

How to Cite:

Hadi, F. A., & Al-Mudallal, S. S. (2022). Detection of 13q deletion in patients with chronic lymphocytic leukemia and its correlation to hematopathological parameters. *International Journal of Health Sciences*, 6(S10), 480–526.
<https://doi.org/10.53730/ijhs.v6nS10.13508>

Detection of 13q deletion in patients with chronic lymphocytic leukemia and its correlation to hematopathological parameters

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Abstract--Background: Chronic lymphocytic leukemia (CLL) is a monoclonal malignancy characterized by an accumulation of small and mature looking B lymphocytes in the blood, bone marrow and other tissues, and is typically characterized by expression CD5+, CD23+, CD22 -, CD79b-, with weak expression of surface immunoglobulin (sIg). Two major clinical staging systems (Rai and Binet staging), that developed to estimate prognosis in CLL. However both these systems are unable to prospectively distinguish the rapidly developing patients from those appreciated to remain with a stable disease for decades. Several publications reported the prognostic importance of 13q deletion in patients with chronic lymphocytic leukemia. 13q deletion is the most important cytogenetic abnormalities detected by Fluorescence In situ Hybridization in CLL patients and represent a good prognostic marker with 60% of patients alive after 5 years as compared with 27% for patients with a normal FISH analysis. Aims of the study: To investigate 13q deletion in patients with chronic lymphocytic leukemia by using FISH technique. To correlate the presence of 13q deletion with hematological and clinical prognostic markers including complete blood picture, absolute lymphocyte count, and modified Rai staging. Patients and Methods: This cross-sectional study was conducted on thirty adult with newly diagnosed and denovo B-CLL patients tested for the expression of 13q deletion using Fluorescence Insitu Hybridization. This was conducted from March 2018 to July 2018 and the diagnosis was documented on the morphology and immune phenotyping of the peripheral blood sample using a four-color flow cytometer in the Nursing Home Hospital / flow cytometry department of the Medical City in Baghdad. The lab work was done in two steps the first step was done in the FISH unit of the nursing Home Hospital in the Medical City and the

second step is done in the postgraduate pathology department in AL-Nahrain Collage of Medicine for detecting the result by using FISH technique. Results: The mean age of all included patients was (63.87±8.01) SD years with more disease predominant in male than female M:F ratio 2:1 . The most common presenting feature of the patients was lymphadenopathy (16 cases). Regarding staging system, the frequency of patient with intermediate risk Rai (43.3%) and (56.7%) patients having high risk Rai . The percentage of expression of 13q deletion in patients included in this study was 23.3%. Conclusion: The frequency of 13q deletion gene was 23.3 %, more frequent in male patients and was an independent risk factor. There was no significant correlation between expression of 13q deletion with hematopathological parameters. The frequency of 13q deletion gene in male patients more than in female patients with male to female ratio 2:1.

Keywords--detection, 13q deletion, patients, lymphocytic leukemia, hematopathological parameters.

Introduction

Chronic lymphocytic leukemia (CLL)

Chronic lymphocytic leukemia (CLL) is a malignant lymphoproliferative disorder of mature B lymphocytes, characterized by the accumulation of a monoclonal population of small mature appearing CD5+B lymphocytes in the blood, bone marrow, and secondary lymphoid organs (lymph nodes and spleen) ⁽¹⁾. CLL is the most common leukemia in adults, with a highly variable clinical course, ranging from very indolent cases to very aggressive and rapidly progressing disease ⁽²⁾. A number of clinical and biological features have been used to separate patients with CLL into subgroups with different prognosis and requirement of different therapeutic approaches ⁽²⁾. In contrast to other B-cell malignancies, CLL is not associated with recurrent balanced chromosomal translocations. For this reason, several biological parameters have been added to the staging system to differentiate prognostic subset ⁽³⁾.

13 q deletion

Many cytogenetic abnormalities were detected in CLL, one of them deletion of 13q14 region which is found in more than 50% of CLL patient ⁽⁴⁾. 13q deletion is the most common cytogenetic abnormality detected by fluorescence in situ hybridization (FISH) and has historically been associated with good prognosis ⁽⁵⁾. During the last years, several studies have revealed some insights in the candidate genes located at 13 q that could be responsible for CLL pathogenesis, as well as in the prognostic heterogeneity of 13q- deleted patients ⁽⁶⁾. One previous study postulated that 13q-x2 may be a more aggressive anomaly than 13q-x1 (Dewald et al., 2003). For initial analysis, patients with a sole 13q- were segregated into one of three groups: heterozygous 13q- (13q-x1), homozygous

13q- (13q-x2), or mosaic deletions with some cells having 13q-x1 and some cells 13q-x2.

Chromosome 13q deletion is a chromosome abnormality that occurs when there is a missing (deleted) copy of genetic material on the long arm (q) of chromosome 13 ⁽⁷⁾. The severity of the condition and the signs and symptoms depend on the size and location of the deletion and which genes are involved ⁽⁶⁾. It is likely that the biological consequence of a unique deletion anatomy is complex, resulting in the disruption of multiple regulatory sequences ⁽⁷⁾. Furthermore, this is likely to be the situation with 13q deletion in other tumor types, such as lymphoma, multiple myeloma, and prostate cancer, as well as deletion events in cancer in general. Here we employ genomic profiling to show that 13q deletion size is associated with disease progression ⁽⁴⁾. In CLL the presence of 13q- conferred a favorable prognosis, with 60% of patients alive after 5 years as compared with 27% for patients with a normal FISH analysis ^(4, 5).

Aims of the study

- To detect 13q deletion in patients with chronic lymphocytic leukemia by using FISH technique.
- To correlate the presence of 13q deletion with hematological and clinical prognostic markers including complete blood picture, absolute lymphocyte count and modified Rai staging.

Literature Review

Definition

Chronic lymphocytic leukemia (CLL) is a disease characterized by the relentless accumulation of CD5+ B lymphocytes in the peripheral blood, bone marrow and secondary lymphoid organs (lymph nodes and spleen). It is usually characterized by an increase in the lymphocyte count in the peripheral blood more than $5 \times 10^9/L$ ^(1, 2). It is the most common leukemia in the Western world, accounting for approximately 30% of all leukemia in Europe and North America ⁽⁸⁾. It is characterized by a marked degree of clinical heterogeneity, ranging from patients that harbor a highly stable disease with a nearly normal life expectancy to patients with a rapidly progressive disease. ⁽³⁾

Epidemiology

The leukemia rank the third of all hematopoietic malignant diseases in Iraq according to the Iraqi cancer registry 2015; it has an average incidence 4.08 cases per 100,000, while the incidence of CLL ranges from <1 to 5.5 per 100,000 people in worldwide ⁽¹⁰⁾. In Iraq, because of its relative indolence, this disease accounts for approximately 1.84% of all cancers and nearly 43.78% of all leukemia ⁽¹¹⁾. In 2011, The American Cancer Society's assaying for leukemia in the United States. There are about 20,110 of new cases CLL and about 4,660 deaths from Chronic lymphocytic leukemia ⁽¹²⁾. It is the most predominant adult leukemia in Western countries, but it is comparatively rare in Asia ⁽¹³⁾. The developing of CLL increased incrementally with the median age that being in 72 year. CLL is very rare below

the age of thirty years, but 20–30 percent of cases present under the age of fifty years. The incidence of CLL cases is more common in whites than blacks and in males than in females; with a male to female ratio of 1.7:1 ^(8, 14).

Historical perspective

The history of CLL is linked with the reports of cases of chronic lymphocytic leukemia that was first recorded in 1845, by Virchow R. and Bennett J.H.. Within a decade thereafter, several other observers also reported similar cases with excessively “white” blood containing colorless corpuscles ⁽¹⁵⁾. Bennett JH. In 1852 called this condition leukocythemia; Virchow R. coined the term leukemia in 1847 and published in 1847 and published a classic paper on this subject in 1856 ⁽¹⁵⁾. The pace of research in leukemia received a major boost at the beginning of the twentieth century, following the introduction of staining methods for blood cells by Ehrlich P. in 1891⁽¹⁵⁾. In 1903 histochemical staining techniques enabled to propose the term lymphomatosis to describe several lymphoproliferative disorders, including CLL. In 1924 provided the first comprehensive description of the clinical features and natural history of CLL, which remained a standard reference for more than four decades ⁽¹⁵⁾. David Galton in 1966 described a proliferative variant that did poorly and a stable variant that did well. He also described CLL as disease of accumulation of long-lived functionally incompetent lymphocytes, a conclusion arrived in independently by William D. in 1967 ⁽¹⁵⁾.

In 1973, Murk Hansen published a series of 189 cases of CLL that had been followed for a long period of time. The immunophenotype of CLL was quickly defined. They found that most cells carried IgD and IgM in 1974 and in 1978 it was found that Surface immunoglobulin density on these lymphocytes was much lower than for normal B cells ⁽¹⁵⁾. Paradoxically, an antigen initially regarded as T-cell specific and later designated CD5 was recognized on the surface of CLL cells by the monoclonal antibodies RFT-1, Leu-1 and OKT-1 at 1982. At the same time as the immunophenotype was being defined, two clinical staging systems for CLL were being developed. In New York, Kanti Ray defined five groups and gave them Roman numerals (1975), while in Paris Jacques-Louis Binet designated three groups alphabetically (1977) ⁽¹⁵⁾. In the late 1980s, Purine analogues, such as Fludarabine and 2-Chlorodeoxyadenosine (Cladribine), were found to be effective for treatment of CLL ⁽¹⁵⁾. In the late 1990s, Campath-1H (Alemtuzumab) was approved for use in patients with refractory disease. New treatment modalities are being examined, including passive or active immunotherapy or ablative chemotherapy with marrow transplantation, because the disease still is not considered curable. In 1999, patients with leukemia cells that express unmutatedIg variable-region genes were recognized in general to have more aggressive disease than patients whose leukemia cells express mutated antibody genes ⁽¹⁵⁾.

Etiology

The exact etiology is uncertain, but there is some risk factors for CLL have been detected, these include:

Gender

Chronic lymphocytic leukemia is further common in males than females and the reasons for this are unidentified. One retrospective study of women noted a trend toward reduced risk of CLL with increasing parity, prompting chance that pregnancy lowers the risk for this disease ⁽¹²⁾. Also, for unknown reasons female patients tend to have a longer survival than male patients ⁽¹⁶⁾.

Environmental factors

There is no documented association of CLL with exposure to radiation, alkylating agents or known leukemogenic chemicals. However, exposure to some chemicals used in agriculture may increase the risk of developing CLL. Associations between CLL and several viruses, including human T-cell lymphotropic viruses I and II (HTLV-I and HTLV-II) and Epstein-Barr virus, have been suggested. However, no conclusive evidence of a causal relationship exists ⁽¹⁷⁾.

Hereditary factors

There are familial risks for development of CLL about 5% -10% of patients are within the same family member. First relatives of CLL patients have risk to develop the same disease between (2-7 times) in younger individuals than with next generations, However the anticipation phenomenon for familial predisposition is accompanied by an early onset of the disease and sever course in the descend generation ^(18, 19).

Race/Ethnicity

In North America and Europe chronic lymphocytic leukemia is more common than in Asia, and Asian people who live in the United States have lower risk than those living in Asia. That is why the experts think the variation in risk are not linked to environmental than genetics factors ⁽²⁰⁾.

Pathogenesis and cell biology

It includes the following:

Defective Apoptosis

In most patients with CLL the cells of origin are clonal B-cells arrested in the differentiation pathway, intermediate between the pre-B cells and mature B-cells. In the peripheral blood, the morphological of these cells look like mature lymphocytes ⁽²¹⁾. In CLL B-cell lymphocytes typically show B-surface antigens, as shown by CD19,CD20dim,CD21,CD23. Because normal CD5+ B-cells are detected in the mantle zone of lymphoid follicles, so B-CLL was most likely a malignancy of a mantle zone population of anergic self-reactive cells dedicated to produce of polyreactive natural autoantibodies ⁽²²⁾. There are several factors are involved in CLL pathogenesis, including: antigen stimulation within specific micro environment, and failure to undergo apoptosis ⁽²³⁾. Studies have showed that the proto-oncogene (bcl2) is overexpressed in B-CLL which is a known suppressor of

apoptosis (programmed cell death); and will result in long life for the cells that are involved, so the majority of CLL cells (more than 90%) are non-dividing cells are arrested at G0 or G1 phase of the cell cycle and are resistant to apoptosis ^(22, 23). The clinical behavior differed between mutated and un mutated cases that relate to continuous response to antigen stimulation, so the un mutated cases have autoreactivity and polyreactivity to certain molecules with more proliferative pattern and aggressive clinical course for the disease and lead to instable genetically, while the mutated cases showed decrease cell division by getting anergic ⁽²⁴⁾. The researchers have been suggested that an excess of B-cells is due to decreased apoptosis and deregulation (cell-cycle control) than increase proliferation rate ^(23, 24).

