Introduction

Acute lymphoblastic leukemia (ALL) is a clonal malignant disease of the bone marrow in which early lymphoid precursors proliferate and replace the normal hematopoietic cells of the marrow **(Jiang et al., 2010)**.

Cytogenetic abnormalities are independent prognostic variables for predicting the outcome of adult ALL patients. Recent genomic studies have provided a refined genetic map of acute lymphoblastic leukemia (ALL) and increased the number of potential prognostic markers **(Moorman et al., 2014).**

Adult and childhood ALL differ markedly in the prevalence of various cytogenetic abnormalities. Philadelphia chromosome (Ph^1) positive ALL, a high-risk cytogenetic subset, accounts for 25-30% of adult ALL cases but occurs in less than 5% of children. Similarly ETV6/RUNX1 (TEL-AML1) fusion and hyperdiploidy, both good risk genetic features, together comprise approximately 50% of childhood ALL, but only approximately 10% of adult ALL **(Pullarkat et al., 2008).**

The ph^1, t(9;22)(q34;q11)(BCR/ABL) results from a translocation involving the breakpoint cluster region of the BCR gene on chromosome 22 and the ABL gene on chromosome 9. One/third of adult ALL patients with a Ph^1 show major (M- BCR) rearrangements, resulting in a 210 - kDa protein, similar to patients with chronic myeloid leukaemia (CML), whereas two - thirds have minor (m - BCR rearrangements), resulting in a 190 - kDa protein **(Jiang et al ., 2010).**

For many years, conventional karyotyping has been used as the sole diagnostic tool for t(9;22). However, it has several limitations that may

lead to failure for detecting BCR/ABL gene rearrangements in relatively high proportion of ALL cases **(Secker-Walker, 1990)**. It is noted that the BCR/ABL is more frequently detected than the corresponding chromosome abnormality t(9;22) **(Moorman et al., 2007).**

In both children and adults ALL, t(9;22)(q34;q11)(BCR/ABL) has the worst prognosis among patients with ALL. Its higher frequency in adult ALL explains in part but not completely the relatively poorer outcome of adults with ALL. In children, the presence of favorable clinical features including age, white blood cell count and response to therapy, is associated with somewhat better outcome **(Mancin et al., 2005 & Schultz et al., 2007).**

The use of a new generation of BCR/ABL FISH probes in interphase nuclei of a large series of BCR/ABL+ve leukemias is associated with the observation of a variable number of different interphase FISH patterns. The most frequently detected patterns with the extra-signal (ES-FISH) probe corresponded to typical BCR/ABLgene rearrangements involving the MBCR and the mBCR breakpoints; as expected, distinction between these two breakpoint regions could not be achieved with the single fusion or double fusion D-FISH probe. Although both major and minor patterns were found in CML and ALL cases, the former was more frequently observed in CML while the latter was usually associated with ALL. Interestingly, additional chromosomal abnormalities (eg; supernumerary Ph, gain or loss of chromosomes 9 and 22, as well as deletions of 9q and 22q) can occur in BCR/ABL+ve CML, ALL and AML patients **(Zhonghua et al., 2010).**

Aim of the Work

This work aims to detect BCR-ABL genes fusion, amplification and deletion in acute lymphoblastic leukemia patients, using extra signal fluorescence in situ hybridization (ES-FISH), and to assess their relation with other standard prognostic factors and therapeutic response.

Chapter (I)

Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is a hematologic malignancy produced by impaired differentiation, proliferation and accumulation of lymphoid progenitor cells in the bone marrow and/or extramedullary sites. ***(Paul Shilpa et al., 2016).***

Epidemiology

Incidence:

The ALL is the most common childhood malignancy, accounting more than quarter of all pediatric cancers ***(Satake et al., 2017).*** ALL has a bimodal distribution; the first peak occurring in individuals around 5 years of age and the second peak at around 50 years of age. It is mainly a pediatric leukemia with 80% of cases occurring in children and 20% occurring in adults.***(Paul Shilpa et al., 2016).***

Incidence of ALL varies among different racial groups, White individuals are more frequently affected than black ***(Satake, 2010)***. In adults there is a slight male predominance with a male to female ratio of 1.6:1.1. Geographic differences in the incidence of ALL are reflected by higher rates in North America and Europe and lower rates in African and Asian populations ***(Coutre, 2014).***

Pathogenesis and aetiology:

Epidemiologic studies show a number of possible risk factors (e.g., environmental, genetic, immunological or infectious) in an effort to determine the etiology of the disease ***(Martin et al.,2006).***

Environmental factors:

Exposure to ionizing radiation or electromagnetic fields or parental use of alcohol and tobacco have not been shown to cause childhood ALL ***(Satake et al., 2017)***.

More relevant to adult ALL is the association between occupational exposure to low dose ionizing radiation and increased risk for leukemia. Cigarette smoking was linked to a small increase in risk for ALL among persons older than 60 years old ***(Coutre,2014).***

Infectious agents:

EBV is a potent transforming agent and has been consistently associated with several human malignancies including pediatric lymphomas. HHV6 has been associated with several hematological malignances, including childhood acute leukemia ***(McNally,2014*).** Parvovirus is incorporated into the DNA of the nucleus; where it is replicated by the cell along with host nuclear DNA ***(Harrison and Foroni., 2002).***

The human T lymphotropic virus I and II are retroviruses that are implicated in some cases of adult T-cell leukemia/lymphoma ***(Foon and Fisher, 2001).*** The main feature of retroviruses is that they contain RNA, direct DNA synthesis from RNA template by the enzyme reverse transcriptase after invading the host. This virus can be transmitted from mother to child ***(Yatgin et al., 2001)***.

Pathophysiology:

The development of ALL is believed to involve a transformation that occurs in a single progenitor cell that has the capability for indefinite clonal expansion. The leukemogenic event may occur in committed lymphoid cells of B- or T-cell lineages or in early precursors, which gives

rise to the different subtypes of ALL based on the stage of lymphoid differentiation of the cell in which the event occurred ***(Esparza and Sakamoto, 2005).***

Genetic abnormalities:

Cytogenetic abnormalities occur in approximately 70% of cases of acute lymphoblastic leukemia (ALL) in adults ***(Seiter, 2011).*** In general, hematological malignancies are characterized by recurrent chromosomal aberrations that lead to the formation of gene fusions and the subsequent expression of chimeric proteins with unique properties ***(Brassesco et al., 2011).*** These include chromosomal translocations that create fusion genes encoding active kinases and altered transcription factors, like t(9;22), deletion, inversion and numerical aberrations. These genetic alterations contribute to the leukemic transformation of hematopoietic stem cells or their committed progenitors by changing cellular functions **(Table 1 & 2)** ***(Hanahan and Weinberg, 2000).***

Altered cellular functions include an enhanced ability of self-renewal, a subversion of control of normal proliferation, a block in differentiation, and an increased resistance to death signals or apoptosis **(Moorman et al., 2007).**

I. Chromosomal translocations:

Self-renewal of hematopoietic stem cells can result from chimeric transcription factors, which arise from genetic translocations that fuse portions of two different transcription factors. These chimeric transcription factors activate diverse transcriptional cascades that converge to modify the normal pattern of expression of family genes, ***(Buske and Humphries, 2000; Ferrando and Look, 2003)***.

Table (1): Common cytogenetic abnormalities in children ALL:

Cytogenetic abnormality	**Target gene**	**Frequency in pediatrics %**
t(1;19)(q23;p13)	E2A-PBX1	4-6
t(9;22)(q34;q11)	BCR-ABL1	3-5
t(4;11)(q21;q23)	MLL-AF4	2-3
High hyperdiploid	-	20-30
Hypodiploid	-	5-6
t(12;21)	ETV6-RUNX1	25
T-ALL t(7;14)(14q;7q34 or 7p14)	TCR Non-TCR NOTCH1, HOX11, JAK1)	60

(Asselin et al., 2013)

The HOX transcription factors bind to DNA and regulate genes involved in the differentiation of both the embryo and the hematopoietic stem cell; they are also important in the self-renewal and proliferation of hematopoietic stem cells ***(Pui et al., 2004 b).***

Table (2): Common cytogenetic abnormalities in adults ALL:

Cytogenetic abnormality	**Target gene**	**Frequency in adults %**
t(1;19)(q23;p13)	E2A-PBX1	<5
t(9;22)(q34;q11)	BCR-ABL1	15-25
t(4;11)(q21;q23)	MLL-AF4	5-10
High hyperdiploid	-	2-15
Hypodiploid	-	5-10
t(12;21) (p12;q22)	TEL-AML1	<1
del(9)(q21-22)	p15, p16	6-30
t(8;14), t(8;22)	C-MYC	5
del(11)(q22-23)	ATM	25-30
t(17;19)	E2A- HLF	<5

(Paul Shilpa et al., 2016).

t(9;22)(q34;q11) BCR/ABL1:

Philadelphia chromosome (ph[1)] results from a reciprocal translocation that fuses the abelson proto-oncogene1 (ABL1) from chromosome 9 to the breakpoint cluster region (BCR) on chromosome 22. The unique biology of Ph[1] positive ALL is attributable to the constitutive expression of oncoprotein BCR/ABL1 with tyrosine kinase activity (**Figure1**) ***(Bachanova.,2017)***.

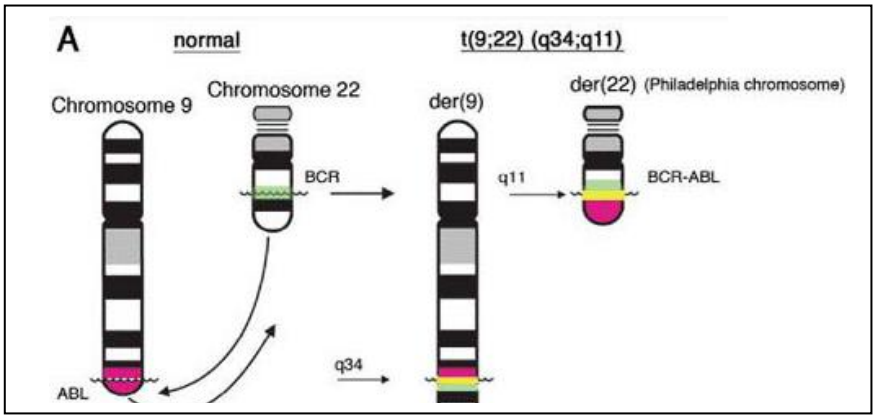


Figure (1): Chromosomal translocation results in formation of BCR-ABL fusion protein ***(George, 2007).***

Genes involved and proteins

Gene Name	ABL
Location	9q34
Protein	giving rise to 2 proteins of 145 kDa; contains SH (SRC homology) domains; N-term SH3 and SH2 - SH1 (tyrosine kinase) - DNA binding motif - actin binding domain C-term; widely expressed; localisation is mainly nuclear; inhibits cell growth

Gene Name	BCR
Location	22q11
Protein	main form: 160 KDa; N-term Serine-Treonine kinase domain, SH2 binding, and C-term domain which functions as a GTPase activating protein for p21rac; widely expressed; cytoplasmic localisation; protein kinase; probable role in signal transduction

(Huret et al., 2013)

Result of the chromosomal anomaly

Hybrid gene

Description	- the crucial event lies on der(22), id est 5' BCR/3' ABL hybrid gene is pathogenic, while ABL/BCR may or may not be expressed; - breakpoint in ABL is variable over a region of 200 kb, often between the two alternative exons 1b and 1a, sometimes 5' of 1b, or 3' of 1a, but always 5' of exon 2; - breakpoint in BCR is either (as in ALL cases): 1- in the same region as in CML, called M-bcr (for major breakpoint cluster region), a cluster of 5.8 kb, between exons 12 and 16, also called b1 to b5 of M-bcr; most breakpoints being either between b2 and b3, or between b3 and b4; transcript is 8.5 kb long; this results in a 210 KDa chimeric protein (P210), with the first 902 or 927 amino acids from BCR; 2- in a 35 kb region between exons 1 and 2, called m-bcr (minor breakpoint cluster region), -> 7 kb mRNA, resulting in a 190 KDa protein (P190), with the 427 N-terminal amino acids from BCR
Transcript	7 or 8.5 kb

(Huret et al., 2013)

Fusion protein

Description	190 or 210 kDa (see above); BCR/ABL has a cytoplasmic localization, in contrast with ABL, mostly nuclear; this may have a carcinogenetic role. The hybrid protein has an increased protein kinase activity compared to ABL: 3BP1 (binding protein) binds normal ABL on SH3 domain, which prevents SH1 activation; with BCR/ABL, the first (N-terminal) exon of BCR binds to SH2, hidding SH3 which, as a consequence, cannot be bound to 3BP1; thereof, SH1 is activated.
Oncogenesis	- proliferation is induced: there is activation by BCR/ABL of Ras signal transduction pathway via it's linkage to son-of-sevenless (SOS), a Ras activator; PI3-K (phosphatidyl inositol 3' kinase) pathway is also activated; MYC as well; - BCR/ABL inhibits apoptosis; - BCR/ABL provokes cell adhesive abnormalities: impaired adherence to bone marrow stroma cells, which allows unregulated proliferation of leukaemic progenitors.

