the information given by DAPI stain. However, the shapes of the nuclei can give an indication of the quality of the sample being studied: if the sample is a follow-up from a patient with chronic myeloid leukemia, then the nuclei that are clearly from mature lymphocytes should be excluded from the score. These cells are likely to be an indication that the bone marrow sample was heavily contaminated with blood. Conversely, if the patient has a diagnosis of chronic lymphocytic leukemia, then the scoring of a bone marrow should exclude cells with polymorphic nuclei.
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Abbreviations

AML: acute myeloid leukaemia
ATM: ataxia-telangiectasia mutated
BAC: bacterial artificial chromosome
BACs: bacterial artificial clones
BCL: B-cell lymphoma
BCR: B-cell receptor
BM: Bone marrow
CBA: conventional banding analysis
CCND2: cell cycle gene encoding cyclin D2
CD: Cluster of differention
CE: clonal evolution
CGH: comparative genomic hybridization
CML: chronic myeloid leukaemia
CpG: Cytocine- phosphate—Guanine
CR: complete remission
FCR: Fludarabine, Chlorambucil, rituximab
FISH: Fluorescence in situ hybridization
GC: germinal centres
IGH: immunoglobulin heavy
IGHD: immunoglobulin heavy chain Diversity
IGHJ: immunoglobulin heavy chain Junction
IGHV: immunoglobulin heavy chain variable
IL: Interleukin
ISCN: International System for Human Cytogenetic Nomenclature
IWCCLL: International Working Party on Chromosomes in CLL
LEU2 gene
LOH: loss of heterozygosity
MDM2: murine double minute 2
MDS: myelodysplastic syndromes
MiR: Micro RNA
MYC: myelocytomatosis
MYCN: V-myc, myelocytomatosis viral related oncogene, neuroblastoma derived (avian)
PAC: bacterial P1-plasmid artificial chromosome
PCR: polymerase chain reaction
PFS: shorter progression free survival
Ph: Philadelphia chromosome
PHA: phytohemagglutinin
PR: partial remission
REL: V-rel, reticuloendotheliosis viral oncogene homolog (avian)
SHM: somatic hyper-mutation
SLL: small lymphocytic lymphoma
SMZL: splenic marginal zone lymphoma
SSCP: strand conformational polymorphism
TP53: Tumor protein
TTCR: Time to chemorefractorines
TTT: Time to treatment
TPA: 12-O-tetradecanoyl-phorbol-13-acetate
UPD: uniparental disomy
VDJ: Variable, Diverse, and Joining
VH: variable heavy
YAC: yeast artificial chromosome
ZAP-70: Zeta associated protein-70