Genomic aberrations and gene mutations

Two genes, named miRNA15a and miRNA16-1, are located at 13q14 and have been shown to encode for a regulatory RNA called microRNA (miRNA). These miRNA genes belong to a family of highly conserved noncoding genes throughout the genome whose transcripts inhibit gene expression by causing degradation of miRNA or by blocking transcription of miRNA ⁽²⁶⁾. Genetic analyses have demonstrated deletion or down regulation of these miRNA genes was found in 70% of 13q deletions and may lead to overexpression of bcl2 proto-oncogene ⁽²⁷⁾. About 10 to 15% of CLL patients have tumor suppressor gene (p-53) which is mutated or deleted that correlates with disease progression and poor prognosis ⁽²⁸⁾.

Cytokines

As lymphocyte survival and growth are intimately related to growth factors, it has been suggested that alterations in the production of and response to cytokines may play major roles in the pathogenesis of CLL. Speculatively, these cytokines may decrease apoptosis or stimulate proliferation of CLL cells but inhibit survival of normal lymphoid and marrow cells leading to the immunosuppression and myelosuppression seen in this disease ⁽²⁷⁾. CLL cells express activation markers such as CD23 and CD27 and produce or at least synthesize the m-RNA for most cytokines (TGFB, IFN-Y, ILs, TNF, Scd23, etc) ⁽²⁶⁾. However, CLL cells undergo apoptosis spontaneously in vitro, a fact that stresses the importance of microenvironment interaction and influences the inhibition of programmed cell death ⁽²³⁾. It has also been suggested that several cytokines inhibit apoptosis in CLL in an autocrine fashion. T-cells also produce some of these cytokines and influence survival of the malignant clone ⁽²²⁾.

Immunological defects

The autoimmune disorders in CLL patients, which result from auto antibodies that are directed toward hematopoietic cell antigens (Ag), such as those Ag found on red blood cells (RBC) or platelets, although other disorder of autoimmune disease that demonstrated more common among chronic lymphocytic leukemia patients than the general population ^(29, 30). In some cases of B-cell, the auto-antibody may be produced by monoclonal, but mostly these auto antibodies are produced by stander non-neoplastic cells, this reflecting a disease-associated with

dys-regulation in immune tolerance to self-antigens (Ag). Some CLL Patients may develop pure red blood cell aplasia or neutropenia due to the autoantibodies development against bone marrow hematopoietic progenitor cells ⁽³¹⁾. The prevalence of direct antiglobulin test (DAT) positive detected in patients with CLL differed from 2% to 35% and its prevalence is higher with disease progression. The autoimmune hemolytic anemia (AIHA) has been revealed clinically in 3% to 37% of B-CLL patients with advanced-stage ⁽³²⁾. Most patients have a warm hemolytic anemia AIHA mediated by IgG anti-erythrocyte antibodies (Ab) directed against antigens of the Rhesus system (RH) on red blood cells (RBC), while the residual have a cold hemolytic anemia AIHA mediated by IgM antibodies rarely have the ability to fix and activated complement. Autoimmune mediated cytopenia may occur at any stage of CLL and could be considered as prognostic significance ⁽³³⁾. The complement pathway in CLL patients are decreased and associated with aberrant binding and activation and these abnormalities present with advanced disease ⁽³⁴⁾. In majority of patients will develop reduction in levels of normal immunoglobulin during the course of their disease. All three classes (IgG, IgA and IgM) are affected, although predominantly (IgG3 and IgG4) ⁽³⁵⁾.

Diagnostic criteria

According to the International Workshop on CLL (IWCLL), the diagnosis of CLL is sustained by the following parameters (36):

- Presence in peripheral blood of more than 5×10^9 / L monoclonal B lymphocytes persisting for more than three months.
- Demonstration of the clonality of the population.
- Characteristic immunophenotype: Smlg weak, CD5+, CD19+, CD20 weak, CD23+. Regarding the immunophenotype, FMC7 (a CD20 epitope) and CD79b are usually absent or weakly expressed.

Based on immunophenotypic characteristics and giving one point to each one of the following: CD5+, CD23+, FMC7 weak, Smlg weak, and CD79 weak. Matutes and Catovsky showed that in patients with a score of 4-5, the diagnosis is virtually always CLL, while in those cases with a score less than 3, the diagnosis of CLL is extremely unlikely. The typical immunophenotype for CLL as accepted in current guidelines should be revisited because of the availability of markers highly characteristic of CLL such as CD200 and RORI ⁽³⁶⁾. CLL and small lymphocytic lymphoma (SLL) are cancers that affect the same lymphocytes. CLL and SLL are essentially the same disease, the only difference being where the cancer primarily occurs. When most of the cancer cells are located in the blood stream and the bone marrow, the disease is referred to as CLL, although the lymph nodes and spleen are often involved. When the cancer cells are located mostly in the lymph nodes, the disease is called SLL ⁽³⁷⁾. Monoclonal B-cell lymphocytosis (MBL) is defined as the presence of a clonal B-cell population in the peripheral blood with fewer than 5×10^9 /L B-cells and no other signs of a lymphoproliferative disorder ⁽³⁸⁾. Now it is known that MBL precedes virtually all cases of CLL/SLL. The updated WHO will retain the current criteria for MBL, but will emphasize that:

- Low count MBL: defined as a PB CLL count of less than $0.5 \times 10^9/L$, must be distinguished from high-count MBL because low count MBL has significant differences from CLL, an extremely limited, if any, chance of progression, and until new evidence is provided, does not require routine follow-up outside of standard medical care.
- High count MBL: require routine/yearly follow up, and has very similar phenotypic and genetic/molecular features as Rai stage 0 CLL, although IGHV mutated cases are more frequent in MBL. Also impacting our diagnostic criteria, the revision will eliminate the option to diagnose CLL with less than $5 \times 10^9/L$ PB CLL cells, in the absence of extramedullary disease even if there are cytopenias or disease related symptoms ⁽³⁷⁾.
- Non-CLL type MBL: at least some of which may be closely related to splenic marginal zone lymphoma, is also recognize. The concept of tissue based MBL of CLL type will be discussed as there are a subset of cases with lymph node involvement by SLL that also do not seem to have a significant rate of progression. In one retrospective study, lymph node with CLL/SLL in which proliferation centers were not observed and patients in whom adenopathy was less than 1.5 cm based on computed tomography scans were the best candidates for tissue based MBL ^(38, 39).

Differential diagnosis of B cell neoplasm

In most patients, the diagnosis of CLL is easily made after a careful review of the peripheral smear and immunophenotyping, although other conditions must be considered (Table 2.1) occasionally, immunohistochemistry or gene rearrangement studies are required to exclude other diagnosis.

Table 2.1
Differential diagnosis of B cell neoplasm ⁽⁴⁰⁾

| |
|--|
| 1. Benign causes of lymphocytosis |
| Bacterial(e.g., tuberculosis) |
| Viral(e.g., infectious mononucleosis) |
| Persistent polyclonal B-cell lymphocytosis |
| Hyperreactive malarial splenomegaly |
| 2. Malignant causes of lymphocytosis |
| B-Cell |
| Monoclonal B-cell lymphocytosis (MBL) |
| Prolymphocytic leukemia (PLL) |
| Leukemic phase of non-Hodgkin lymphomas |
| Mantle cell lymphoma |
| Follicular lymphomas |
| Marginal zone lymphomas including splenic lymphoma with villous lymphocytes (SLVL) |
| Diffuse large cell lymphoma |
| Hairy cell leukemia |
| Waldenström macroglobulinemia |
| T-Cell |
| Pro lymphocytic leukemia |

| |
|---|
| Adult T-cell leukemia/lymphoma |
| Sézary syndrome |
| Large granular lymphocytic (LGL) leukemia |

A general approach to the workup of lymphocytosis is suggested in figure (1.1)

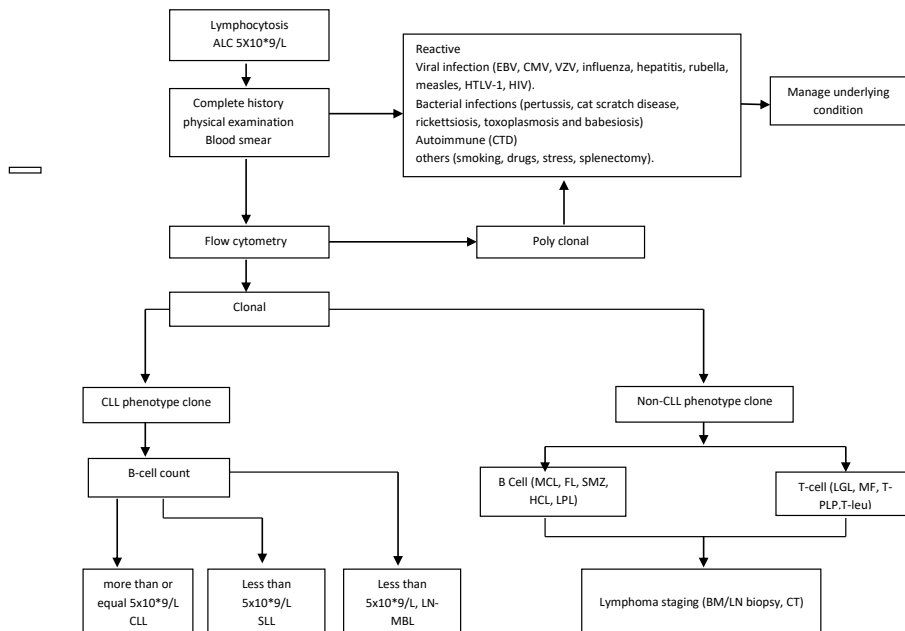


Figure 2.1. General approaches to the workup of lymphocytosis

BM, bone marrow; CMV, cytomegalovirus; CTD, connective tissue disease; EBV, Epstein- Bar virus; FL, follicular lymphoma; HCL, hairy cell leukemia; HTLV, human T-lymphotropic virus; LGL, large granular lymphoma; LN, lymph nodes; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MF, mycosytosis fungoides; T-PLP, T prolymphocytic leukemia; SMZ, splenic marginal zone lymphoma; T-cell leukemia; VZV, varicella zoster virus. If the clinical and laboratory evaluation point toward a neoplastic origin, clonality should be evaluated through flow cytometry. A variety of clonal B-cell disorders can be identified based on surface protein markers with such analysis. The management of clonal disorders of CLL phenotype is the focus of the remainder of this review. The detection of clonal B cells with a non-CLL phenotype (non-CLL MBL) or t-cell monoclonal lymphocytosis should warrant further testing, including computed tomography (CT) imaging, bone marrow biopsy, molecular and genetic studies according to the suspected lymphoproliferative disorder.

Table 2-2
Immunophenotype of common clonal B-cell disorders (40)

| | CD5 | CD19 | CD20 | CD23 | CD10 | CD103 | Dual CD11c/22 | sIg | CD200 | Genetic defect |
|-----|-----|--------|--------|------|------|-------|------------------|--------|-------|-------------------|
| CLL | + | + | Dim | + | - | - | - | Dim | + | - |
| MCL | + | + | Bright | Dim | - | - | - | Bright | - | t(11,14) |
| FL | - | + | + | +/- | +/- | - | - | + | - | t(14,18) |
| MZL | - | + | Bright | - | - | - | + | + | - | 7q- |
| HCL | - | Bright | Bright | - | - | + | Bright | + | + | - |
| LPL | +/- | + | + | +/- | - | - | - | Dim | + | - |

Investigations

Morphology

The complete blood count (CBC) is one of the key tests in the diagnostic process and is the first step. The widespread availability of CBC analysis explains the increasing detection of CLL as an “incidental” finding. The full blood count reveals the increase in the lymphocytes in the blood but cannot tell whether they are B-, T- or NK-cells ⁽⁴¹⁾. Patients with CLL may develop anemia secondary to leukemic marrow infiltration, the myelosuppressive effect of chemotherapy and inhibiting cytokines, autoimmunity directed against red cell antigens, hypersplenism, and/or a poor nutritional status that leads to deficiency of folic acid, vitamin B12, or iron. The nature of the blood findings vary depending on the factor(s) responsible for the anemia ⁽⁴²⁾. During the most advanced disease stage, patients have thrombocytopenia because of marrow replacement and hypersplenism. At any stage, however, patients can develop immune thrombocytopenia because of antiplatelet antibodies. It has been estimated that immune thrombocytopenia can complicate the course of CLL in approximately 2% of patients ⁽⁴²⁾. The diagnosis of CLL requires the presence of at least $5 \times 10^9/L$ B lymphocytes in the peripheral blood.