(Huret et al., 2013)

BCR/ABL variants

The BCR-ABL transcript depending on whether the break in M-BCR occurs in the intron between exons “e13” and “e14”, or in the intron between exons “e14” and “e15”. A break in the former intron yields an “e13a2” mRNA junction and a break in the latter intron yields an “e14a2” junction (It should be noted that exon e13 was previously termed exon b2 and exon e14 was previously termed b3; thus the two ribonucleic acid junctions were known as b2a2 and b3a2, respectively) (**figure 2**) (***Goldman and Mughal., 2011***).

According to the location of the breakpoint in BCR, three types of fusion protein can be formed. As a result, there is excessive tyrosine phosphorylation of many intracellular proteins including the BCR-ABL itself. Although not all interactions of BCR-ABL with other proteins are phosphotyrosine dependent, it is clear from mutational analysis of PTK activity is an absolute requirement for malignant transformation, it cannot be complemented by any downstream effector. In contrast, less clear which of the various signaling pathways activated by BCR-ABL and

activators of transcription ,and phosphatidylinositol 3′-kinase is essential for transformation **(Mrózek *et al*., *2009*).**

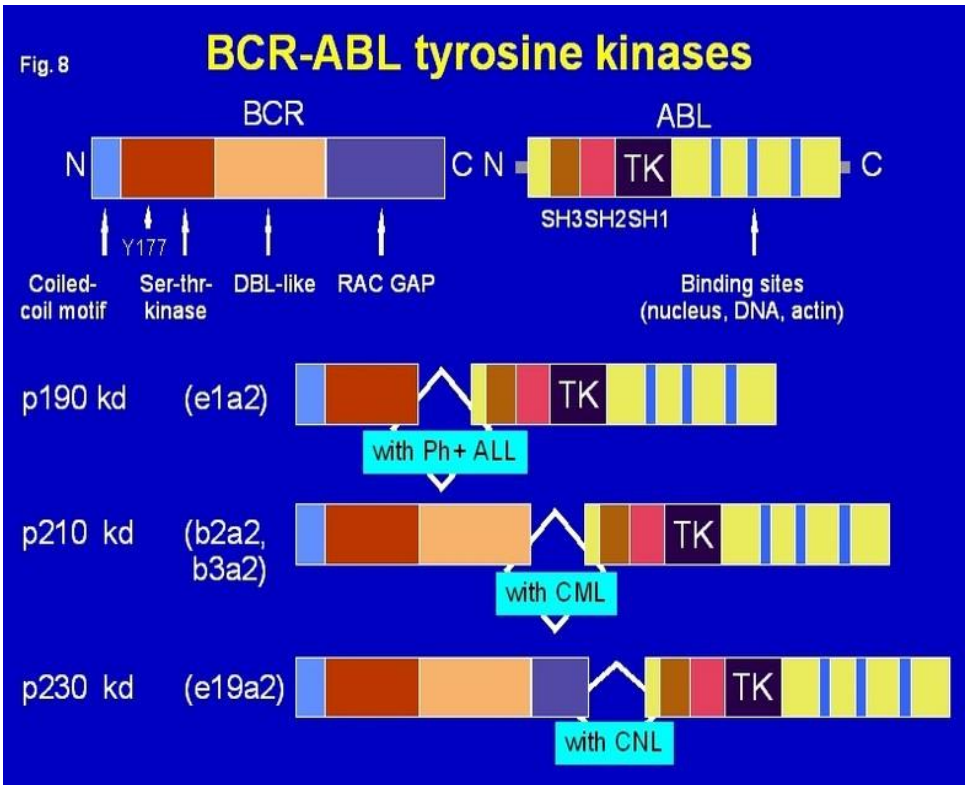


Figure (2): Locations of the breakpoints in the BCR-ABL genes and structure of the chimeric mRNAs derived from the various breaks**.** **(*Goldman and Mughal, 2011*)**.

In two-thirds of Ph positive ALL patients, the genomic breakpoint occurs in the first intron of the BCR gene [minor breakpoint cluster region (m-BCR)]. The BCR/ABL gene results from fusion of the first exon “e1” of the BCR gene with the second exon “a2” of the ABL gene. The mRNA is designated “e1a2” and encodes a protein of 190 kDa ($p190^{BCR-ABL}$). The 190 kDa BCR-ABL protein has higher tyrosine kinase activity than does the 210 kDa protein, resulting in a greater potential to induce cancer, this may explain the acute phenotype associated with Ph chromosome-positive ALL **(*Barrett and Yong, 2010*).**

The TEL-AML1 fusion gene:

The fusion gene translocation TEL/AML1, resulting from the translocation t(12; 21) (p13;q22), is present in 20–30% of childhood ALL ***(Schmidt et al., 2009).***

TEL gene is an important regulator of hemopoietic-cell development, essential for definitive hemopoiesis ***(Hock et al., 2004),*** it is almost the entire coding region of another transcription factor gene, and is required for the homing of hematopoietic progenitor cells to the bone marrow, whereas AML1, encodes the α subunit of core binding factor (also called CBFα), a master regulator of the formation of definitive hematopoietic stem cells ***(Pui et al., 2004 b)***. The chimeric TEL-AML1 transcription factor retains an essential protein–protein interaction domain of TEL and the DNA-binding and transcriptional regulatory sequences of AML1 ***(Loh and Rubnitz, 2002; Speck and Gilliand, 2002)***.

A prominent effect of the TEL-AML1 fusion protein is inhibition of the transcriptional activity that is normally initiated when AML1 binds to a DNA region termed the core enhanced sequence ***(Hiebert et al., 1996).*** The binding of AML1 to the core enhanced sequence recruits other transcription factors and coactivators to this region, and the resulting protein complex regulates transcription. These changes in the normal AML1-mediated transcriptional cascade alter both the self-renewal capacity and the differentiation capacity of hematopoietic stem cells ***(Speck and Gilliland, 2002; Downing, 2003).***

TEL-AML1 occurs as a 1st hit already in utero and leads to the expansion of a pre leukemic clone. A 2nd hit, usually postnatal, is required to fully transform these cells leading to the clinical manifestation of the leukemia. The pre leukemic clone may survive treatment thereby providing

the reservoir for other secondary mutations that give rise to subsequent leukemia - clinically diagnosed as a relapse ***(Grümayer, 2007).***

Translocations involving the MLL gene:

The HOX regulatory pathway is the mixed-lineage leukemia (MLL) protein, a nuclear protein that maintains the expression of particular members of the HOX family. Leukemia-associated translocations of MLL result in chimeric proteins consisting of the N-terminal portion of MLL fused to the C-terminal portion of 1 of more than 40 partners. This genetic alteration occurs in more than 80 percent of infants with ALL and in most therapy-induced leukemias caused by topoisomerase II inhibitors ***(Ernst et al., 2002)***.

The MLL fusion proteins have a dominant gain of function effect that enhances their transcriptional activity. This alteration disrupts the normal pattern of expression of HOX genes, causing a change in the self-renewal and growth of hematopoietic stem cells and committed progenitors ***(Pui et al., 2004b).***

II. Genetic and chromosomal mutations:

Chromosomal aberrations are a hallmark of ALL but alone fails to induce leukaemia ***(Mullighan et al., 2007).*** Instead, genetic alterations that impair differentiation, such as those described above, probably cooperate with a second class of mutations that alter the proliferation and survival of hematopoietic progenitors ***(Speck and Gilliand, 2002).***

There are deletion, amplification, point mutation and structural rearrangement in genes encoding principal regulators of B lymphocyte development and differentiation in 40% of B-progenitor ALL cases ***(Mullighan et al., 2007)***. Here are several genes involved in the second type of mutations.

The FLT-3 gene:

Over expression of FLT-3, a receptor tyrosine kinase important for the development of hematopoietic stem cells, occurs in cases of ALL with either MLL rearrangements or hyperdiploidy involving more than 50 chromosomes ***(Armstrong et al., 2002; Yeoh et al., 2002).***

Normally, the FLT-3 ligand triggers the tyrosine kinase activity of FLT-3 ***(Gilliland and Griffin, 2002)***, but in these subtypes of leukemia, the kinase is constitutively turned on by activating mutations, autocrine secretion of the FLT-3 ligand, or self-activation induced by the overexpression of FLT-3. Continuous signaling by the receptor contributes to the abnormal growth of leukemic cells ***(Armstrong et al., 2003; Armstrong et al., 2004).***

The Retinoblastoma gene mutation:

Another frequently altered regulatory network in ALL consists of the interrelated pathways controlled by the tumor suppressor retinoblastoma protein (RB). The principal role of RB is to control entry into the cell cycle ***(Sherr and McCormick, 2002).***

Despite the rarity of inactivating mutations or deletions of RB in ALL ***(Krug et al., 2002),*** functional inactivation of the RB pathway through the deletion or epigenetic silencing of $P16^{INK4a}$ and $P15^{INK4b}$ occurs in nearly all cases of childhood T-cell ALL and in a small proportion of cases of B-cell–lineage ALL ***(Omura et al., 2000)***.

Tp53 gene mutation:

The Tp53 gene, which encodes the p53 transcription factor and regulates apoptosis and cell cycle arrest in G1, is itself rarely altered in ALL; however, components of the p53 pathway are frequently mutated in ALL ***(Vousden and Lu, 2002)***.