Flow cytometry

The clonality of the circulating B lymphocytes needs to be confirmed by flow cytometry ⁽⁴³⁾. In all lymphocytosis cases Immunophenotyping should be done to confirm the diagnosis that suspected by their morphology and to rule out the other cell disorders (B and T) cells. Moreover cases with low lymphocyte counts have particular value to confirm for the diagnosis of chronic lymphocytic leukemia and to exclude the reactive lymphocytosis. Typically, CLL cells express the T-cell antigen CD5 and B-cell surface antigens (CD19, CD20, and CD23), and low levels of surface immunoglobulin often IgM with or without IgD, CD20, and CD79b are characteristically low compared than found on normal B-cells. FMC7, is a monoclonal Ab that binds to the epitope of CD20 when this surface antigen is present at high density, typically this will not react with chronic lymphocytic leukemia cells, so reflecting the low level expression of (CD20) in most patients with CLL. Each clone is restricted to expression of either kappa or lambda Ig light chains. Moreover CD43 and CD200, can provide another information in differentiating chronic lymphocytic leukemia from certain B-NHL, so CD43 helps to distinguish chronic lymphocytic leukemia (CD43+ve) from follicular lymphoma

(CD43-ve) ; while CD200 will help to distinguish chronic lymphocytic leukemia (CD200+) from Mantle cell lymphoma which is (CD200-ve) ⁽⁴⁴⁾.

Table 2.3
The Scoring system for chronic lymphocytic leukemia diagnosis ⁽⁴⁵⁾

| Antigen | 0 points | 1 point |
|---------|----------|---------------|
| CD79b | Strong | Weak/Negative |
| CD23 | Negative | Positive |
| CD5 | Negative | Positive |
| FMC7 | Positive | Negative |
| SmIg | Strong | Weak/Negative |

A score more than or equal 4 is indicative of CLL. A score of less than or equal 3 should prompt consideration of an alternative diagnosis.

Bone marrow examination

Although marrow examination is not usually essential for the diagnosis of CLL , the presence of proliferation centers and absence of para trabecular foci and cyclin D1 nuclear staining support a diagnosis of CLL in cases with atypical morphology and a low immunophenotype score. Marrow examination is also valuable for determining the cause of cytopenias, providing prognostic information and assessing the response to therapy ⁽⁴⁶⁾. In CLL, characteristically more than 30% of the nucleated cells in the aspirate are lymphoid ⁽⁴⁷⁾. Marrow infiltration in CLL may be interstitial, nodular, mixed (nodular and interstitial) or diffuse, with mixed being the most common and nodular the least common. Diffuse involvement, in which there is effacement of the fat spaces by tumor, carries the worst prognosis. The marrow involvement is random and contrast with follicular lymphoma, in which paratrabeular involvement is the rule. In contrast to marrow, involvement of the lymph node is diffuse. Proliferation centers paraimmunoblast are typically seen in both marrow and lymph nodes ⁽⁴⁸⁾. The value of BM trephine biopsy in CLL ⁽⁴⁹⁾

- **Prognostic feature**
 - Diffuse pattern: packed bone marrow has poor prognosis
- **Clarify the nature of cytopenias (before and after therapy)**
 - Low platelets count
 - Red cell aplasia
 - Myelodysplastic changes
- **Differential diagnosis from low-grade NHL**
 - Paratrabeular pattern not seen in CLL
 - More proliferative centers in CLL/PL
- **To assess response to treatment**
 - Nodular partial remission seen only on biopsy (needs immunostaining)
 - Hypocellular bone marrow, without CLL infiltrates, and low blood counts

Cytogenetic/Molecular analysis Deletions in Band 13q14

Deletion of (13q14) is the most common structural aberration in the long arm of chromosome in CLL, and have a good prognosis due to the mechanism of regulatory tumor suppressor in controls the expression of the whole region ⁽⁵⁰⁾.

Deletions of ataxia telangiectasia-mutated gene (ATM) (11q22–q23)

It rarely occurred in early- stage of the disease, and about 1/4 of patients with advanced CLL have (11q23) deletions in the long arm of chromosomes 11 ⁽⁵⁰⁾. Mostly the patients displayed more brisk disease and extensive lymphadenopathy (LAP). In all cases this region has the ataxia telangiectasia-mutated (ATM) gene ⁽⁵¹⁾. The ATM protein kinase is a central element of the DNA breaks and functions directly in the repair of DNA- DSBs by maintaining DNA ends in the repair complex ⁽⁵²⁾.

Trisomy 12

It occurs in 10%– 20% in CLL patients and it is a frequent aberrations and less common than deletions in band 13q14. The genes that involved in the pathogenesis of trisomy 12 are unknown and it is associated with poor outcome ⁽⁵³⁾.

Deletions in Band 17p13 or (TP53 Mutations)

It occurred in 4–9% of chronic lymphocytic leukemia. Deletion of 17p13 may be observed at diagnosis or at initiation of the therapy. The 17p13 deletion usually includes the short arm of chromosome 17p where the TP53 (tumor suppressor) is located ⁽⁵⁴⁾. In one hand, the patients who had chronic lymphocytic leukemia cells with chromosomal aberrations del(11q) and del(17p) had an poor outcome compared to patients who had normal karyotype or del(13q) as the only genetic abnormality in CLL ⁽⁵⁵⁾.

Prognosis

Prognostic factors of CLL

There are many factors that help to predict the patient outlook. There are variable Classical prognostic markers that have been identified in CLL which include the following ⁽⁵⁶⁾.

- Age and gender.
- Clinical staging.
- Lymphocytes doubling time.
- Blood lymphocytes doubling time.
- Marrow histology and cell morphology.
- Cytogenetics and P53 expression.
- Some biological prognostic markers.

Age and gender

- **Age:** the influence of age is greater in Binet stage A, in which up to 50% of patients may die of causes unrelated to CLL. Overall, one third of patients may die from causes other than CLL, such as other neoplasms or cardiovascular accidents, and these are significantly influenced by age (56).
- **Gender:** female patients survive significantly longer than males and to respond better to treatment. In addition, females have lower incidence of the disease. The biology behind the better prognosis of women is not as yet obvious. The proportion of CLL with mutated IGHV genes is higher in females, and this may provide a possible explanation (56).

Clinical staging

There are two widely accepted systems for staging methods that used in both patient care and clinical trials: the Rai system (1975) and Binet system (1981). The original Rai classification staging system takes the view that CLL cells accumulate first in the blood and bone marrow; then in lymph nodes and spleen, finally, leading to bone marrow failure. The patient surviving chances will depend mostly on the stage at which patient was presented. It is mostly used in the United States, while Binet system is used more broadly in Europe. The Rai system was modified to decrease the number of prognostic groups from (5 - 3). Additionally, both systems now prescribe three major subgroups with separated clinical results. Both system depended just on a physical examination, standard (laboratory tests) and do not need ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) (57).

Rai Staging System

Table 2.4
Rai and Modified Rai staging system (58)

| Risk Group | Stage | Description |
|--------------|-------|---|
| Low | 0 | Lymphocytosis only |
| Intermediate | I | Lymphocytosis plus enlarged nodes |
| | II | Lymphocytosis plus enlarged liver or spleen with or without enlarged nodes |
| High | III | Lymphocytosis plus anemia(Hb less than 11) with or without enlarged nodes,liver,or spleen |
| | IV | Lymphocytosis plus thrombocytopenia(platelet count less than $100 \times 10^9/L$) with or without anemia,enlargednodes,liver or spleen |

It is important to remember that when anemia or thrombocytopenia is immune in etiology, the patient should not be assigned to stage III/IV, because these stages refer to cytopenias that result from bone marrow failure rather than autoimmune destruction (57, 58).

The Binet staging system ^(58, 59)

| | |
|---|---|
| A | less than 3 lymphoid areas* |
| B | more than or equal 3 lymphoid areas* |
| C | Hb less than 10g/dl or platelet less than $100 \times 10^9/L$ * |

*The five possible lymphoid areas comprise: unilateral or bilateral cervical, axillary, and inguinal lymphadenopathy, hepatomegaly, and splenomegaly.

Lymphocyte doubling time

Is a useful indication of disease progression. Taking longer to double the lymphocyte count (more than 12 months) implies a favorable prognosis, while aggressive cases show a shorter doubling time (less than 12 months). Clinically, since there are many factors affecting the growth rate of cells, relying on a single prognostic parameter is not recommended (60).

Marrow Histology and cell morphology.

Biopsy can reveal characteristic patterns of marrow infiltration, defined as nodular, interstitial, mixed, or diffuse. A diffuse replacement of the marrow is associated with a worse prognosis than is a nodular or interstitial pattern. The marrow biopsy is more reliable than is the aspirate in distinguishing patients with favorable disease (nodular and/or interstitial) versus non favorable disease (diffuse) independent of clinical stage. In addition, marrow biopsy appears more sensitive than the aspirate in detecting marrow infiltration of leukemia cells ^(42, 43)

Cytogenetics

Approximately 80% of individuals with CLL have acquired chromosomal abnormalities within their malignant clone and can be categorized into five prognostic groups accordingly: deletion 13q (median survival, 133 months); deletion 11q (median survival, 79 months); trisomy 12 (median survival, 114 months); normal cytogenetics (median survival, 111 months); and deletion 17p (median survival, 32 months) ^(61, 62).

13 q deletion

Deletion of (13q14) is the most common structural aberration in the long arm of the chromosome in CLL, and have a good prognosis due to the mechanism of regulatory tumor suppressor in controls the expression of the whole region ⁽⁶²⁾. Among chromosomal abnormalities, deletion of the long arm of chromosome 13 (13q) is detected in more than 50% of cases of chronic lymphocytic leukemia as well as in other B-cell malignancies including de novo and transformed diffuse large B-cell lymphomas (DLB-CL) and multiple myeloma (MM). Deletion of 13q is the most common cytogenetic abnormality in CLL. This deletion represents early clonal aberration and suggests the loss of a tumor suppressor gene ⁽⁶²⁾. CLL patients with deletion 13q show a better prognosis and a longer overall survival than CLL patients with deletion (11q23) or deletion (17p) (63).

Also the percentage of CLL cells with deletion of (13q) is associated with survival ,i.e., a high percentage (more than 80%) of deletion (13q) cells results in a shorter survival compared to patients with a lower percentage (less than 80%) of these cells(63). In addition, it has been shown that patients with a higher percentage of del (13q) cells have a higher lymphocyte count and tend to exhibit a diffuse pattern of BM infiltration and splenomegaly. Deleted in leukemia 1 (DLEU 1) and 2 (DLEU 2) are two genes transcribed head to head in a 30kb region located in the long arm of chromosome 13 (13q14), which is lost in more than 50% of patients with CLL and that predict a poor prognosis(63). The homozygous loss of this region has great effects on the regulation and control of normal CD5+ B lymphocytes and their homeostasis. With regard to biologic basis underlying 13q deletion, miR-15a and miR16-1, located in the minimal deleted region (MDR), have been described to exhibit a tumoral suppressor function in CLL patients (6, 7).

However, miR-15a and miR16-1 are not invariably included in 13q deletions, and although their expression is decreased in several CLL patients, a clear correlation with the number of deleted 13q alleles has not been found (62). Thereby, besides these microRNAs, other genes located in 13q, such as DLEU7, could cooperate in the tumoral suppressor activity. In addition, it has been extensively demonstrated that large 13q losses involving RB1 (type 1 deletions) gene are related to shorter time to first treatment (TTFT) and overall survival (OS) than those small deletions encompassing only miR-15a and miR16-1 (type II) (63, 64). Results indicated that both percentage of deleted nuclei and presence of larger deletions involving the RB1 locus cooperated to refine the prognosis of del. 13q only cases. In particular, CLL carrying less than 70% of 13q deleted nuclei with deletions not comprising the RB1 locus were characterized by particularly long time to treatment. Conversely CLL with 13q deletion in less than 70% of nuclei but involving the RB1 locus, or CLL carrying 13q deletion in more than or equal 70% of nuclei with or without RB1 deletions, collectively experienced shorter time to treatment (63).

In contrast to other recurrent abnormalities in CLL ,the presence of biallelic losses in 13q has been described in nearly 30% of 13q-deleted CLL patients (65). Biallelic 13q deletions are characteristically small and do not involve Retinoblastoma -1 (63); nevertheless their clinical impact has been controversial. Some authors hypothesized that they result from an evolution of the monoallelic losses and therefore represent a more aggressive abnormality (66, 67). However, we and others did not find significant differences in the baseline characteristics and clinical outcome among CLL patients with monoallelic or biallelic 13q deletions (66-69). It is worth noting that 13q14 region can be inactivated by other mechanisms such as copy neutral loss of heterozygosity and epigenetic silencing by DNA methylation of CpG islands or histone deacetylation (70, 71). Thus, it is feasible to assume that the potential effect of the biallelic 13q losses on the prognosis could be masked either by the size of the deleted region or the inactivation of the remaining allele by other mechanisms (65).

Regarding the size of abnormal clone detected by FISH, it has been described that those patients with a higher percentage of altered nuclei have a significantly shorter TTFT and OS. The optimal cut-off point that defines the poorer outcome of 13q deletion differs between published studies (72, 66, 67, 68, 72, 73). Indeed, although the cut-off described ranged from 65.5% to 90%, the percentage of 13q deletion

had predictive value as a continuous variable (66, 73). Thus those patients with isolated 13q deletion can be risk stratified according to the percentage of altered cells by FISH (66,67).

Biologic heterogeneity underlying clinical differences observed among 13q-deleted patients has been also demonstrated by gene expression profiling and miRNA analysis (74, 75). Specific transcriptional profiles have been correlated with two subgroups of 13q deletion based on the size of deleted area (short/biallelic versus wide/monoallelic). Thus, those patients with large 13q losses showed down regulation of ten genes including TPT1/TCTP, which is involved in prosurvival and growth signaling through inhibition of BAX-induced apoptosis and overexpression of 53 genes. Most of the up-regulated genes (AMF, GPI, BSG, LGALS1, PAK2, PARVB and VIM) were involved in cell motility and adhesion, regulation of cell proliferation, tumor cell migration, metastasis, angiogenesis, and apoptosis (64,65,66).

Interestingly, deregulation of many relevant cellular pathways has also been shown in those patients with higher percentages of 13q deletion (above 80%) (66). Among them are remarkably the deregulation of several important miRNAs and overexpression of genes mainly involved in BCR signaling (e.g., SYK and CD79b), NFkB signaling and pro-survival and anti-apoptotic pathways (RAS signaling) (75). Because of the high frequency of such deletion, a suggestion has been made that chromosome 13q14 region may harbor some tumor suppressor genes. Several groups have used positional cloning to identify the gene or genes targeted by the deletions. A region of more than 1 Mb has been fully sequenced and characterized in detail (33, 76).

So far, a total of eight genes have been identified and screened for alterations at the DNA and/or RNA level in sporadic and familial cases of CLL: Leu-1 (BCMS or EST70/Leu-1), Leu 2 (ALT1 or 1B4/Leu-2), Leu 5 (CAR), CLLD6, KPNA3, CLLD7, LOC51131 (putative zinc finger protein NY-REN-34 antigen) and CLLD8. However, detailed genetic analysis, including extensive loss of heterozygosity (LOH), mutation, and expression studies, have failed to demonstrate the consistent involvement of any of the genes located in the deleted region (33, 34, 35, 50, 51, 52, 61, 76). On the other hand a number of researchers found 51.7% with chromosome abnormalities. The most frequent one was deletion 13q14 in 34.5% of cases. It was associated to other alterations in 17.2%. 17p13 deletions were found in 17.2% and trisomy 12 in 13.8% (in isolation in 6.9% and associated to del 13q14, in 6.9% of the cases) (67).

An 11q22 deletion was found in one case associated to a 13q14 deletion. To better evaluate the relationship between chromosome aberrations and other prognostic factors in CLL, two cytogenetics groups were considered: favorable (13q deletion in isolation and no alteration) and unfavorable outcomes (trisomy 12, 17p13 deletion, 11q22 deletion and two simultaneous alterations). The unfavorable alterations were more frequently seen among young individuals (less than 60 years) (68). In relation to the Binet staging system, patients with unfavorable cytogenetic alterations tended to be B and C stages, while in the favorable group prevailed patients in stage A. Additionally, patients with poor prognostic cytogenetics tended to express CD38 and ZAP70 proteins (68).

P53 expression

P53 is a very important protein molecule that is activated when there is damage to the cell's DNA. Such damage happens spontaneously and can also be deliberately induced by chemotherapy or radiotherapy. Once activated, p53 stops the cell dividing so the damage can be repaired. If the damage is beyond repair, p53 triggers the cell to commit suicide by a process called apoptosis. In this way, p53 keeps cancer cells in check and also helps chemotherapy and radiotherapy to work. Cells in which p53 not working properly are genetically unstable and are not killed effectively by radiation or chemotherapy. The gene encoding p53 is located on the short arm of chromosome 17 and is deleted in 17p- CLL ⁽⁵⁸⁾.

Some biological prognostic markers

Serum markers (77,78)

- LDH: serum levels are also of prognostic significance. High serum level, which is a measure of tumor burden and turnover, indicates poor prognosis ⁽⁴⁾. It was found that the overall survival time in group of normal levels of LDH.
- Serum Thymidine-Kinase activity (sTK) in CLL patients is probably related to the number of dividing neoplastic cells, reflecting tumor mass and rate of tumor cell proliferation. The ability of sTK levels to detect a subgroup of patients with early CLL at risk of rapid disease progression seems particularly useful. sTK levels inversely correlate with survival.
- B2microglobulin: shows a positive correlation with clinical stages and has been found to be a strong prognostic marker in predicting survival after chemo or chemo-immunotherapy.
- Soluble CD23: high levels of the sCD23 have also been linked to adverse prognostic features such as diffuse bone marrow infiltration, rapid doubling time and disease progression in early stage CLL ^(77, 78).

Role of CD38 in CLL (77, 78)

CD38 is an enzyme that present on the cell surface, and involved in trans membrane signaling, cell adhesion, transduction, and calcium regulation. The extracellular nucleotides metabolized by CD38 for example: ATP and NAD, into nucleosides, so can be taken up by cells. CD38 plays a role in increasing cytoplasmic calcium concentrations ⁽⁷⁷⁾. In conjunction with chemokines and their receptors, CD38 also influences cell migratory responses. These considerations are the rationale for devising a CLL therapy that uses CD38 as the target. The use of reagents specifically blocking the molecule might provide a new approach for interfering with deleterious growth circuits, therefore increasing the susceptibility of leukemic cells to conventional chemotherapy ⁽⁷⁸⁾. Excess CD38 expression associated with increase the vascular density in lymph nodes, consensual with increased lymphocyte proliferation and this lead to disease progression ⁽⁷⁷⁾.

Fluorescence In Situ Hybridization Principles of FISH

It is a powerful technique that merges cytogenetics and molecular genetics by utilizing fluorescent -labeled DNA probes to detect genetic aberrations that are generally beyond the resolution of conventional chromosome banding studies ⁽⁷⁹⁾. FISH is based on the principle that a single -stranded DNA molecule will recognize and bind to its complementary sequence on a metaphase chromosome or in an interphase nucleus ⁽⁸⁰⁾. The major advantage of FISH, however, is the utilization of a DNA probe labeled with a fluorescent dye, which results in a highly sensitive, simple, and rapid assay ⁽⁷⁹⁾. FISH can be used to target genomic sequences in non dividing cells, which allows identification of chromosomal aberrations irrespective of cell -cycle stage ^(79, 80).

This technique, known as interphase FISH (iFISH), is a powerful cytogenetic tool that can be applied to a wide variety of clinical specimens to enumerate chromosomes and identify chromosome rearrangements ⁽⁸¹⁾. With iFISH, the most common single chromosome abnormality is a deletion of 13q14 found in 55% – 65% of cases followed by trisomy 12 in 15% –25%, deletion of 11q22/ ATM in 11% –18%, deletion of 17p13/p53 in 7% –8%, and deletion of 6q in 5% –6% ⁽⁸²⁾. Of interest, all deletions are monoallelic, with the exception of the 13q14 locus where biallelic deletions are observed in 25% –45% of cases ^(81, 83). Therefore, FISH using a panel of disease - specific probes is increasingly being offered as an adjunct to conventional G -banding to assess the genetic defects in CLL/ SLL ⁽⁸⁴⁾.

Indications for FISH testing in hematolymphoid disorders (79,80)

- Confirmation of chromosome abnormalities detected by conventional cytogenetics and establishment of FISH signal pattern for follow -up study.
- Detection of chromosome abnormalities when clinical and morphological findings are suggestive of a specific chromosome abnormality, such as t(11;14) in mantle cell lymphoma.
- Characterization of genetic aberrations using a panel of disease - specific FISH probes for risk stratifications and therapeutic management, such as in acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), and plasma cell myeloma.
- Detection of cryptic or masked translocations when chromosome analysis is inconclusive or yields a normal karyotype, such as the t (12;21) in ALL or t(4;14) in myeloma.
- Detection of lymphoma -associated translocations in paraffin - embedded tissue sections.
- Quantitation of minimal residual disease (MRD) and detection of cytogenetic remission and relapse through analysis of a large number of both dividing and non dividing cells.
- Monitoring of cross -sex bone marrow transplantation patients for engraftment status (chimerism).
- Rapid detection of PML/RARA gene fusion in acute promyelocytic leukemia where quick diagnosis is necessary to initiate treatment.

Advantages of FISH analysis (79,80)

- Can be performed on metaphase cells or interphase nuclei (non dividing cells) and on fresh or fixed tissue samples.
- Targets genetic aberrations that pinpoint candidate genes involved in leukemogenesis.
- Can simultaneously assess chromosome aberrations, cellular phenotype, and tissue morphology (paraffin FISH).
- Analysis is highly specific and sensitive, reproducible, and provides for objective interpretation.
- Rapid turnaround time.
- Can simultaneously assess multiple genomic targets.
- Provides superior resolution (interphase FISH ≥ 20 kbp, metaphase FISH ≥ 100 kbp) compared with standard karyotyping (> 10 Mb megabase pairs).
- Detects specific cryptic chromosome abnormalities.

Limitations of FISH analysis (79,80)

- Does not provide a genome -wide assessment of chromosomes.
- The choice of probes utilized is guided by clinical suspicion (differential diagnosis).
- Requires a high -quality fluorescence microscope with multiple filters, a charge -coupled device camera that can detect low -level light emission, and sophisticated imaging software.
- Less sensitive than quantitative procedures, such as real -time polymerase chain reaction (RT -PCR) for detection of minimal residual disease (1:100 versus 1:100,000).

Patients, Materials and Methods

Patients

This cross-sectional study was conducted on thirty adult with newly diagnosed and denovo CLL patients tested for the expression of 13q deletion using Fluorescence In situ Hybridization. This was conducted from March 2018 to July 2018 and the diagnosis was documented on the morphology and immunophenotyping of the peripheral blood sample using a four-color flow cytometer in the Nursing Home Hospital / flow cytometry department of the Medical City in Baghdad. The lab work was done in two steps the first step was done in the FISH unit of the Nursing Home Hospital in the Medical City and the second step was done in the postgraduate pathology department in AL-Nahrain Collage of Medicine for detecting the result by using FISH technique. From each patient a verbal consent was acquired for accepting to take the peripheral blood samples. For each patient a questionnaire form was arranged, which was shown in the appendix1 including: name, age, sex, the main symptoms and physical signs especially the presence of lymphadenopathy, splenomegaly, hepatomegaly and B symptoms including (fever, weight loss and night sweating).

Inclusion criteria

- All patients were diagnosed as typical CLL depending on the morphology of their peripheral blood sample and confirmed by flowcytometry criteria which should be 4-5 score ⁽⁴⁵⁾.
- Patients were newly diagnosed and denovo CLL and did not receive any medication before the time of collecting blood samples.
- Patients were randomly selected regarding age and sex, duration and the stage of the disease ⁽⁷⁹⁾.

Clinical and laboratory information regarding age, sex, CBC, percentage of lymphocyte in peripheral blood were obtained from patients hospital records at diagnosis. Rai and modified Rai staging-system were applied for staging the patients with CLL. Accordingly they were classified into 4 groups: low; Intermediate I and Intermediate II; high risk groups.