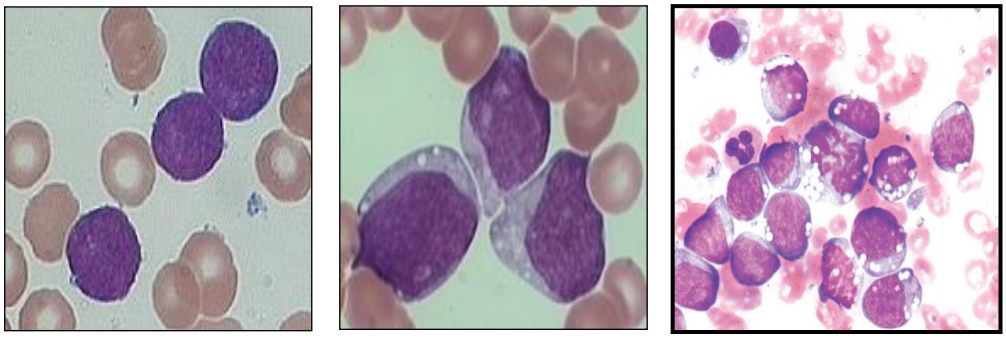
The activity of p53 is harnessed by HDM2, a protein that binds to p53 and induces its degradation; HDM2, in turn, is inhibited by the $p14^{ARF}$ tumor suppressor. Deletion or transcriptional silencing of $p14^{ARF}$ is a frequent event in ALL, whereas overexpression of HDM2 or silencing of the p53 transcriptional target $p21^{CIP1}$ occurs in approximately 50 percent of cases of ALL ***(Pui et al., 2004b).***

Classification:

Classification of the ALL requires morphological, cytochemical, immunological, cytogenetic and molecular analysis ***(Christ and Smithson, 2000).***

FAB-classification:

ALL was based on the French-American-British morphological criteria that described 3 subtypes of ALL (L1, L2, and L3) based on cell size, cytoplasm, nucleoli vacuolation, and basophilia **(Figure 3)** ***(Paul Shilpa et al., 2016).***



L1 blast L2 blast L3 blast

Figure(3): Morphology of L1, L2 and L3 blasts ***(Jabbour et al.,2005).***

WHO classification:

The information derived from cytogenetic studies has resulted in a new classification of tumors of the hematopoietic and lymphoid tissues, developed by the World Health Organization (WHO) and based on genetic subtyping of diseases. WHO's recent classification of myeloid and lymphoid neoplasms utilizes morphology, immunophenotype, genetics, and clinical features to define disease entities of clinical significance **(Table 3)** ***(Tansatit.,2017).***

Immunological classification:

European Group for the Immunological Classification of Leukemias (EGIL) has proposed that acute leukaemia be classified on the basis of immunophenotype alone. This classification has the strength that it suggests standardized criteria for defining a leukaemia as myeloid, T lineage, B lineage, or biphenotypic **(Table 4)**. It also suggests criteria for distinguishing biphenotypic leukaemia from AML with aberrant expression of lymphoid antigens, and from ALL with aberrant expression of myeloid antigens ***(Hoelzer and Gokbuger, 2002).***

Table (3): WHO (2016) classification of acute lymphoblastic leukemia

Acute leukemias of ambiguous lineage
Blastic plasmacytoid dendritic cell neoplasm
Acute leukemias of ambiguous lineage
Acute undifferentiated leukemia
Mixed phenotype acute leukemia (MPAL) with t(9;22)(q34.1;q11.2); BCR-ABL1
MPAL with t(v;11q23.3); KMT2A rearranged
MPAL, B/myeloid, NOS
MPAL, T/myeloid, NOS
B-lymphoblastic leukemia/lymphoma
B-lymphoblastic leukemia/lymphoma, NOS
B-lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities:
B-lymphoblastic leukemia/lymphoma with t(9;22)(q34.1;q11.2);BCR-ABL1
B-lymphoblastic leukemia/lymphoma with t(v;11q23.3);KMT2A rearranged
B-lymphoblastic leukemia/lymphoma with t(12;21)(p13.2;q22.1); ETV6-RUNX1
B-lymphoblastic leukemia/lymphoma with hyperdiploidy
B-lymphoblastic leukemia/lymphoma with hypodiploidy
B-lymphoblastic leukemia/lymphoma with t(5;14)(q31.1;q32.3) IL3-IGH
B-lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3);TCF3-PBX1
B-lymphoblastic leukemia/lymphoma, BCR-ABL1–like
B-lymphoblastic leukemia/lymphoma with iAMP21
T-lymphoblastic leukemia/lymphoma
Early T-cell precursor lymphoblastic leukemia
Natural killer (NK) cell lymphoblastic leukemia/lymphoma

(Arber et al.,2016)

Mixed phenotype acute leukemia (MPAL):

Leukemia formerly designated as bilineal acute leukemia and biphenotypic acute leukemia are now collectively considered as mixed phenotype acute leukemia (MPAL). The WHO classification not only places acute leukemias of ambiguous lineage in a chapter distinct from those of AML and ALL, but has significantly altered the criteria used to define the largest subset of these cases; those that express antigens of more than one lineage. Cases with no lineage-specific markers are designated as acute undifferentiated leukemia (AUL). Such cases often express CD34, HLA-DR, and/or CD38, and sometimes TdT, but lack specific myeloid or lymphoid antigens. Leukemias with blasts that coexpress certain antigens of more than one lineage on the same cells or that have separate populations of blasts that are of different lineages are referred to as mixed phenotype acute leukemia (MPAL). These cases may be further designated as B-myeloid or T-myeloid, irrespective of whether one or more than one population of blasts is found. The requirements for assigning specific lineages to the blasts are given in **(Table 5)**. Only a limited number of antigens are used in defining the pattern of lineage involvement ***(Arber et al., 2016).***

Table (4): European Group for the Immunological characterization of Leukemias (EGIL) classification of ALL.

Precursor B-lymphoblastic leukemia

(HLA-DR+, TdT+, CD19+, and/or CD79a+, and/or CD22+, and/or CD34+). This type of ALL accounts for around 75% of adult cases and is subdivided into the following groups:

a. Pro B-ALL expresses HLA-DR, TdT, and CD19. CD10-, cytoplasmic immunoglobulin negative; represents approximately 10% of adult ALL.

b. Common ALL is characterized by the presence of CD10, cytoplasmic immunoglobulin negative; comprises greater than 50% of adult cases of ALL.

c. Pre B-ALL is characterised by the expression of cytoplasmic immunoglobulin
and CD10; this subtype of ALL is identified in nearly 10% of adult cases.

d. Mature B-ALL is found in approximately 4% of adult ALL patients. The blast
cells express surface antigens of mature B cells, including surface membrane immunoglobulin (SmIg+). They are typically TdT and CD34 negative and have
L3 morphology. This category overlaps with Burkitt lymphoma, which is included under the mature B-cell neoplasms.

Precursor T-lymphoblastic leukemia

Cells are TdT+ in addition to cytoplasmic CD3+ and CD34+. This type of ALL
accounts for around 25% of adult cases and is subdivided into:

a. Pro T-ALL CD2-, CD7+, CD4-, CD8- seen in around 7% of adult ALL.

b. Pre T-ALL CD2+, CD7+, CD4-, CD8-.

c. Cortical T-ALL or Thymic ALL (Thy ALL) is CD1a+ and accounts for 17% of
adult ALL CD7+, CD2+, CD5+, CD4+, CD8+

d. Mature T-ALL are surface CD3+, CD2+, CD7+, CD4 or 8, and

TdT/CD34/CD1a- and make up approximately 1% of adult ALL.

(Hoelzer and Gokbuger, 2002).

Table (5): Requirements for assigning more than one lineage to a single blast population in mixed phenotype acute leukemia (MPAL):

Myeloid lineage Myeloperoxidase (flow cytometry, immunohistochemistry, or cytochemistry) Or Monocytic differentiation (at least 2 of the following: nonspecific esterase, CD11c, CD14, CD64, lysozyme)
T lineage Cytoplasmic CD3 (flow cytometry with antibodies to CD3 epsilon chain; immunohistochemistry using polyclonal anti-CD3 antibody may detect CD3 zeta chain, which is not T cell–specific**)** Or Surface CD3 (rare in mixed phenotype acute leukemia)
B lineage (multiple antigens required) Strong CD19 with at least 1 of the following strongly expressed: CD79a, cytoplasmic CD22, CD10 Or Weak CD19 with at least 2 of the following strongly expressed: CD79a, cytoplasmic CD22, CD10

(Arber et al., 2016).

Chapter (II)

Prognostic Factors of ALL

Many clinical, biological, genetic, and molecular features have been identified as having prognostic significance affecting the outcome of patients with ALL ***(Stams et al., 2005)***. Patients with ALL are usually treated according to Prognostic risk assessment includes clinical features (age and white blood cell [WBC] count at diagnosis), biologic characteristics of the leukemic blasts, response to the induction chemotherapy, and minimal residual disease (MRD) burden. Based on these criteria, patients can be effectively stratified into low risk, average or standard risk, high risk, and very high-risk ***(Satake et al., 2017).***

The intensity of treatment required for favorable outcome varies substantially among subsets of Patients with ALL. Risk-based treatment assignment is utilized in patients with ALL so that those with favorable clinical and biological features who are likely to have a very good outcome with modest therapy can be spared more intensive and toxic treatment, while a more aggressive, and potentially more toxic, therapeutic approach can be provided for patients who have a lower probability of long term survival **(Table 6 & 7)** ***(Itakura and Coutre, 2009).***

Table (6): Prognostic factors in childhood ALL:

Determinants	**Favorable**	**Unfavorable**
- white blood cell count	< 10 x 10^9 /L	>200 x 10^9 /L
- Age	1-10 years	<1 - >10 years
- Sex	Female	Male
-Node,liver,spleen enlargment	Absent	Present
- Testicular enlargement	Absent	present
- CNS leukemia	Absent	Overt(blasts+pleocytosis)
-FAB morphologic features	L1	L2
- Immunophentype	Pre-B or T	Early T-cell precursor
-Ploidy	Hyperdiploidy>50	Hypodiploidy<44
- Genetic markers (examples)		
B lineage	Trisomies 4 and 10	iAMP 21
	t (12;21)(p13;q22)	IKZF1 deletions/mutations
		t (4;11)(q21;q23)
T lineage	t (1;14)(p32;q11)	t (10;11)(p13;q14)
	t (10;14)(q24;q11)	t (5;14)(q35;q32)
	t (11;14)(p15;q11)	t (7;7)or inv7 (p15q34)
	t (11;19)(q23;p13)	
- Time of remission	<8d	>28d
-Minimal residual disease (day 28-56)	<10^{-4}	>10^{-4}

(Raetz et al., 2014).

Table (7): Prognostic factors in adults ALL:

Patient Features	**Prognostic Factor**
Age (y) <25, <35 >35, >55, >70	 Favorable Unfavorable
White blood cell count(x 10^9 /L) <30 ≥30 (≥100 for T cell)	 Favorable Unfavorable
Immunophenotype Thymic T Early T (CD1a-, sCD3-) Mature T (CD1a-, SCD3+) Pro B (CD10-)	 Favorable Unfavorable Unfavorable Unfavorable
Cytogenetics Hyperdiploidy >50 Hypodiploidy <44 9p abnormality deletion 6q Normal Complex (≥5 abnormalities)	 Intermediate to favorable Unfavorable Intermediate to favorable Intermediate Intermediate Unfavorable
B Lineage (e.g.,) t (12;21)(p13;q22) t (4;11)(q21;q23) t (1;19)(q23;p13) t (9;22)(q34;q11) IKZF1 deletions/mutations	 Favorable Unfavorable Unfavorable Unfavorable Unfavorable
T Lineage (e.g.,) NOTCH1/FBXW7 mutations TLX1 or t (10;14)(q24;q11) t (10;11)(p13;q14) t (5;14)(q35;q32)	 Favorable Favorable Unfavorable Unfavorable
Response to therapy Complete remission within 4wk Persistent minimal residual disease	 Favorable Unfavorable

(Coutre ,2014).