Work program

Each patient was assessed at time of admission in the outpatient clinic of the Baghdad Medical City. Cases that were suspected CLL from morphology and physical examination were sent for laboratory investigation to confirm CLL depending on peripheral blood count, morphology and immunophenotyping. Blood samples from CLL patients were collected in a sodium heparinized tubes to evaluate of q13 deletion by FISH technique. The lab work was done in two steps , the first step was to separate lymphocytes. It was done in the FISH unit of the Nursing Home Hospital in the Medical City. The second step was slide preparation and determination of the deletion by FISH technique. It was done in the post graduate lab of the Pathology Department in AL-Nahrain Medical Collage. Clinical and laboratory information regarding age, sex, CBC count, percentage of lymphocyte in peripheral blood were obtained from patients hospital records at diagnosis, and also physical examination regarding hepatosplenomegally, lymphadenopathy and the presence of B symptoms (fever, weight loss, night sweating) were done at time of the taking the blood samples.

Laboratory tests

Blood sampling

From each patient included in this study, a total 3ml of venous blood sample was obtained by venipuncture from anti cubital fossa under aseptic condition into a sodium heparinized tube. The blood samples was kept at room tempreture and was processed within less than one hour in the FISH unit of the Nursing Home Hospital in the Medical City.

Flowcytometric immunophenotyping

After cases that were suspected to be CLL by morphology in the teaching laboratories of the Medical City, the samples were transferred in cool box within 6 hours since obtaining the sample to assess the scoring of CLL. According to the International Workshop on CLL (IWCLL), the diagnosis of CLL is sustained by the following parameters:

- Presence in peripheral blood of more than 5×10^9 monoclonal B lymphocytes persisting for at least 3 months.
- Demonstration of the clonality of the population.
- Characteristic immunophenotype Smlg weak, CD5+, CD19+, CD20 weak, CD23+. Regarding the immunophenotype, FMC7 (a CD20 epitope) and CD79b are usually absent or weakly expressed.

Based on immunophenotypic characteristics and giving one point to each one of the following: CD5+, CD23+, FMC7 weak, Smlg weak and CD79b weak. Matutes and Catovsky showed that in patients with a score of 4-5, the diagnosis is virtually always CLL, while in those cases with a score less than 3, the diagnosis of CLL is extremely unlikely. The typical immunophenotype for CLL as accepted in current guidelines should be revisited because of the availability of markers highly characteristic of CLL, such as CD200.

Table 3.1
The Scoring system for diagnosis of chronic lymphocytic leukemia ⁽⁴⁵⁾

| Antigen | Expression | Score |
|---------|---------------|-------|
| SmlgM | Weak/Mod. | +1 |
| CD5 | Positive | +1 |
| CD23 | Positive | +1 |
| CD79b | Negative/Weak | +1 |
| FMC7 | Negative | +1 |

Material

Instruments and Equipments

In the present study we used the following instruments and equipments in table (3.2)

Table 3.2
Instruments and equipments used in the present study

| Instrument and equipment | Manufacture company | Origin |
|---------------------------|---------------------|---------|
| 1-Cover slip | Marienfeld | Germany |
| 2-Distal water | Local made | Iraq |
| 3-Electric oven | Gallenkamp | England |
| 4-Fluorescent microscope | Meta system | Germany |
| 5-Freezer(-20) | Sony | Japan |
| 6-Glass staining jar | Fisher | USA |
| 7-Gloves | Marienfeld | Germany |
| 8-Graduated cylinder | Marienfeld | Germany |
| 9-Hot plate | Photax | England |
| 10-Humid chamber | Locally made | Iraq |
| 11-Micro centerfugate | Memert | Germany |
| 12-Micro pipet | Gilson | France |
| 13-Microscope with camera | Genex | USA |

| | | |
|-------------------------------|-------------------|---------|
| 14-PH meter | Meret | Germany |
| 15- Centrifuge tube | Proway | China |
| 16-Na heparinized tube | Mareinfeld | Germany |
| 17-Positive charge tube | Fisher scientific | USA |
| 18-Refrigerator | Sony | Japan |
| 19-Sterile tips | Marienfeld | Germany |
| 20-Pasture pipit | Marienfeld | Germany |
| 21-Incubator | Gallenkamp | England |
| 22-slid holder(ten slid each) | Marienfeld | Germany |

Chemical materials used

The chemical materials that used in the presented study as the following in the table (3.2)

Table 3.3
Chemical materials used in the current study

| Material | Manufacture company | Origin |
|--------------------------------------|-----------------------------|---------|
| 1-2xSSC saline sodium citrate buffer | Al basher scientific bureau | India |
| 2-Alcohol100% | BDH | England |
| 3-DAPI/anti fade | Meta system | Germany |
| 4-Glacial acetic acid | Gce | UK |
| 5-Phosphate buffer saline | Locally made | India |
| 6-Potassium chloride | SDFCL | India |
| 7-Methanol | BDH | England |
| 8- Pepsin powder | BDH | England |
| 9-Fix gum (cement) | Meta system | Germany |
| 10- Black box | Local made | Iraq |
| 12-Probe10ml (DELEU/LAMP probe set). | Meta system | Germany |

Solution

Table 3.4
Solutions used in blood preparation and slide preparation

| Solutions | Preparation | Dilution | Stored temperature |
|-------------------------------------|---|----------------------------------|--------------------|
| 1-PBS (phosphate buffer saline) | 40gm NACL+1gm KCL+5.75gm sodium phosphate dibasic dehydrate NA ₂ HPO ₄ +1 gm potassium phosphate mono basic KH ₂ PO ₄ all of them dissolve in 500 ml distal water | 5ml of PBS in 45 distal water | Room temp. |

| | | | |
|-------------------------------------|---|--|-----------|
| 2-Caryos | Glacial acetic acid +methanol | 37.5 glacial acetic acid +12.5 methanol | -4c |
| 3-KCL | Potassium chloride | 2.5gm of potassium chloride dissolve in 500ml distal water | 37c |
| 4-20xSSC (saline sodium citrate) | 3M sodium chloride +300 mM trisodium citrate (adjusted to ph 7.0 wit HCL) | 20ml of 20XSSC in 200 ml of DW | Room temp |
| 5-Pepsine buffer | Pepsine powder and BPS | 0.154mg +200ml BPS | Room temp |
| 6-70% ethanol 6 | Absolute ethanol 100% and distal water | 140 ml absolute ethanol and 60ml distal water | Room temp |
| 7-85% ethanol | Absolute ethanol 100% and distal water | 170 ml absolute ethanol and 30 ml distal water | Room temp |
| 8-100% ethanol | 100% ethanol | 100% ethanol | Room temp |
| 9-0.4 2SSC | 20SSC and distal water | 4ml OF 20XSSC In 100 ml distal water | Room temp |

Methods

Fluorescent in situ hybridization technique

Compromise;

- blood preparation
- slide preparation
- slid reading

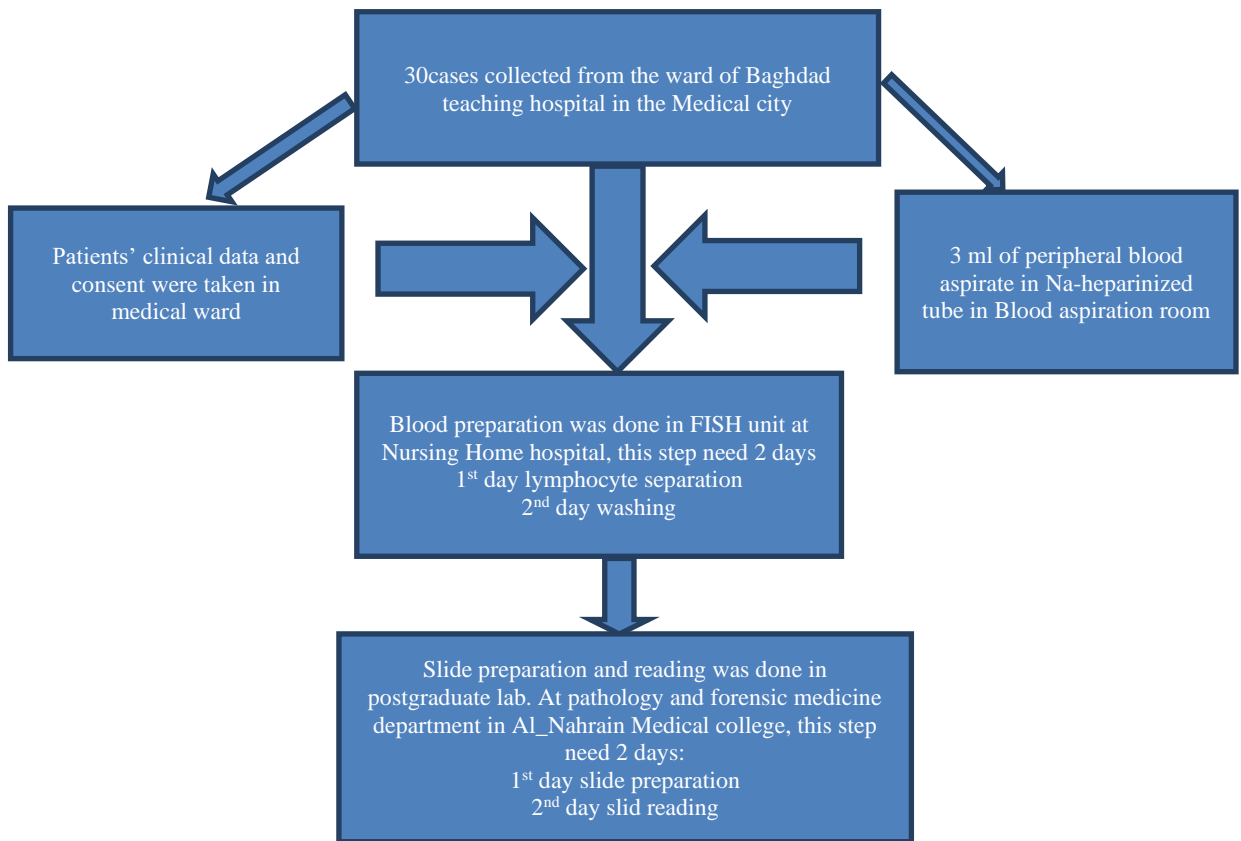


Figure 3.1. work program of 13q deletion in patients with chronic lymphocytic leukemia

Blood preparation

Laboratory precaution

- Wearing a clean lab coat and sterile gloves when handling samples
- Changing a glove whenever contamination was suspected
- Separated area dedicated equipment and supplies were maintenance for samples preparation and slide preparation
- Cleaning lab benches and equipment period toy with freshly diluted 10% chlorine bleaching solution

Procedure

Preparation of uncultured blood:

- 750 microliters of blood added by pasture pipet to 5ml of PBS in centrifugate tube mixing well by pasture pipet
- Solution stored at 4°C in refrigerator for 1 hours

- Solution centrifugated at room temperature for 10 min at 1000 Rpm and discard the supernates by sucking it off carefully with pasture pipet (1 ml of supernatant was left in the tube to avoid loss of material).
- For hypotonic treatment, the pellet was resuspended in 5ml of KCL (37°C) and incubated at 37 °C for 20 min.
- One ml of caryos (fixative material) slowly added to tube, mix carefully and leave it in refrigerator 4°C for 30 min .
- Solution centrifugated in (10 min and 1000RPM), discard the supernatant, five ml of caryos added to solution and stored in refrigerator at (4°C) overnight.
- Second day start with washing to remove all RBC so centrifugate the solution, discard the supernatant and added 5ml of caryos .
- Step seven repeated three time, then five ml of caryos added to solution and stored in refrigerator (4°C) until slide preparation start.

Slide preparation

Procedure

- The tube ,that was stored in refrigerator centrifugated at 1000 rpm for eight minute to concentrate the pellate .
- The supernatant removed by pasture pipet, spot cell taked from pellet by micro pipet, put it on positive charge slide, air drying ,check cell distribution under light microscope and try to avoid crowding cell .
- 10 slides together puted in slide holder, incubated in
- 2SSC jar for 5min
 - In pepsin buffer jar for 10min
 - In BPS jar for 5min
 - 75% alcohol for 2 min
 - 85% alcohol for 2 min
 - 100% alcohol for 4min
 - Then air drying for 5min
- Probe centrifugated for concentration and mixing with vertex, 10 ml of probe added to each slid, cover with coverslip (22*22mm²)
- Denaturation
Slides putted in hot plate (72°C) for 10 min .
- Hybridization
Slides incubated in hummed chamber at 37°C for over night .
- Post hybridization wash; involve
 - Cover slip and all glue material removed carefully
 - Slides washed in 0.4SSC (ph 7.0) at (72°C) for 2 min
 - Slides drained and washed in 2SSC at room temp.
 - Slides rinsed in distal water to avoid crystal formation and let air drying.
- Counter stain involve;
 - Apply ten ml of DAPI/Anti fade and overlay with coverslip.
 - Allow penetration for 10 min.