I-Patients related prognostic factors

Age:

The cytogenetic and morphologic distribution of adult leukemia differs significantly from childhood ALL, which appears to be one of the

main reasons for the better outcome of children however, even with the same genetic aberrations, adults have an inferior outcome; infant ALL is associated with a high WBC, hepatosplenomegaly, and CNS involvement. CD10 negativity and co-expression of myeloid markers are also common ***(Kanerva et al., 2004).***

Young adult patients with ALL between 15 and 20 years of age with ALL represent a unique epidemiologic group in that they may be treated by either adult or pediatric hematologists ***(Foa et al., 2011).***

In one study the prognosis was better in patients younger than 25 years; another study found a better prognosis in patients younger than 35 years. These findings may, in part, be related to the increased incidence of the Ph1 in older ALL patients, a subgroup associated with poor prognosis ***(Greer et al.,2014).***

Gender:

Male gender has consistently been an adverse prognostic feature than females; that is due to the testis functions as a so-called “sanctuary site” with the blood–testis barrier protecting leukaemic cells from anti-cancer drugs. Subsequently, dissemination to the bone marrow occurs, resulting in a relapse ***(Plasschaert et al., 2004).***

Race:

Survival rates in black and Hispanic patient with ALL have been somewhat lower than the rates in white patient with ALL ***(lee et al., 2011).***

Asian patient with ALL fare slightly better than white one the reason for better outcome in white and Asian patient compared with black and

Hispanic one is not known , but it cannot be completely explained based on known prognostic factors ***(Tijchon et al., 2013).***

Pharmacogenetics:

Genetic polymorphisms in genes that encode drug-metabolizing enzymes, transporters, receptors, and drug targets result in wide differences among patients in terms of drug disposition and pharmacologic effects ***(Evans and Relling, 2004).***

Patients who inherit homozygous or heterozygous deficiency of thiopurine methyltransferase, the enzyme that catalyzes the S-methylation (inactivation) of mercaptopurine, have a significantly increased risk for hematopoietic toxic effects, but they tend to have a better treatment response than do patients without this inherited deficiency, possibly because they receive a higher dose intensity of mercaptopurine ***(Stanulla et al., 2005).***

The null genotype of glutathione S-transferases, enzymes that catalyze the inactivation of many antileukemic agents, has been associated with a reduced risk of relapse. A tandem repeat polymorphism within the enhancer region of the thymidylate synthase gene, one of the major targets of methotrexate, has been linked to increased expression of the enzyme and an increased risk of relapse ***(Rocha et al., 2005).***

II-Disease related prognostic factors

Leukemic burden

WBC is reflective of tumor burden , although the underlying biological mechanisms that account for the adverse outcomes associated with an elevated WBC are uncertain other features associated with high tumor

burden, such as hepatosplenomegaly and mediastinal mass, are also associated with a greater risk of relapse ***(Tijchon et al., 2013).***

Laboratory criteria

WBC count at diagnosis:

The WBC count at presentation is one of a highly significant prognostic variable. Children with WBC of less than 50,000/μL at presentation, lack unfavorable cytogenetic features, show a good response to initial chemotherapy***.(Satake et al., 2017).*** In adults, the cutoff value is not clear but is probably lower than that for children ***(Jorge and Hagop, 2007).***

The tumor burden of the leukemia is also a marker of its biological characteristics patient with high WBC at the first presentation have a "rapid disease" with a high proliferation rate of the leukemic blasts. These patients often have near normal hemoglobin and platelet levels ***(Whitlock et al., 2010).***

Hb level and platelet count:

Anemia is common in patients with newly diagnosed ALL, lower Hb levels were more often diagnosed in leukemia subtypes associated with a favorable outcome (*TEL-AML1*$^{+,}$ hyperdiploid karyotype). In contrast, more aggressive leukemia subtypes (T-cell leukemia and BCR-ABL^{+} precursor B-cell leukemia) were associated with higher Hb levels ***(Möricke et al., 2008).***

Immunological assay:

Reduced level of one or more serum immunoglobulin (IgG, IgM, and IgA) at diagnosis is predictor of induction failure ***(Potopnev et al., 2004).***

Immunophenotype (IPT):

To detect surface immunoglobulin on leukemic blasts (diagnosis of mature B-cell leukemia) or the expression of T-cell–associated surface antigens (diagnosis of T-lineage ALL). ***(Satake et al., 2017).***

The major immunophenotypic subgroup with prognostic value and therapeutic importance is the mature B-cell neoplasm. The mature B-cell phenotype is found in 5% of adult ALL cases ***(coutre, 2014).*** And occurs in only 1 to 2% of children ***(patte et al., 2001).*** Approximately three quarters of patients with B-precursor ALL have the best prognosis ***(Moricke et al., 2004)***.

Central nervous system (CNS) status at diagnosis:

CNS status at diagnosis has prognostic significance. Patients who have a non-traumatic diagnostic lumbar puncture may be placed into one of three categories according to the number of WBC/µL and the presence/ absence of blasts on cytospin as follows ***(Whitlock et al., 2010).***

- CNS 1: Cerebrospinal fluid (CSF) that is cytospin negative for blasts regardless of WBC count.
- CNS 2: CSF with fewer than five WBC/µL and cytospin positive for blasts.
- CNS 3 : CSF with five or more WBC/µL and cytospin positive for blasts. ***(Itakura and Coutre, 2008).***

The adverse prognostic significance associated with CNS 2 status, if any, may be overcome by the application of more intensive intra-thecal therapy, especially during the induction phase ***(lee et al., 2008).***

A traumatic lumbar puncture (≥10 erythrocytes/µL) that includes blasts at diagnosis appears to be associated with increased risk of CNS relapse and indicates an overall poorer outcome ***(lee et al., 2008).***

Chromosomal abnormalities:

Both numerical and structural cytogenetic abnormalities appear to have an important prognostic significance. They are essential for proposed risk classification and for detection of minimal residual disease **(Tables 8&9)** ***(Rubintz and Look, 2000).*** Many of these can be detected using standard cytogenetic analysis, while some more recently discovered structural changes are diagnosed through other techniques, such as fluorescence in situ hybridization (FISH) on reverse transcriptase-polymerase chain reaction (RT-PCR), which can be performed on samples that are inadequate for cytogenetic analysis and are quicker, more sensitive and specific than karyotyping ***(Friedmann and Weinstein, 2000).***

Table (8): Cytogenetics and molecular genetics of ALL:

Chromosome Aberration/Genes Involved	**Children Frequency**	**Children Clinical outcome**	**Adults Frequency**	**Adults Clinical outcome**
High hyperdiploidy	23%-30%	Favorable	7%-8%	Favorable Intermediate
Hypodiploidy	6%	Intermediate	7%-8%	Adverse
Near-haploidy	0.4%-0.7%	Adverse	Rare	Not determined
t(12;21)(p13;q22)/ETV6-RUNX1(TEL-AML1)	22%-26%	Favorable	0%-4%	Not determined
t(9;22)(q34;q11.2)/BCR-ABL1	1%-3%	Adverse	11%-29%	Adverse
t(4;11)(q21;q23)/MLL-AFF1(AF4)	1%-2% 55% of infants	Adverse	4%-9%	Adverse
t(1;19)(q23;p13.3)/der(19) t(1;19)(q23;p13.3)/TCF3 (E2A)-PBX1	1%-6%	Favorable Intermediate	1%-3%	Favorable Intermediate Adverse
t(10;14)(q24;q11)/TCRA/ TCRD-TLX1(HOX11)	Rare	Not determined	0.6%-3%	Favorable Intermediate
del(6q)	6%-9%	Not prognostic	3%-7%	Intermediate
Abnormal 9p	7%-11%	Notprognostic Adverse	5%-15%	Favorable Intermediate
Abnormal 12p	3%-9%	Not prognostic	4%-5%	Faorabvle
Normal karyotype (no aberration detected)	31%-42%	Relatively favorable	15%-34%	Relatively favorable Intermediate

(Mrózek et al., 2009)

Table (9): Common genetic subtypes in ALL with their associated features

Subtype	**Associated Features**
Hyperdiploidy (>50 chromosomes)	Predominant B cell precursor phenotype; low leukocyte count; favorable age group (1–9 years) and prognosis in children
Hypodiploidy (<45 chromosomes)	Predominant B cell precursor phenotype; increased leukocyte count; poor prognosis
TEL-AML1 fusion	CD13±/CD33± B cell precursor phenotype; pseudodiploidy; age 1–9 years; favorable prognosis
t(1;19)(q23;p13.3) with *E2A-PBX1* fusion	CD10±/CD20–/CD34– pre-B phenotype; pseudodiploidy; increased leukocyte count; black race; CNS leukemia; prognosis depends on treatment
t(9;22)(q34;q11.2) with *BCR-ABL* fusion	Predominant B cell precursor phenotype; older age; increased leukocyte count; dismal outcome in adults and in children with poor early response to induction or with leukocyte counts >50 x 10^9/liter; improved prognosis with transplant from a matched related donor
t(4;11)(q21;23) with *MLL-AF4* fusion	CD10±/CD15±/CD33±/CD65± B cell precursor phenotype; infant and older adult age groups; hyperleukocytosis; CNS leukemia; dismal outcome
t(8;14)(q24;q32.3)	B cell phenotype; L3 morphology; male predominance; bulky extramedullary disease; favorable prognosis with short-term intensive chemotherapy including high-dose methotrexate, cytarabine, and cyclophosphamide
HOX11 overexpression	CD10+ T cell phenotype; favorable prognosis with chemotherapy alone

(Faderl et al., 2003; Mrózek et al., 2009)

III. Treatment related prognostic factors:

Response to induction therapy considerably influences the outcome of the disease. It includes the time to achieve CR, as well as the level of MRD. In all age groups, a slow early response to therapy, defined as 25%

or more residual blasts in the marrow on day 7 or day 14 of induction is associated with inferior event free survival ***(*Ribera *et al., 2010).***

Early response to therapy:

The rapidity with which leukemia cells are eliminated following onset of treatment is associated with long-term outcome, as is level of residual disease at the end of induction. Because treatment response is influenced by the drug sensitivity of leukemic cells and host pharmacodynamics and pharmacogenomics, early response has strong prognostic significance ***(Cario et al., 2007)***.

Various ways of evaluating the leukemia cell response to treatment have been utilized, including the following:

A- Day 7 peripheral blood and day 14 bone marrow responses:

Patients who have a rapid reduction in leukemia cells to less than 5% in their bone marrow within 7 or 14 days following initiation of multi-agent chemotherapy have a more favorable prognosis than do patients who have slower clearance of leukemia cells from the bone marrow ***(Cario et al., 2007)***.

B-Peripheral blood response to steroid prophase:

Patients with no circulating blasts on day 7 have a better outcome than those patients whose circulating blast ***(lee et al., 2008).***

C-Peripheral blood response to multi- agent induction therapy:

Patients with persistent circulating leukemia cells at 7 to 10 days after the initiation of multi-agent chemotherapy are at increased risk of relapse compared with patients who have clearance of peripheral blasts within 1 week of therapy initiation. Rate of clearance of peripheral blasts

has been found to be of prognostic significance in both T-cell and B-lineage ALL ***(Gökbuget et al., 2012).***

D- Induction failure:

Patients at highest risk of induction failure include those with T-cell phenotype (especially without a mediastinal mass) and patients with B-precursor ALL with very high presenting leukocyte counts and/or the Philadelphia chromosome ***(Garand et al., 2013).***

Relapse:

Although five-year survival rates for childhood ALL are now over 80% in most industrialized countries, not all children have benefited equally from this progress **(Yang et al,2011)**.

Relapse is defined as reappearance of the leukemic cells in any site in the body. The marrow remains the most common site of relapse in ALL. Leukocytosis, leucopenia, anemia, thrombocytopenia, enlargement of liver or spleen, fever, bone pain, or sudden decrease in tolerance to therapy may signal the onset of relapse. Marrow relapse has a poor outcome especially if it occurs while on therapy or shortly after initial remission ***(Rivera et al., 2005)***.

The German Berlin-Frankfurt-Munster (BFM) group has developed risk stratification for relapsed ALL **(table 10 & 11)**. In this risk stratification, duration of first complete remission and immunophenotype are associated with outcome ***(Sramkova et al., 2007).***

Table (10): BFM relapse risk group assignment for precursor B- ALL

	Extramedullary Relapse	**Combined Bone Marrow and Extramedullary Relapse**	**Marrow Relapse**
Very early relapse (<18 months from diagnosis)	Intermediate	High	High
Early relapse (>18 months from diagnosis and <6 months from completion of therapy)	Intermediate	Intermediate	High
Late relapse (>6 months from completion of therapy)	Standard	Intermediate	Intermediate

(Roy et al., 2005)

Table (11): BFM relapse risk group assignment for T- ALL.

	Extramedullary Relapse	**Combined Bone Marrow and Extramedullary Relapse**	**Marrow Relapse**
Very early relapse (<18 months from diagnosis)	Intermediate	High	High
Early relapse (>18 months from diagnosis and <6 months from completion of therapy)	Intermediate	High	High
Late relapse (>6 months from completion of therapy)	Standard	High	High

(Roy et al., 2005)

Assessment of minimal residual disease (MRD):

The detection of measurable residual disease (MRD) is an important marker of an increased risk of relapse in pediatric and adult acute lymph-oblastic leukemia .***(Torra et al., 2017).***

MRD in ALL is generally measured either by multiparametric flow cytometry (MFC), polymerase chain reaction (PCR) of the IgH and/ or TCR gene rearrangements or leukemia-specific fusion transcripts (eg, BCR-ABL in Philadelphia chromosome‑positive ALL). ***(Torra et al., 2017).***

Testing for MRD by PCR has been investigated as a predictor for relapse. It is an extremely sensitive and specific method of detecting leukemic cells ($1x10^6$) rather than morphologic examination of blast cells, in patients who are considered in remission ***(Ziegler, 2005)***. Patients with immunological or molecular remission, which is defined as leukemic involvement or MRD of less than 0.01% nucleated BM cells on completion of induction therapy have a good outcome, while those with MRD equal to or more than 1% after induction therapy have a high relapse risk even if they are in morphologic remission ***(Pui et al., 2001)***.

Several ongoing regimens include treatment intensification for children with higher MRD. Treatment de-intensification for patients with early MRD clearance is also being tested ***(Campana, 2008)***.

Other prognostic criteria:

I Cell cycle regulators and apoptosis-related molecules:

Some cell cycle regulators can influence both cell division and programmed cell death.

*** p73 gene**

The p73 gene is a homologue of the p53 tumor suppressor. p73 is involved in the regulation of cell cycle, cell death and development. Hypermethylation and subsequent inactivation of the p73 gene are the most common findings in malignant lymphoproliferative disorders, especially ALL and non-Hodgkin's lymphomas. Assessment of p73 methylation provide important prognostic information, as was confirmed in patients with ALL ***(Pluta et al., 2006)***

*** Retinoblastoma protein**

Stock and his colleages estimated that patients with low pRB expression are more liable to relapse and have shorter overall survival. So, it was proposed that low pRB expression is an unfavorable prognostic predictor in initial and relapsed childhood ALL ***(Stock et al., 2000)***.

***p15, p16 genes**

p15 and P16 are cyclin dependent kinase inhibitors. They inhibit activated cyclin-D complexes which control the cell cycle by phosphorylation and inactivation of Rb, leading to release of transcriptional factors necessary for entry into S-phase (synthesis phase). Genetic aberratios, usually deletion, lead to suppression of p15 and p16 and thus are associated with poor prognosis in ALL ***(Bertin et al., 2003).***

*** p 53 gene**

In childhood acute leukemias, p53 mutations are infrequent at diagnosis, but tend to be associated with poor outcome. Recently, it was reported that altered expression of p53 at diagnosis was more common in childhood leukemias associated with early treatment failure, compared to a group of

children who remained in long-term continuous remission ***(Addeo et al., 2005).***

*** Ras gene**

Ras is one of the most frequently deregulated genes in leukemias and estimated to be mutated in at least 30% of cases. Patients with N-Ras mutations have poor response to chemotherapy and low remission rates ***(Liang et al., 2006).***

*** Survivin protein**

Survivin is a member of the inhibitor of apoptosis proteins (IAP) family which cause impaired apoptosis, and is thought to contribute to leukemic cell survival. Very high levels of survivin have been described in a number of different tumors. Overexpression of survivin was found to correlate with poor prognosis in a variety of cancers including hematologic malignancies**.** Overexpression of survivin in BCP-ALL identifies patients with a high risk of early relapse ***(Troeger et al., 2007).***

II Multidrug resistance proteins and cellular drug resistance:

Various cellular mechanisms can give rise to multidrug resistance (MDR). Best studied is the transmembrane protein-mediated efflux of cytotoxic compounds that leads to decreased cellular drug accumulation and toxicity. Several MDR-related efflux pumps have been characterised, including P-glycoprotein (Pgp), multidrug resistance-associated protein 1 (MRP1) and lung resistance protein (LRP) ***(Swerts et al., 2006).***

*** P-glycoprotein**

P-glycoprotein (P-gp) is 170KD intra membranous protein that is an energy dependent efflux pump which has increased expression on drug-resistance cells ***(Nikougoftar et al., 2003).***

Ilaria and his colleagues found P-gp to be independent prognostic factors with regard to DFS. P-gp represents sensitive indicators of clinical outcome, and potential target of novel molecule aimed at overcoming chemoresistance and recurrent relapses ***(Ilaria et al., 2003)***.

Shman and his colleagues showed that the survival of patients with ALL was lower in cases with increased expression and function of P-gp ***(Shman et al., 2006)***.

*** Multidrug resistance protein**

Cellular resistance in childhood acute leukemias might be related to profile and function of multidrug resistance proteins and apoptosis regulating proteins ***(Styczynski et al.2007.)*** Evaluation of MRP1 expression at diagnosis of childhood ALL may contribute to the early identification of patients at risk of treatment failure ***(Kourti et al., 2007).***

***Lung resistance related-protein (LRP)**

LRP is encoded by the 1rp gene located on the short arm of chromosome 16. It's over expression might be one of the mechanisms involved in drug resistance particularly in children with pre-B ALL. Its expression was found to be higher in multiple relapse- ALL cells compared with initial ALL cells ***(Huh et al., 2006).***

Chapter (III)

Methods of Cytogenetic Analysis

A cytogenetic analysis provides important prognostic and predictive information and guides therapeutic decisions by setting the basis for individual treatment options that target cancer- specific genetic abnormalities or their products. ***(Tansatit,2017).***

Characterization of these abnormalities has not only provided insights into the mechanisms of leukemogenesis, but has led to more precise classification of blast cells and better estimates of the relapse hazard in individual patients, thus contributing to advances in risk directed therapy ***(Coco et al.,2014).***

Investigations using karyotyping and fluorescence in situ hybridization, loss of heterozygosity analysis, single nucleotide polymorphism arrays, and, most recently, massively parallel sequencing have challenged this view. In particular, comparison of diagnostic and relapse samples, modeling in transgenic mice, and whole-exome and whole-genome sequencing have indicated that wide spread genomic heterogeneity, which is masked by adominant clone, may be present in AML and ALL ***(Paulsson,2013).***

Techniques used for detection of chromosomal abnormalities are divided into two main categories:

I-Conventional karyotyping

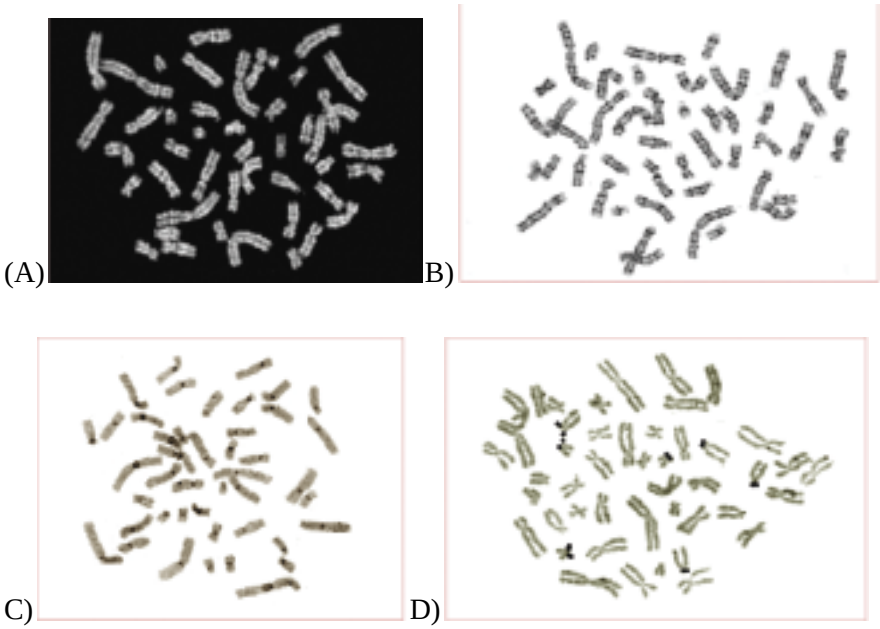
II-Cytogenetic and molecular techniques

I. Conventional karyotyping:

Chromosome banding techniques:

Chromosomes are stained by so called banding techniques to produce a specific banding pattern in which darkly stained proportions of the chromatin alternate with lighter stained regions ***(Mancini et al., 2014).***

The banding pattern of each chromosome pair is unique allowing positive identification of every chromosome. Development of the Q-banding technique in 1970 improved visualization of the genome and lead to the stratification of the genetic regions based on the intensity of the bands that were later called as euchromatin and heterochromatin. Induecourse, several other banding techniques were developed such as, G-,R-,C-and NOR- banding. G-banding, which is carried out by staining the chromosomes with Giemsa solution, became the most frequently used method **(Figure 4, Table 12)** ***(Sheth et al.,2014).***



Figure(4): Chromosome banding techniques. (A)Quinicrine [Q] banding, (B)Giemsa [G] banding, (C)Centromere [C] banding and (D)Nucleolar organizing region (NOR) banding ***(Sheth et al.,2014)***.

Table (12): The main types of chromosome banding methods:

Banding methods	Type	Principal use
Trypsin - induced Giemsa stain	G	Differentiates light and dark bands
AT – specific fluorochrome (quinacrine, Hoechst 33 25B)	Q	Light fluorescence in the region of dark G-bands, some centromere regions, distal long arm of the Y chromosome
Reverse bands	R	Opposite of G
Centromere stain	C	Centromere region darkly stained
Bromodeoxy uridine (BrdU) for two cell cycles	SCE	Differential staining of sister chromatids (Sister chromatid exchanges) (SCE)
Distamycin A-DAPI	DA / DAPI	Light fluorescence in the short arm of chromosome 15, centromere regions of 1, 9 and 16, distal long arm of Y
Silver nitrate stain	NOR	Short arms of all acrocentric chromosomes
Giemsa 11	G 11	Centromere of chromosome 11

(Shang-Ju et al., 2013).