Slide reading

The specimen were studied in rundown order by meta system fluorescents microscope. For purposes of this paper ,orange signals are referred to as O ,green signals are referred to G. For each specimen, each microscopic scored 100 consecutive qualifying interphase nuclei from different area of the same slide .samples were considered deleted positive 4 cell showed the presence of deletion nuclei in which probe was fused. An abnormal cell with the deletion would show:

- One green (1G) signal and one orange (O) signal.
- Two green (2G) signals and one orange (1O) signal.
- Two orange (2O) signals and one green (1G) signal.

Types of FISH probes routinely used in hematological disorders.

There are primarily three types of probes used in clinical FISH testing

- Centromere -enumeration probes (CEP),
- Locus specific identifier probes (LSI),
- Whole -chromosome paint (WCP) probes as show in figure (2-5)

The CEP probes, labeled in one color, recognize a highly repetitive alpha -satellite DNA sequence located at the centromere of each chromosome. These probes give a large, bright signal and are useful for chromosome enumeration in both interphase and metaphase cells. The LSI probes hybridize to single -copy DNA sequences in a specific chromosomal region or gene. These probes are used to identify fusion gene products generated from a reciprocal translocation, chromosome inversion, and gene deletion or amplification. On metaphase cells, the LSI probes give two small, discrete signals per chromosome. The gain of LSI signals within a nucleus is consistent with duplications or amplifications, while loss of LSI signal indicates a deletion. The design of LSI probes that target specific translocations has evolved considerably, minimizing the false -positive and false -negative rates. Dual color, dual -fusion (DCDF) LSI probes are designed to span both sides of the breakpoints in two different chromosome regions/genes involved in a reciprocal translocation, resulting in a remarkable improvement of the specificity. To assess the rearrangement of a gene that may be associated with multiple translocation partners, a dual -color break -apart (DCBA) LSI probe has been designed. The DCBA probe is a combination of two differently labeled probes that bind to sequences that flank the 5 ' and 3 ' ends of the breakpoint within the involved chromosome region. The separation of the two colors is indicative of rearrangement.

WCP probes are cocktails of unique sequence DNA probes derived from flowsorted chromosomes, chromosome specific libraries, or chromosome micro dissected regions that recognize specific sequences that span the length of a chromosome. In normal metaphase preparations, this gives the effect that both chromosome homologues are painted WCP probes are useful to identify marker chromosomes and to detect cryptic translocations; however, their utility in interphase nuclei is limited as chromosomes are widely spread out during interphase and WCP probes yield only diffuse staining. A variety of FISH probes have become commercially

available to detect various chromosome abnormalities, permitting widespread clinical application of this technology to genetical characterize leukemias and lymphomas .

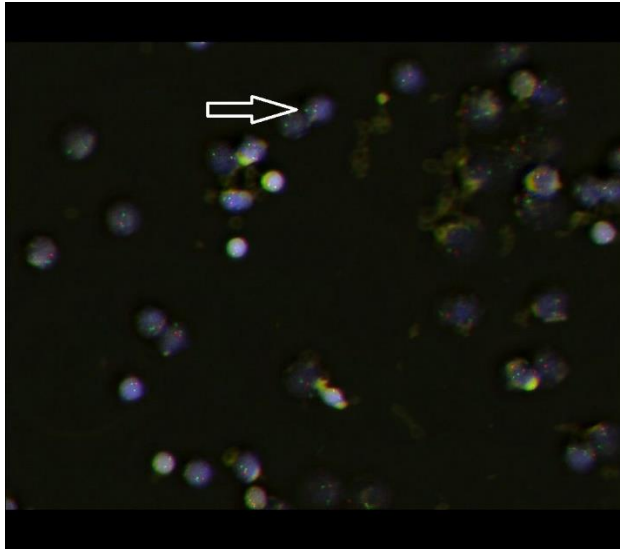


Figure 3.1. leukemic cells with deletions, 2 green signals and one orange signal.

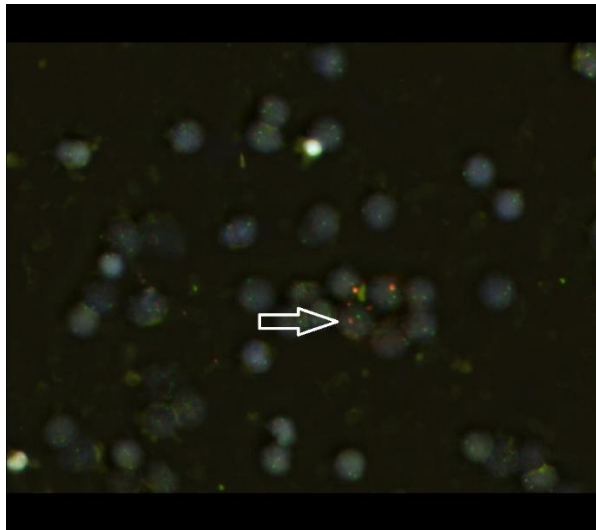


Figure 3.2. leukemic cells with deletions, 2 orange signals and one green signal

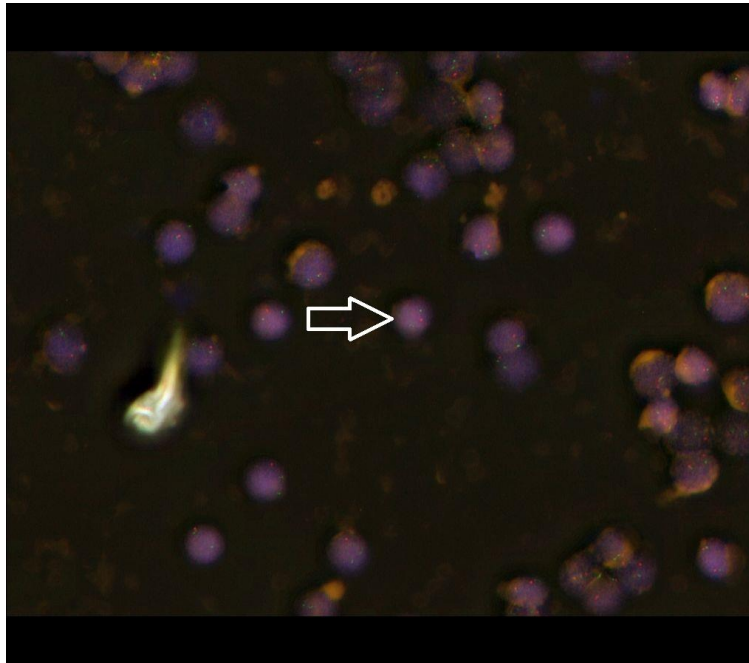


Figure 3.3. leukemic cell with deletion, one green signal and one orange signal

Fluorescence Microscope

Fluorescence microscope was used to visualize the FISH probe signals.

Image Capturing

The analysis was performed using the metasystems automated image acquisition and analysis system (Altlusheim, Germany), area involved by leukemia through the nuclear DAPI appearance. Program used for image capturing integrated spectrographic innovative software (ISIS) version 5.5 which uses a set of premade experiment types i.e appropriate setting for different FISH probe fluorescent combination

Capture Fluorescent Image

In this study, we used the experiment type which use Blue, Green, Aqal /DEAC, images are captured with a monochrome charge coupled device (CCD) camera (Metasystems), the images are taken according to the following steps:

- Separate image was captured for each fluorochrome through a specific
- fluorescent filter set (dapi, fitc, taksaa red, trits).
- All images have been captured in the different color channel.
- They were superimposing to generate the full gene image.
- Reach integrated time was adjusted manually using tools found on capture window in the program.

Sources of autofluorescence

- Red blood cell
- Protein
- Contamination

Statistical analysis

The statistical analysis of this cross sectional study performed with the statistical package for social sciences (SPSS) 21.0 and Microsoft Excel 2013 programs. Numerical data were described as mean and standard error. Analysis of variance (ANOVA) was used for comparison among three groups. While, categorical data described as count, percentages, Chi-square test used to estimate the association between variables. For the tables with frequencies, range, mean & standard deviation values were considered statistically significant difference when p -value < 0.05 .

Results

Demographic characteristics

The statistical analysis in the current study was based on enrollment of 30 patients with CLL. The mean age of patients was 63.87 ± 8.01 years and the age has ranged from 46 to 75 years. Patients 70 years or younger have accounted to 25 out of 30 (83.3 %) and they have been categorized as following: 4 (13.3%) within the age interval 46-50, 0 (0.0%) within age interval 51-55, 3 (10.0%) within the age interval 56-60, 9 (30.0%) within the age interval 61-65 and 9 (30.0%) within the age interval 66-70, whereas, patients older than 70 years have accounted to 5 out of 30 (16.7 %), table 4.1; thus, majority of patients with CLL (60.0 %) were between 60 and 70 years, figure 4.1. According to gender the study included 20 males and 10 females, accounted for 66.7 % and 33.3 %, respectively; the male: female ratio was 2:1, table 4.1.

Table 4.1
Demographic characteristics

| Characteristic | Value | |
|----------------|-----------------|------------------|
| Age (years) | Mean \pm SD | 63.87 \pm 8.01 |
| | Range | 46-75 |
| | 46-50 | 4 (13.3%) |
| | 51-55 | 0 (0.0%) |
| | 56-60 | 3 (10.0%) |
| | 61-65 | 9 (30.0%) |
| | 66-70 | 9 (30.0%) |
| | > 70 | 5 (16.7%) |
| Gender | Male, n (%) | 20 (66.7%) |
| | Female, n (%) | 10 (33.3%) |

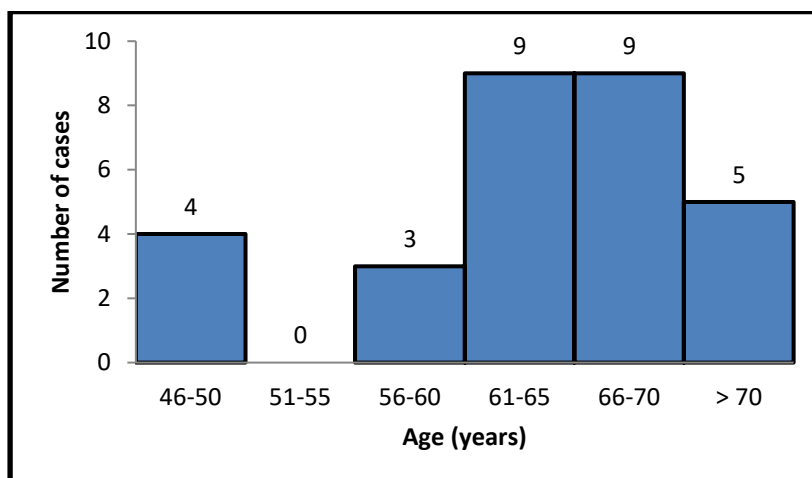


Figure 4.1. Histogram showing the frequency distribution of CLL patients according to 5 years age interval

Distribution of CLL patients according to clinical signs

Patients with CLL were categorized according to physical findings into those with Lymphadenopathy, those with splenomegaly and those with hepatomegaly, 16 (53.3 %), 9 (30 %) and 5 (16.7 %), respectively, as shown in table 4.2.

Table 4.2
Distribution of CLL patients according to clinical signs

| Clinical Sign | <i>n</i> | % |
|-----------------|----------|------|
| Lymphadenopathy | 16 | 53.3 |
| Splenomegaly | 9 | 30 |
| Hepatomegaly | 5 | 16.7 |

Distribution of CLL patients according to hematological parameters

The level of hemoglobin in CLL patients has been in the range of 6 to 15 g/dl and the mean was 10.80 ± 2.04 g/dl. Anemia ($Hb < 10$ g/dl) has been reported in 17 patients (56.7 %). The leukocyte count has been in the range of 9000 - 30000 and the mean was 19783.00 ± 7583.65 . The mean platelet count has been $166.93 \pm 82.26 \times 10^9$ and the range has been from 40 - 330×10^9 ; patients with thrombocytopenia have accounted for 5 (16.7 %). The absolute lymphocyte count has been in the range of $8 - 185 \times 10^9/L$ and the mean has been 69.34 ± 44.11 ; patients with absolute lymphocyte count $> 50 \times 10^9/L$ have accounted for 18 (60.0 %), table 4.3.