Advantages of conventional karyotyping:

The major advantage is its non-directed nature. There does not need to be asuspicion for aparticular genetic change. This makes the banded chromosome analysis the most useful technique when it is unclear what abnormality might be found ***(Anastasi,2003)***.

Limitations of conventional karyotyping:

-Requirement of specimens that contain viable dividing cells which limits the examination to asmall number of cells that can be successfully

arrested in metaphase and exclude from analysis larger number of cells that remain in interphase.

-The conventional chromosomal analysis is not possible in terminally differentiated cells and is difficult in cells with alow mitoticrate.

-In specimens with a mixed population of cells conventional karyotyping can be confounded by overgrowth of the tumor cells by non malignant components ***(Wang,2002)***.

-The conventional chromosomal analysis relies on morphological features which can sometimes be inaccurate, ***(Anastasi,2003)***, due to low chromosome band resolution, poor banding quality, and condensed or fuzzy appearance of the chromosomes **(Figure 5)** ***(Salvi et al.,2013).***

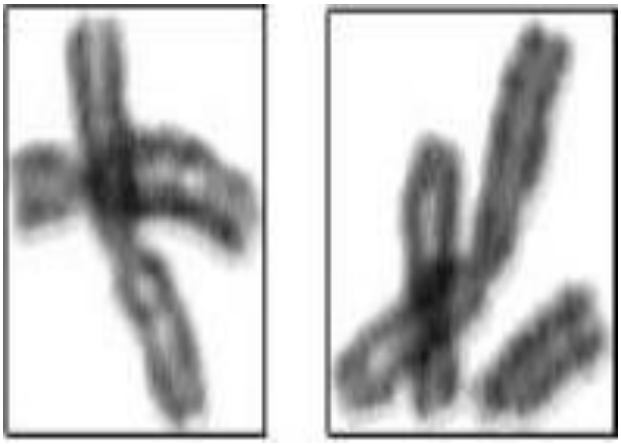


Figure (5): Over lapping chromosomes**.** ***(Yan and Bai,2013).***

It can miss cryptic translocations ***(Anastasi,2003)***. Banding techniques (BT) may be insufficient to determine the actual loss of a complete chromosome, especially incomplex karyotypes ***(Wawrzyniak et al.,2013).***

In order to over come these limitations, molecular genetic methods such as fluorescence in situ hybridization(FISH), quantitative real-time polymerase chain reaction(Q-RT-PCR), and array comparative genomic

hybridization(CGH) have emerged for diagnosis of hematologic malignancies ***(Salvi et al.,2013).***

II. Molecular techniques:

Molecular analysis is almost routinely used now in the evaluation of patients with malignant diseases. Although the findings from genetic studies are not contributory in all cases, in many instances they can be integrated with morphology, cytochemical features and immune-phenotypic studies to confirm a diagnosis. Also they help resolve a difficult differential diagnosis, recognize disease subsets or identify pathways involved in the pathogenesis of the disease process ***(Anastasi, 2003)***.

The molecular techniques used in detection of chromosomal abnormalities in leukemias are:

Fluorescence in Situ Hybridization (FISH)

The development of fluorescence in situ hybridization technology represents an important advancement in cytogenetics. FISH is a merge of classical cytogenetics with molecular technologies and has versatile applications. While many laboratories still utilize traditional special stains in specific circumstances, FISH techniques have replaced special stains in many laboratories ***(Gersen and Keagle, 2013).***

These powerful techniques allow us to detect and physically map on interphase nuclei, chromatin fibers, or metaphase chromosomes probes derived from single copy genes to repetitive DNA sequences. Other variants of the technique enable the co-localization of genes and the overall comparison of the genome among individuals of the same species or of different taxa. A further variant detects and localizes bacteria on tissues and

cells. Overall, this offers are markable multiplicity of possible applications ranging from strict physical mapping, to clinical and evolutionary studies, making it a powerful and informative complement to other molecular, functional, or genomic approaches ***(Pita et al., 2014).***

Principle:

The technique of FISH is based on the same principle as any DNA hybridization method that uses the ability of single-stranded DNA to anneal to complementary DNA. In the case of FISH, the target DNA, which may be metaphase chromosomes, interphase nuclei or tissue sections, is attached to a glass microscope slide. The advent of FISH represented an important step in the field of cytogenetics and since its development , it has been used extensively in both research and diagnostics ***(Gersen and Keagle, 2013)*** .

Advantages of FISH technique:

It is particularly important to denote that in the short time since the development of FISH technique, it has had a major impact on the capabilities of cytogenetic and pathology laboratories because of the following characteristics: ***(Dewald,2002).***

- Rapid technique; minimizing the turn around time to just 24 hours.
- Efficiency of hybridization and detection is high.
- Sensitivity and specificity are very high as it allowed greater resolution compared to the standard G-banding technique ***(Tansatit,2017).***
- Also FISH can identify chromosomal rearrangements in approximately 80% of patients, whereas CC can identify chromosomal aberrations in only approximately 40-50% of patients ***(Hu et al., 2014).***
- Large number of cells can be analyzed in a short time increasing sample sizes and enhancing statistical power. ***(Tansatit,2017).***

- Cytogenetic data can be obtained from tumor cells with a low mitotic index (e.g. lymphoid malignancies) or from poor samples that contain too few cells from routine cytogenetic studies ***(Sheth et al., 2014).***
- Permits the direct correlation of cytogenetic and cytologic/morphologic features, enabling pathologists to differentiate malignant from benign conditions in equivocal cases. It is widely used to detect submicroscopic deletions and characterization of chromosomal aberrations ***(Sheth et al., 2014).***
- Major advantages of FISH are that it can be performed on non-dividing interphase cells and the ease with which a large number of cells can be scored. ***(Tansatit,2017).***

Currently, FISH is widely used for detecting specific genomic aberrations, providing important information for disease diagnosis, risk stratification and prognosis. Further more, FISH is the gold standard for evaluating some key biomarkers, such as BCR/ABL1, HER2 and ALK rearrangements, and plays a critical role in guiding targeted therapies. So that, FISH has evolved to become a vital diagnostic tool for personalized medicine ***(Hu et al., 2014).***

Limitations:

FISH testing does not usually screen all chromosomes for aberrations; the cytogenetic data can be obtained only for the target chromosomes. Therefore, FISH is not a good screening tool for unusual chromosomal aberrations. ***(Tansatit,2017).***

FISH applications:

With the discovery of numerous disease related genes in recent years, the applications of FISH broadened to include more genetic diseases, hematologic malignancies, and solid tumors. This allows more accurate and

advanced molecular profiling for individual patients; enabling better disease stratification and prognosis, leading to tailored therapeutic regimens. Apparently, a new era of personalized medicine has arrived much earlier than most of us expected***. (Hu et al., 2014).***

Detection of numerical & structural chromosomal abnormalities:

In many cases, FISH is a widely used method to assess fixed tissues or isolated cells for numerical and structural chromosome aberrations and it has correlated the interpretation of chromosomal abnormalities that was based on cytogentic analysis alone. Chromosomal translocation can be identified in interphase or metaphase cells by using LSP, so it is applied in diagnosis of leukemia. ***(Weier et al., 2013).***

Monitoring the effects of therapy and detecting MRD:

FISH provides a rapid means of targeting the analysis to detect residual leukemia cells. With the development of new treatment strategies in acute leukemia and MDS, it may be useful to define the lineage involved in the neoplastic condition and to monitor the involvement throughout the therapy ***(Löwen berg, 2003).***

Identification of the origin of marrow cells following marrow transplantation:

In allogenic bone marrow transplantation patients who received opposite sex donor cells, the success of engraftment can be monitored by FISH studies. Differentially labeled X and Y specific probes can be used to detect the proportions of XX to XY cells in bone marrow or peripheral blood nuclei in a dual color FISH procedure ***(Pichler et al., 2014).***

Identification of the lineage of neoplastic cells:

One of the advantages of FISH is that one can combine this method with morphologic or immunohistochemical assays to examine the cytogenetic pattern of specific cell populations and so, identification of lineages involved in neoplastic process or evaluation of cells induced to differentiate by growth factor therapy ***(Levsky and Singer, 2003).***

Prenatal screening for aneuploidies:

In prenatal cases with advanced maternal age, abnormal ultrasonography markers or an abnormal result in triple marker screening, pre natal diagnosis to screen for aneuploidies of chromosomes 13, 18, 21, X and Y on uncultured cells from amniotic fluid and chorionic villi sampling is commonly per formed using FISH ***(Sheth et al., 2014).***

Detecting specific biomarkers in solid tumors:

As for lung, breast and prostatic cancers, also in melanomas**,** for instance, there were several reports of the successful automated evaluation of HER2 gene amplification using breast cancer specimens, ***(Hu et al., 2014).***

Chromosome specific probes:

Fluorescence in situ hybridization (FISH) is a cytogenetic technique developed in the early 1980s. FISH uses fluorescent DNA probes to target specific chromosomal locations within the nucleus, resulting in colored signals that can be detected using a fluorescent microscope***.(Hu et al.,2014).***

Probes for repeated DNA sequences (repetitive sequence probes):

The most widely used repetitive sequence probes are for the alpha satellite sequences located at the centromeres of human chromosomes.

Alpha satellite DNA is composed of tandem repeated monomers, thus the sequences targeted by the probes are present in several hundreds or thousands of copies, producing strong signals. Each chromosome's alpha satellite sequence (with the exception of chromosomes 13 and 21 and chromosomes 14 and 22) is sufficiently divergent to allow for the development of centromere specific probes ***(Gersen and Keagle, 2013).***

These probes are particularly useful for detection of aneuploidy in both metaphase and interphase cells. Other types of repetitive sequences for which probes have been developed include the beta satellite sequences (located in the short arms of the acrocentric chromosomes), "classical" satellite sequences (found at various locations including the heterochromatic region of the Y chromosome), and telomeric repeat sequences (TTAGGG) that mark the physical ends of each human chromosome. These latter probes are not as routinely used in the clinical setting but are valuable for the study of structural aberrations ***(Gersen and Keagle, 2013).***

Locus-specific (Unique sequence) probes:

These probes are generated from regions of the genome that are either cloned into various vectors (e.g., cosmids, yeast artificial chromosomes [YACs] , bacterial artificial chromosomes [BACs]) or are made by PCR using sequence-specific primers. Some probes include extraneous repetitive sequences, and Cot-1 DNA must be added to the hybridization mixture to block non specific binding so that only the unique sequences are visualized. Other probes, termed single-copy probes, are designed and developed based on genomic sequences that are devoid of repetitive sequences . Unique sequence probes, which range in size from approximately 1 kilobase (Kb) to >1 megabase (Mb), may be used to examine a particular area for copy number or location ***(Gersen and Keagle, 2013).***

Types of locus-specific probes:

1) Single fusion – dual colour: (Figure 6)

The single-copy genomic probes target distinct chromosomal regions (not repetitive sequences). They are used to detect the presence of amplification (increased number of copies of a specific locus).***(Gorczyca, 2008).*** Or to detect the presence of rearrangement, for example; the identification of the t(9;22) known as the Philadelphia translocation. Single fusion – dual color probes has high specificity but low sensitivity, because probes of this type yield a relatively high number of false-positive fusion signals (2–6 %) with a cut off value ≥6%. ***(Tansatit,2017).***

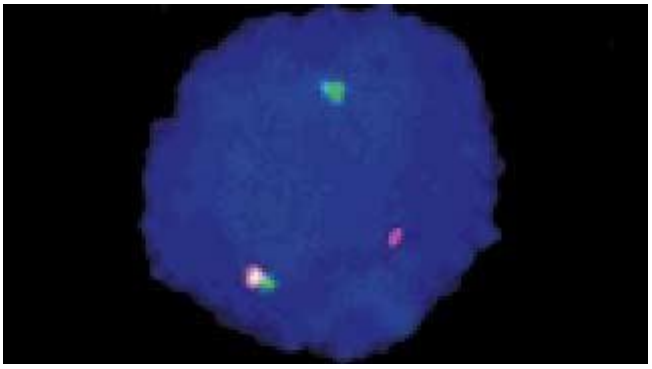


Figure (6): Signal pattern positive for BCR/ABL fusion gene by LSI BCR/ABL single fusion, dual color probe. One red, one green and one fusion (1R1G1F) signal pattern is observed ***(http:// www.abbottmolecular.com /LSI BCRABL DualColor SingleFusion TranslocationProbe_5362.aspx).***

2) Double fusion – dual colour (D-FISH): (Figure 7)

Dual-color double-fusion probes greatly reduce the number of normal nuclei exhibiting abnormal signal patterns and are optimal for detecting low numbers of nuclei possessing a simple balanced translocation. Two labeled large probes spanning regions of the two translocation break-points on different chromosomes allow the simultaneous visualization of a fusion signal on both derivative

chromosomes, significantly reducing the impact of false-negative results, a source of concern in single-fusion probes. ***(Tansatit,2017).***

A dual-fusion probe consists of a pair of probes labeled with two different colors (fluorochromes), green e.g. fluorescein isothiocyanate (FITC) and red e.g. rhodamine (TRITC) directed against translocation breakpoint regions in the two different genes involved in a reciprocal translocation. In a normal cell there are two green and two red signals corresponding to two separate loci that are not in close proximity (no translocation). In cells with translocation between the targeted loci, there is one green and one red signal (normal chromosome) and two yellow signal indicating the fusion between two loci. Variant and complex patterns may also be identified and provide additional clinical information on the underlying chromosomal changes ***(Gorczyca, 2008).***

3) Extrasignal FISH (ES-FISH): (Figures 8 & 9)

The first generation of BCR/ABL single fusion FISH probes detected the fusion gene with high specificity (false positive rate 5%) but with a low sensitivity. A new generation of FISH probes has been developed with the rational to define the t(9;22) by two FISH events: a fusion signal and an extra signal (ES) corresponding either to the remaining probe on the 9q+ or to a second fusion on the 9q+, according to the breakpoint localization in the BCR gene. With these new ES probe, the cutoff rate for false positives has dropped significantly to 3%. The pattern of interphase FISH (iFISH) signals observed is distinct for the MBCR or mBCR genes ***(Albano et al., 2007).***

Amplification is usually determined by a comparison of the gene copy number to the number of centromeres in the same cell. The ratio of gene to centromere number is used frequently in reporting whether a

tumor is amplified or deleted for a particular gene.Some of the common uses of these probes in clinical cytogenetics are for the diagnosis of microdeletion and microduplication syndromes ***(Tansatit,2017).***

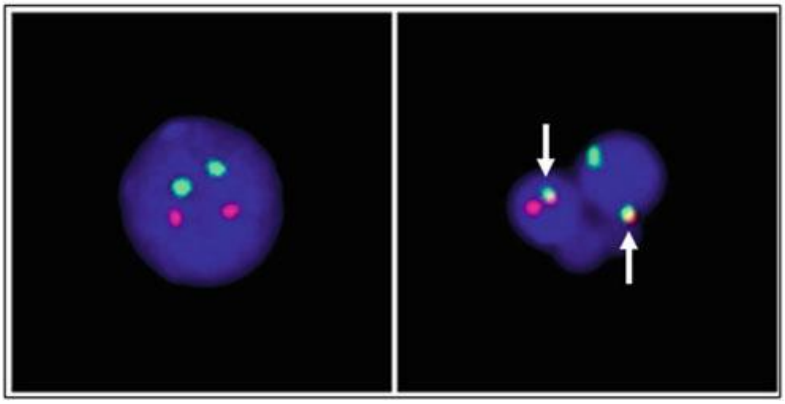
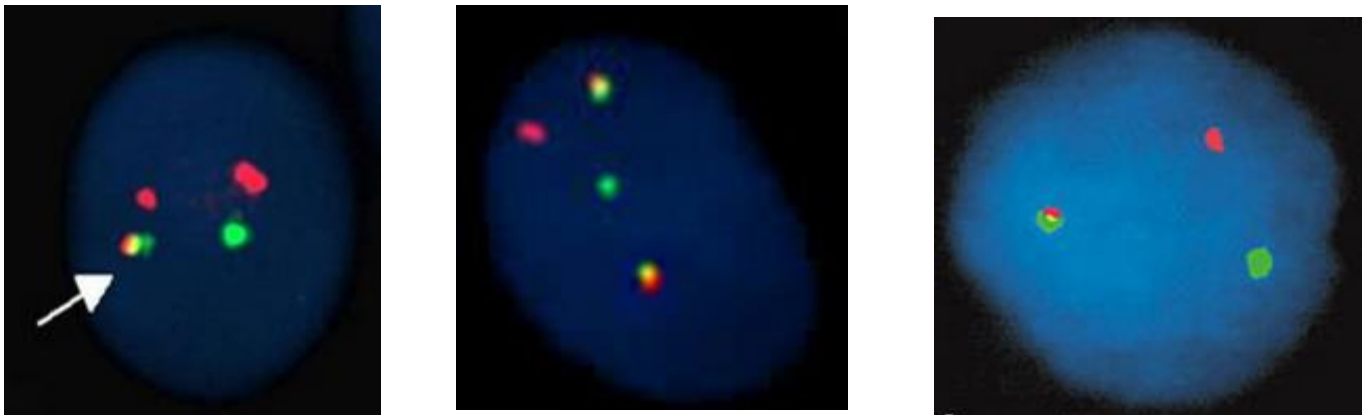


Figure (7): Signal pattern positive for BCR/ABL fusion gene by LSI BCR/ABL double fusion, dual colour probe. One red, one green and two orange/green fusion signals are observed (1R1G2F). ***(Tansatit,2017).***



(a) **(b)** **(c)**

Figure (8): Signal pattern positive for BCR/ABL fusion gene by LSI BCR/ABL extra signal, dual color probe. One green (native BCR), one large red (native ABL), one smaller red (ES) and one fused orange/green (2R1G1F) signal pattern **(a):** Major BCR/ABL fusion . ***(b):***minor BCR/ABL fusion. ***(c):***Abl deletion. ***(http://www.abbottmolecular.com/LSIBCRABLESDualColorTranslocationProbe_5370.aspx*).**

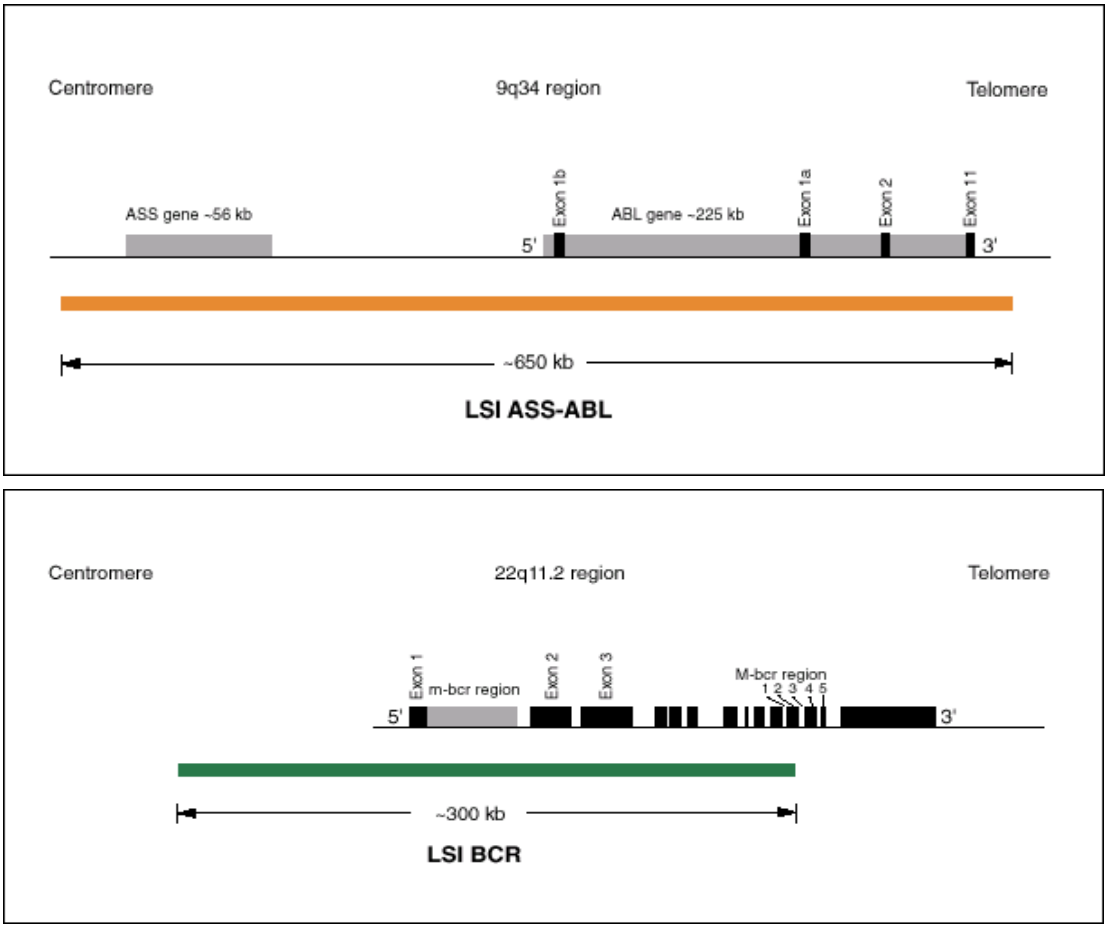


Figure (9): Schematic representation of extra signal, dual color LSI BCR/ABL fusion gene probe.***(http://www.abbottmolecular.com/LSIBCRABLESDualColorTranslocationProbe_5370.aspx).***

Whole-Chromosome Painting robes (WCP):

These probes are composed of unique and moderately repetitive sequences from an entire chromosome or chromosomal region. The generation of this type of probe requires that DNA from a particular chromosome be isolated from the rest of the genome. This may be accomplished using flow sorting, somatic cell hybrids containing a single human chromosome or area of a chromosome, or microdissected chromosomes and subsequent amplification of the dissected DNA sequences via the polymerase chain reaction (PCR) . WCPs are commercially available for each human chromosome and are most frequently used for the study of structural

aberrations. For example, WCPs may be used to identify the chromosomal origin of additional unknown material of derivative chromosomes and also to confirm the cytogenetic interpretation of translocations. ***(Gersen and Keagle, 2013).***

Modifications of FISH technique:

There are several modifications of FISH technique which are discussed below:

Multicolor WCP or Spectral Karyotyping (M-FISH and SKY):

Multicolor fluorescence in situ hybridization, or FISH, is a widely used method to assess fixed tissues or isolated cells for numerical and structural chromosome aberrations ***(Weier et al., 2013).***

Advanced techniques such as spectral karyotyping or multicolor FISH (M-FISH) allow all of the 24 human chromosomes to be labeled in different colors using a combination of five flourochromes in a single preparation. The diagnostic utility of single, dual and multi-color FISH has been evaluated for cancer genetics, characterization of marker chromosomes, breakpoint characterization in structural aberrations, in pre natal diagnosis and screening for micro-deletion / duplication in syndromic cases. ***(Sheth et al., 2014).***