Table 4.3
Hematological characteristics of patients with CLL

| Characteristic | Statistic | Value |
|---|---|------------------|
| Hb g/dl | Mean \pm SD | 10.80 \pm 2.04 |
| | Range | 6 - 15 |
| | Anemia (Hb <11), <i>n</i> (%) | 17 (56.7 %) |
| WBC X10 ⁹ /L | Median (IQR) | 22500 (9250) |
| | Range | 8000 -30,000 |
| PLT X10 ⁹ /L | Median (IQR) | 148.5 (103.0) |
| | Range | 40 -330 |
| | Thrombocytopenia (platelet < 100), <i>n</i> (%) | 5 (16.7 %) |
| Absolute lymphocyte count X10 ⁹ /L | Median (IQR) | 62.30 (47.35) |
| | Range | 8 -185 |
| | ALC > 50, <i>n</i> (%) | 18 (60.0 %) |

HB: Hemoglobin; WBC: white blood cells; PLT: platelet; SD: standard deviation; IQR: inter-quartile range ; ALC: absolute lymphocyte count

Categorization of CLL patients based on Rai and modified Rai staging system

Stage I disease has been identified in 10 (33.3 %), stage II disease has been seen in 3 (10.0 %), stage III disease has been reported in 12 (40.0 %) and stage IV disease has been observed in 5 (16.7 %), table 4.4. Therefore, intermediate risk (stages I and II) has been seen in 13 (43.3 %), while high risk (stages III and IV) has been identified in 17 (56.7 %), table 4.4.

Table 4.4
Rai staging of CLL patients

| Stage | Risk | <i>n</i> | % |
|--------|--------------|----------|------|
| 0 | Low | 0 | 0 |
| I &II | Intermediate | 13 | 43.3 |
| III&IV | High | 17 | 56.7 |

13q14 deletion in CLL patients

According to XL DLEU/LAMP (13q14 deletion), patients were classified in to those with positive marker expression and those with negative marker expression, 7 (23.3 %) and 23 (76.6 %), respectively, as shown in figure 4.2.

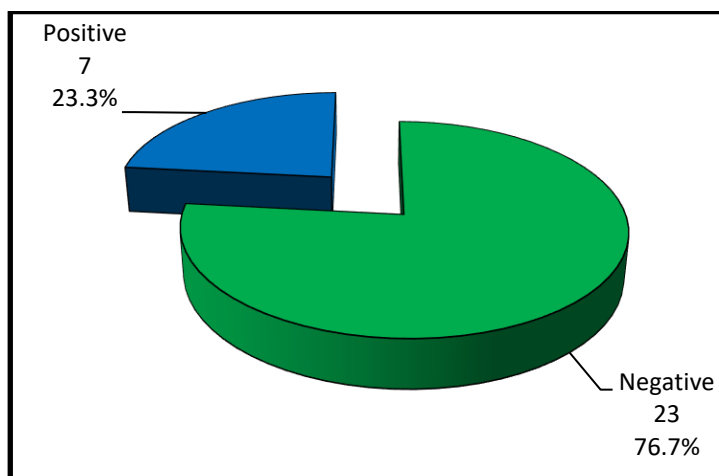


Figure 4.2. Pie chart showing XL DLEU/LAMP (13q14 deletion) in CLL patients

Association between age and gender of patients with CLL and 13q14 deletion

There was no significant difference in mean age between CLL patients with positive 13q14 deletion and those with no deletion, 61.57 \pm 6.37 years versus 64.57 \pm 8.45 years ($P = 0.396$). Moreover, when CLL patients were categorized into those ≤ 70 years and those > 70 years, there was no significant difference in proportion of patients with positive 13q14 deletion and those with no deletion ($P = 0.304$), as shown in table 4.5. There was also no significant association between gender and 13q14 deletion in patients with CLL as shown in table 4.1.

Table 4.5
Association between age of patients with CLL and 13q14 deletion

| Characteristic | | 13q14 deletion | | P |
|----------------|--------------------------|---------------------|----------------------|---------------|
| | | Positive $n = 7$ | Negative $n = 23$ | |
| Age (years) | Mean \pm SD | 61.57 \pm 6.37 | 64.57 \pm 8.45 | 0.396 † NS |
| | ≤ 70 years, n (%) | 0 (0.0 %) | 5 (21.7 %) | 0.304 ¥ NS |
| | > 70 years, n (%) | 7 (100.0 %) | 18 (78.3 %) | |
| Gender | Male, n (%) | 5 (71.4 %) | 15 (65.2 %) | 1.000 ¥ NS |
| | Female, n (%) | 2 (28.6 %) | 8 (34.8 %) | |

n : number of cases; SD: standard deviation; †: Mann Whitney U test; ¥: Fischer exact test; NS: not significant at $P \leq 0.05$

Association between clinical signs and 13q14 deletion in patients with CLL

When CLL patients with splenomegaly were contrasted against CLL patients with Lymphadenopathy, there was no significant difference in proportion of patients with positive 13q14 deletion and those with no deletion ($P = 1.000$); in addition, when CLL patients with hepatomegaly were contrasted against CLL patients with Lymphadenopathy, there was no significant difference in proportion of patients

with positive 13q14 deletion and those with no deletion ($P = 1.000$), as shown in table 4.6.

Table 4.6
Association between clinical signs and 13q14 deletion in patients with CLL

| Sign | Total $n = 30$ | 13q14 deletion | | | | P ¥ |
|--------------|-------------------|---------------------|------|----------------------|------|-------------|
| | | Positive $n = 7$ | | Negative $n = 23$ | | |
| | | n | % | n | % | |
| LAP | 16 | 4 | 25.0 | 12 | 75.0 | Reference |
| Splenomegaly | 9 | 2 | 22.2 | 7 | 77.8 | 1.000 NS |
| Hepatomegaly | 5 | 1 | 20.0 | 4 | 80.0 | 1.000 NS |

n : number of cases; LAP: Lymphadenopathy; ¥: Yates correction; NS: not significant at $P \leq 0.05$

Association between anemia and 13q14 deletion in CLL patients

There was highly significant difference in mean Hb between CLL patients with positive 13q14 deletion and those with no deletion ($P = 0.001$), being higher in patients with positive 13q14 deletion than those without deletion, 12.96 ± 1.41 g/dl versus 10.14 ± 1.74 g/dl, respectively. In addition, all CLL patients with positive 13q14 deletion were free of anemia, whereas anemia (Hb < 11 g/dl) was seen in 17 (73.9 %) of patients with no deletion; the variation in 13q14 deletion according to anemia was highly significant ($P = 0.001$), as shown in table 4.7.

Table 4.7
Association between anemia and 13q14 deletion in CLL patients

| Hb | Positive $n = 7$ | Negative $n = 23$ | P |
|---------------------------|---------------------|----------------------|---------------|
| Mean \pm SD | 12.96 ± 1.41 | 10.14 ± 1.74 | 0.001 † HS |
| Anemia (Hb < 11), n (%) | 0 (0.0%) | 17 (73.9 %) | 0.001 ¥ HS |
| No anemia, n (%) | 7 (100.0 %) | 6 (26.1 %) | |

n : number of cases; SD: standard deviation; †: Mann Whitney U test; ¥: Fischer exact test; HS: highly significant at $P \leq 0.01$

Association between 13q14 deletion and total WBC and absolute lymphocyte counts in CLL patients

There was no significant difference in mean total WBC count between CLL patients with positive 13q14 deletion and those with no deletion, 24000 (6000) versus 21000 (10000) ($P = 0.597$). In addition, there was no significant difference in mean absolute lymphocyte count between CLL patients with positive 13q14 deletion and those with no deletion, 40.5 (64.9) versus 63.8 (42.6) ($P = 0.898$).

Moreover, when CLL patients were categorized into those with absolute lymphocyte count ≤ 50 and those with absolute lymphocyte count > 50 , there was no significant difference in proportion of patients with positive 13q14 deletion and those with no deletion ($P = 0.392$), as shown in table 4.8.

Table 4.8
Association between 13q14 deletion and total WBC and absolute lymphocyte counts in CLL patients

| Characteristic | | 13q14 deletion | | P |
|----------------------------|--------------------------|--------------------------|---------------------------|---------------|
| | | Positive <i>n</i> = 7 | Negative <i>n</i> = 23 | |
| WBC | Median (IQR) | 24000 (6000) | 21000 (10000) | 0.597 † NS |
| | Median (IQR) | 40.5 (64.9) | 63.8 (42.6) | 0.898 † NS |
| Absolute lymphocyte counts | ≤ 50 , <i>n</i> (%) | 4 (57.1 %) | 8 (34.8 %) | 0.392 ¥ NS |
| | > 50 , <i>n</i> (%) | 3 (42.9 %) | 15 (65.2 %) | |

n: number of cases; IQR: inter quartile range; †: Mann Whitney U test; ¥: Fischer exact test; NS: not significant at $P \leq 0.05$

Association between 13q14 deletion and platelet count in CLL patients

There was no significant difference in mean platelet count between CLL patients with positive 13q14 deletion and those with no deletion, 150.0 (146.0) versus 147.0 (100.0) ($P = 0.734$). Moreover, when CLL patients were categorized into those with thrombocytopenia (platelet < 100) and those without thrombocytopenia (platelet count ≥ 100), there was no significant difference in proportion of patients with positive 13q14 deletion and those with no deletion ($P = 1.000$), as shown in table 4.9.

Table 4.9
Association between 13q14 deletion and platelet counts in CLL patients

| Platelet count X1000/cc | 13q14 deletion | | P |
|--------------------------|--------------------------|---------------------------|---------------|
| | Positive <i>n</i> = 7 | Negative <i>n</i> = 23 | |
| Mean \pm SD | 150.0 (146.0) | 147.0 (100.0) | 0.734 † NS |
| Thrombocytopenia < 100 | 1 (14.3 %) | 4 (17.4 %) | 1.000 ¥ NS |
| ≥ 100 | 6 (85.7 %) | 19 (82.6 %) | |

n: number of cases; IQR: inter quartile range; †: Mann Whitney U test; ¥: Yates correction; NS: not significant at $P \leq 0.05$

Association between 13q14 deletion and Rai staging in CLL patients

The positive 13q14 deletion was significantly higher in patients with intermediate stage than patients with high stage, 46.2 % versus 5.9 %, respectively ($P = 0.032$),

table 4.10. On the other hand, positive 13q14 deletion according to stage of disease was as following: stage I (40.0 %), stage II (66.7 %), stage III 0 (0.0 %) and stage IV (20.0 %), that is, the lower the stage, the more likely is 13q14 deletion, table 4.10; however, Chi-square was not valid because more than 20 % of cells had expected count less than 5; therefore, Spearman correlation test was carried out instead and the results were shown in figure 4.3; in which the correlation was negative ($r = 0.332$) and the level of significance was border line ($P = 0.073$) which is very close to 0.05.

Table 4.10
Association between 13q14 deletion and Rai staging in CLL patients

| Characteristic | | Total $n = 30$ | 13q14 deletion | | P |
|----------------|--------------|-------------------|---------------------|----------------------|---------------|
| | | | Positive $n = 7$ | Negative $n = 23$ | |
| Risk | Intermediate | 13 | 6 (46.2 %) | 7 (30.4 %) | 0.032 ¥ |
| | High | 17 | 1 (5.9 %) | 16 (69.6 %) | NS |
| Stage | I | 10 | 4 (40.0 %) | 6 (26.1 %) | 0.039 £ NV |
| | II | 3 | 2 (66.7 %) | 1 (4.3 %) | |
| | III | 12 | 0 (0.0 %) | 12 (52.2 %) | |
| | IV | 5 | 1 (20.0 %) | 4 (17.4 %) | |

n : number of cases; †: Mann Whitney U test; ¥: Yates correction; £: Chi-square test; NS: not significant at $P \leq 0.05$; NV: Not valid since more than 20 % of cells have expected count < 5

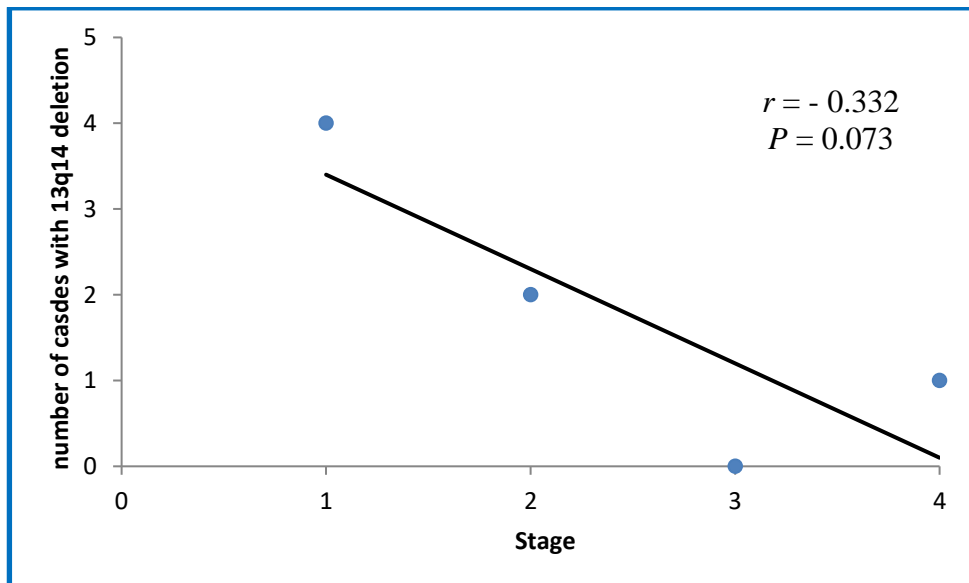


Figure 4.3. Correlation between Rai stage and marker expression

Discussion

Demographic characteristics

In the present study, the mean age of CLL patients was 63.87 ± 8.01 years and the age has ranged from 46 to 75 years; majority of cases has been between 60 and 70 years. It is well known that this disorder is a disease of elderly. Many studies have stated that the median age at time of diagnosis was in the range of 65 to 70 years (85-88); however, some authors have reported a median age of 72 (89). Moreover, in the current study, only 13.3% were younger than 55 years of age; this is in accordance with the National Cancer Institute's Surveillance and Epidemiology End Results (SEER) database, which revealed that 11% of all patients diagnosed with CLL in 2009 were less than 55 years of age (90). In a recent Iraqi study (91), it has been found that the median age of patients with CLL was 65 years which is approximately similar to that of the present study. Moreover, Iraqi cancer registry has postulated that the leukemia rank the third of all hematopoietic malignant diseases in Iraq according to the Iraqi cancer registry 2015; it has an average incidence 4.08 cases per 100,000, while the incidence of CLL ranges from <1 to 5.5 per 100,000 people in worldwide (10). In Iraq, because of its relative indolence, this disease accounts for approximately 1.84% of all cancers and nearly 43.78% of all leukemia (11).

In 2015, The American Cancer Society's assaying for leukemia in the United States are About 20,110 of new cases CLL and about 4,660 deaths from Chronic lymphocytic leukemia (12). It is the most predominant adult leukemia in Western countries, but it is comparatively rare in Asia (13). The developing of CLL increased incrementally with the median age that being in 72 year. CLL is very rare below the age of thirty years, but 20–30 percent of cases present under the age of 50 years. The incidence of CLL cases is more common in whites than blacks and in males than in females; with a male to female ratio of 1.7:1 (8, 14). In the this study, two thirds of patients were men and one third were women with a male to female ration of 2:1, indicating that the disease in our community is more common in men than in women. Indeed, this result is similar to the trend that appeared in most published articles which have pointed to the fact that the disease is more common in men than in women with a ratio of 2:1 (92-94). In a recent Iraqi study, the male to female ratio was 2:1 which is in agreement with the finding of the current study (91).

Distribution of CLL patients according to clinical signs

In the current study lymphadenopathy has been identified in 53.3 % of cases, splenomegaly has been reported in 30 % and hepatomegaly has been observed in 16.7 %. It has been stated that lymphadenopathy may be observed in approximately 80% of cases often with cervical and axillary lymph nodes bilaterally and symmetrically being affected. Splenomegaly is usually mild to moderate and is observed in approximately 50% of cases; hepatomegaly is less frequent (95). In an Indian study, Lymphadenopathy, splenomegaly and hepatomegaly were seen in 52 (55%), 63 (66%) and 60 (63%) patients, respectively (96). In a review article about CLL, it has been mentioned that the most common physical findings with CLL include lymphadenopathy (87%), splenomegaly (54%),

and hepatomegaly (14%)⁽⁹⁷⁾. Despite the presence of difference in the percentages of lymphadenopathy, splenomegaly and hepatomegaly between the present study and some previous reports, a common denominator existed that the most common finding is lymphadenopathy followed by splenomegaly and then hepatomegaly⁽⁹⁷⁾.

Distribution of CLL patients according to hematological parameters

In the current study, the level of hemoglobin in CLL patients was in the range of 6 to 15 g/dl and the mean was 10.80 ± 2.04 g/dl. In one study, the mean baseline hemoglobin level in patients with CLL was 9.8 g/dl and the range was from 7.2 to 11 g/dl⁽⁹⁸⁾. In another study, the mean hemoglobin level of CLL patients was 9.5 g/dl and the range was from 3.9 to 15.2 g/dl⁽⁹⁹⁾. In a further study, mean hemoglobin level at baseline was 10.4 g/dl⁽¹⁰⁰⁾. Therefore, the level of hemoglobin in patients with CLL, enrolled in the current study, is comparable to that reported by previous studies. In addition, anemia, defined as Hb < 10 g/dl, has been identified in 56.7 % of CLL cases participating in this study. In one study, anemia was seen in 26 % of cases⁽⁹⁶⁾; a figure that is lower than that reported in the current study. In general, anemia may be observed in 15 to 30% of patients with CLL and it often results from bone marrow infiltration, even though it can also be attributed to an autoimmune phenomenon⁽⁹⁵⁾.

The identification of anemia is important clinically since prognosis in CLL patients is partly determine by the existence and severity of anemia, therefore, anemia is one of the clinical parameters that have been incorporated within the two widely used clinical staging system for CLL, namely Rai and Binet⁽⁹⁵⁾. In the current study, the mean platelet count was $166.93 \pm 82.26 \times 10^9/L$ and the range has been from 40 -330 $\times 10^9/L$; patients with thrombocytopenia have accounted for 5 out of 30 patients (16.7 %). Those results were comparable to many studies^(95, 101, 102). In one study, thrombocytopenia was identified in 18 % of patients with CLL⁽⁹⁶⁾, a figure that is approximately similar to that of the present study; however, it is lower than that described by Hasan *et al.* who stated that thrombocytopenia was identified in 60 % of cases⁽⁹¹⁾. Thrombocytopenia was usually attributed to bone marrow failure.

In this study, the leukocyte count was in the range of 9000 -30000 $\times 10^9/L$ and the mean was 19783.00 ± 7583.65 . According to several authors, high leukocyte count is associated with poor prognosis^(101, 103). The absolute lymphocyte count was in the range of 8 -185 $\times 10^9/L$ and the mean has been 69.34 ± 44.11 ; patients with absolute lymphocyte count > 50 have accounted for 18 (60.0 %). In one study, the mean lymphocyte count in CLL patients was $186.68 \times 10^9/L$ and it ranged from 5.03 to 869 $\times 10^9/L$ ⁽⁹⁹⁾. Absolute lymphocyte count has been used as a prognostic factor and high lymphocyte count of more than 50,000/L has been linked to poor prognosis^(104, 105). Therefore, identification of patients with high lymphocyte count may aid in better disease staging and hence strict prognostic directed treatment options.

Categorization of CLL patients based on Rai staging

In the current study, stage I disease has been identified in 10 (33.3 %), stage II disease has been seen in 3 (10.0 %), stage III disease has been reported in 12 (40.0 %) and stage IV disease has been observed in 5 (16.7 %), whereas no patients was identify in stage 0. Therefore, intermediate risk (stages I and II) has been seen in 13 (43.3 %), while high risk (stages III and IV) has been identified in 17 (56.7 %). This fact reveals that more than halve of patients enrolled in the current study have advanced stage disease.

XL DLEU/LAMP marker expression in CLL patients

The XL DLEU/LAMP probe detects deletions on chromosome 13q. The orange labeled probe hybridizes to the DLEU locus region at 13q14.2, including D13S319 and the green probe hybridizes to the LAMP locus at 13q34⁽¹⁰⁶⁾. In the present study, chromosome 13q deletion was identified in 7 (23.3 %) of patients, whereas 23 (76.6 %) CLL cases have no deletion. It has been found that deletion in the 13q14 region was detected in more than half of CLL patients, being the most common chromosomal abnormality in CLL^(107, 108). The prognosis and clinical course of CLL are heterogeneous. Conventional banding techniques in CLL are hampered by the low mitotic index of the neoplastic cells. The introduction of interphase cytogenetics using fluorescent *in situ* hybridization (FISH) has greatly increased the sensitivity of cytogenetic analyses. The most frequently deleted region in B-CLL is located in 13q14.3 distal to RB1.

Association between age of patients with CLL and marker expression

In the current study, there was no significant difference in mean age and gender between CLL patients with chromosome 13q14 deletion and those without such deletion ($P = 0.396$). Some authors have linked survival to age at diagnosis and have found that prognosis is better in younger patients^(109, 110); however, we failed to find an association between age and chromosome 13q14 deletion following thorough search in available published articles dealing with CLL⁽¹¹¹⁻¹¹³⁾.

Association between clinical signs and marker expression in patients with CLL

In the current study there was no significant association between chromosome 13q14 deletion and hepatomegaly or splenomegaly, however splenomegaly and hepatomegaly were detected more frequently in negative patients. Knowing that the presence of splenomegaly and hepatomegaly indicates progression of the disease toward more advancing stage and is correlated with less favorable prognosis. Most of studies describing the prognostic value of chromosome 13q14 deletion in CLL have linked this type of deletion to survival rate rather than to other prognostic factors such as splenomegaly or hepatomegaly⁽¹¹¹⁻¹¹³⁾.

Association between anemia and marker expression in CLL patients

In the current study, there was highly significant difference in mean Hb concentration between CLL patients with chromosome 13q14 deletion and those

without deletion ($P = 0.001$), being lower in patients without deletions. In addition, all CLL patients with chromosome 13q14 deletion were free of anemia, whereas anemia ($Hb < 11 \text{ g/dl}$) was seen in 17 (73.9 %) of patients without with chromosome 13q14 deletion. Anemia is a poor prognostic factor and therefore, indirectly, one can conclude that chromosome 13q14 deletion in CLL is associated with favorable prognosis. Several studies have pointed to similar results ⁽¹¹¹⁻¹¹³⁾.

Association between marker expression and total WBC and absolute lymphocyte counts in CLL patients

In the current study, there was no significant difference in mean total WBC count and mean absolute lymphocyte count in correlation to chromosome 13q14 deletion. High leukocyte count and high absolute lymphocyte counts have been linked to less favorable prognosis by some authors ^(101, 103-105); however, for the best of our knowledge none of the authors dealing with chromosome 13q14 deletion in CLL have linked such deletion to leukocyte or lymphocyte counts ⁽¹¹¹⁻¹¹³⁾.

Association between marker expression and platelet count in CLL patients

In the present study, there was no significant difference in mean platelet count between CLL patients with 13q14 chromosomal deletion and those without, further more there was no significant association between thrombocytopenia and 13q14 chromosomal deletion. The lack of association between 13q14 chromosomal deletion and thrombocytopenia may indicate that such deletion is an independent prognostic factor since a number of authors have shown favorable survival rate in association with 13q14 chromosomal deletion ⁽¹¹⁴⁻¹¹⁷⁾.

Association between marker expression and Rai grade in CLL patients

The current study revealed that CLL patients with chromosome 13q.14 deletion have better Rai staging than those without so that 16 out of 17 patients in the high risk had no deletion. Therefore, indirectly, one can suppose that chromosome 13q.14 deletion carry good prognosis in CLL patients; however, it appears better to link such deletion to survival rate in order to get better idea about its prognostic significance.

Conclusion

- The frequency of 13q deletion gene in sample of Iraqi chronic lymphocytic leukemia patient , was 23% which is the highest reported frequencies among studies which were established.
- The frequency of 13q deletion gene in male patients more than female patients.
- The presence of 13q deletion gene was independent risk factor with hematological parameters including HB,WBC,Platelet and absolute lymphocyte count.
- The frequency of 13q deletion was much less in high risk patients of the modified Rai staging system.

Recommendation

- Implementation of FISH as a part of routine work-up for chronic lymphocytic leukemia patients . Its relative low cost makes it possible to be acquired by clinical laboratories with limited resources and thus improve the ability to accurately diagnosis.
- Further studies with larger numbers of patients are recommended to confirm our results with further cytogenetic abnormalities for CLL patients.

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