Quantitative Multigene (QM-FISH):

The FISH method that employs multiple probes, called quantitative multi-gene FISH (qmFISH) has recently become popular. Abbott's Multi Vysion PB multi-color probe kit, a five-color FISH kit that detects chromosomes 13, 16, 18, 21 and 22 was developed to assist in pre implantation diagnosis (PGD) by polar body analysis. A four-color FISH assay, targeting chromosomes 1, 2, 6, 9, 7, 17, the loci 3p24pter, and

3p13p14 has been used for the early diagnosis of renal carcinoma in biopsies of uncertain renal masses ***(Sheth et al., 2014).***

LA Vysion FISH, a four-color FISH kit for simultaneously detecting chromosome 6 and the 5p15, 7p12 (EGFR gene), and 8q24 (MYC gene) loci was developed to assist in the differential diagnosis of ambiguous lung cancers. In recent years, qm FISH has been used in genetic variegation and clonal evolution studies of both hematological and non-hematological cancers ***(Hu et al., 2014).***

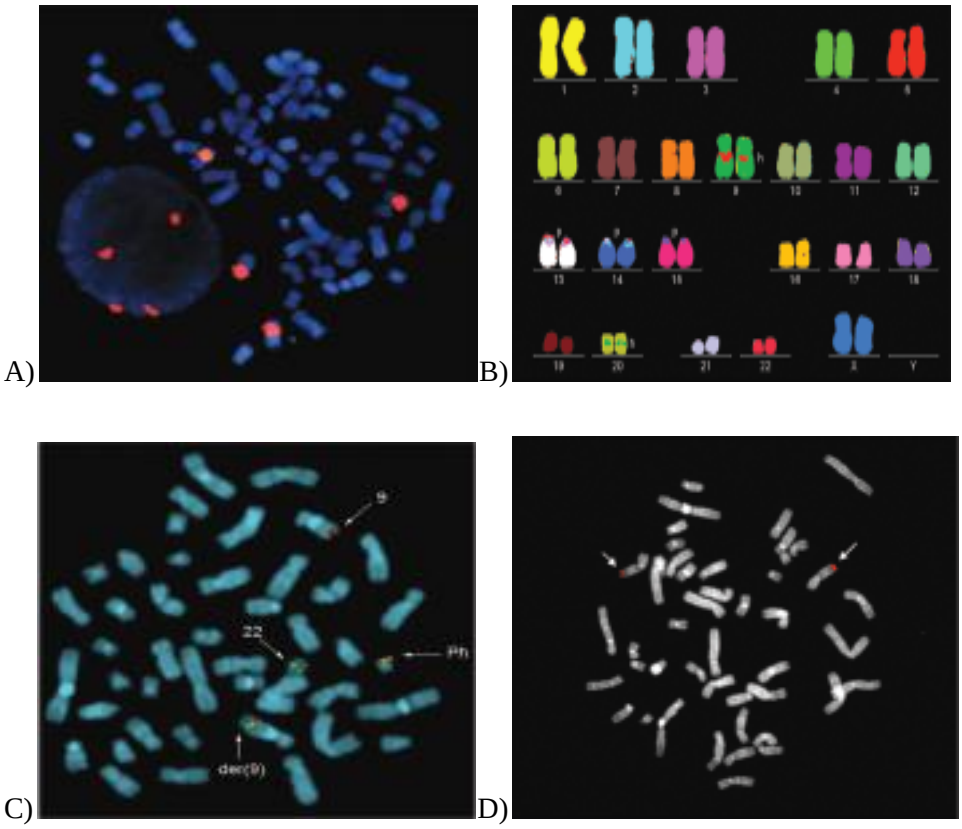


Figure (10): Various applications of FISH technique. (A)Interphase FISH and centromeric FISH confirming tetrasomy of chromosome 8 in apatient with CML, (B)Multiplex[M] FISH karyotype (CourtesyLiehrT.), (C)Detection of Philadelphia chromosome using locus specific probes and (D)Presence of subtelomeric region of chromosome 11 detected using subtelomeric FISH probes ***(Sheth et al., 2014).***

Hypermetaphase FISH (HMF):

The technique of HMF relies on the accumulation of large numbers of metaphase cells produced by exposure of a BM culture to 24 hrs of colcemid or 24 – 27 hrs to different hematopoietic growth factors (G-CSF, GM-CSF) and other mitogens (IL2, TNF and TPA) ***(Chase et al., 2001).***

The advantage of HMF is the ability to view the chromosome morphology, which goes some way to alleviate the problem of false positives which has dogged interphase FISH. The method has been reported to yield up to 2000 metaphases, giving a sensitivity of 1 × 105. However, post-treatment BM often has a low mitotic index and such high numbers of cells are rarely achievable ***(Kearney, 2001).***

High through put FISH analysis (HTFA):

A new technology called HTFA is different from conventional cytogenetics techniques and able to detect more than 1 deletion duplication region at the same time with high resolution at 300-500 kb. It is a bacterial artificial chromosome (BAC) array platform introduced for use in 2010. HTFA panels contain 31 regions on somatic chromosomes that are related to hematologic malignancies. HTFA might be though to fas a kind of array CGH technology, but it is not accepted as a traditional glass microarray platform due to the fact that multi-FISH probes are designed with BAC ***(Salvi et al., 2012).***

Polymerase Chain Reaction:

PCR is a simple in vitro chemical reaction that permits the synthesis of essentially limitless quantities of a targeted nucleic acid sequence. This is accomplished through the action of a deoxyribonucleic acid (DNA) polymerase that, under the right conditions, can copy a strand of DNA***(Nolte et al., 2017).***

A PCR cycle consists of three steps: denaturation, annealing, and extension. At the end of each cycle, the PCR products are theoretically doubled.***(Nolte et al., 2017).***

Principle of PCR technique: (Figure 11)

- DNA template:

Prior to initiating the PCR reaction, the template DNA must be isolated from the sample to be tested.

- Denaturation of the DNA template:

The template DNA is heated to 95 ºC resulting in two single strands of DNA.

- Annealing of the oligonucleotide primers:

After denaturation, the temperature is reduced to 50 – 60 ºC to allow primers to bind with high specificity to their complementary base on the template DNA.

- Synthesis of new DNA:

After annealing the primers, the temperature is increased to 72 ºC, the optimal temperature for DNA polymerase activity which results in synthesis of two double stranded DNA molecules identical to the initial template DNA.

- The chain reaction:

The three step process of denaturation, annealing, and synthesis is typically repeated for 25 – 30 cycle. At the end of each cycle the template DNA is amplified again, after 30 cycles, there will be over 1 billion double stranded DNA molecules identical to the initial template DNA fragment.

- Product analysis:

The PCR product has an expected size determined by the region of DNA that is flanked by the primer annealing sites. The product can be visualized by gel electrophoresis and ethidium bromide staining. ***(Wittwer and Kusukawa, 2006)***.

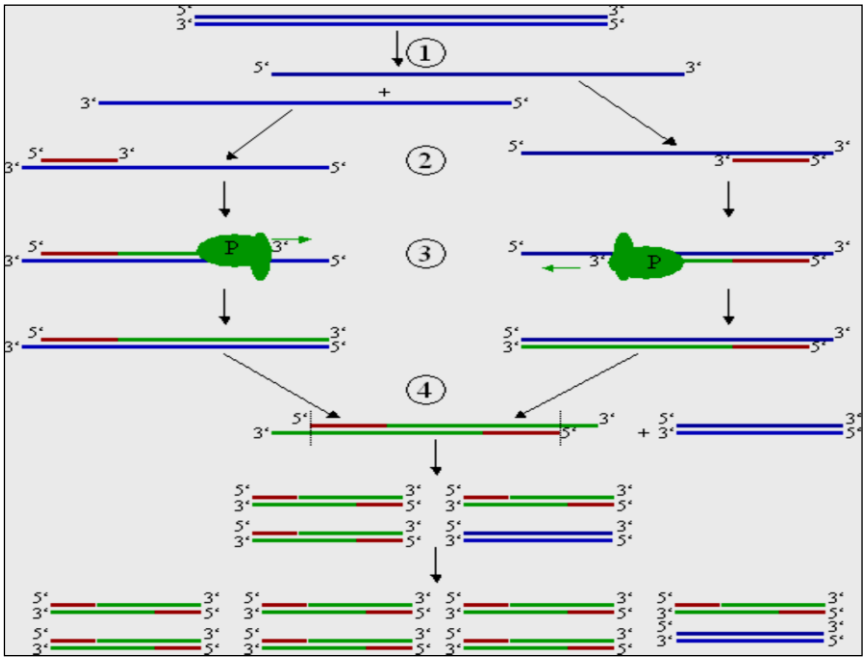


Figure (11): Schematic drawing of the PCR cycle ***(Gorczyca, 2008).***

Advantages and disadvantages of PCR:

Advantages of PCR:

- PCR has high sensitivity.

- PCR is simple, automated, in vitro method for DNA amplification.

- PCR technique can be completed in one working day, providing rapid results.
- PCR is highly specific due to the specificity of primer annealing.
- A single sample can be simultaneously amplified for several different markers and this has important economic implications ***(Gorczyca, 2008).***

Disadvantages of PCR:

- Minute amount of contamination can lead to false positive results**.**
- False negative results may occur due to degradation of template DNA, the presence of enzyme inhibitors or deterioration of reagents.
- PCR technique is expensive.
- Like FISH, PCR analysis is a directed analysis, that is suspicion for a specific abnormality is required ***(Gorczyca, 2008)***.

Reverse-transcriptase PCR (RT-PCR):

Reverse-transcriptase PCR (RT-PCR) was developed to amplify ribonucleic acid (RNA) targets. In this process, complementary DNA (cDNA) is first produced from RNA targets by reverse transcription, and then the cDNA is amplified by PCR. ***(Nolte et al., 2017).***

Real time Quantitative PCR (RQ-PCR):

RQ-PCR quantitates the initial amount of the template most specifically, sensitively and reproducibly. This is based on the detection and quantitation of a fluorescent signal which increases in direct

proportion to the amount of PCR product in a reaction ***(Spitzack and Ugaz, 2006).***

Real-time PCR offers a much wider dynamic range of up to 10^7-fold (compared to 1000-fold in conventional real-time PCR). It follows that the broader the dynamic range the more accurate the quantitation **(Pfaffl, 2004).**

Subjects and Methods

I. Subjects:

This study was carried out on 39 newly diagnosed acute lymphoblastic leukemia (ALL) patients who were attending the hematology oncology clinics of Ain Shams University Hospitals. They were 24 males (61.5%) and 15 females (38.5%) with a male to female ratio of 1.6:1 and their age was ranged from 19 year to 71 years.

This study had been submitted for approval by Research and Ethical committee at faculty of medicine of Sohag University and informed consent was obtained from patients to use their samples in this study.

All patients were subjected to the following:

A. History and clinical examination laying stress on the presence of hepatomegaly, splenomegaly, lymphadenopathy and CNS infelteration.

B. Laboratory investigations, which included:

1. Complete blood count using Sysmex XN-1000 & SA-01.

2. Examination of Leishman stained PB smears laying stress on differential leucocytic count , assessment of blast cell number and morphology.

3. Bone marrow aspiration and examination of Leishman stained smears.

4. Immunophenotyping on BM or PB samples, performed on EPICS XL Coulter Flow cytometer, USA.

5. Fluorescence in situ hybridization using the following probes:

- LSI dual color single fusion and double fusion BCR/ABL probes for detection of t(9;22)(q34; q11).

- LSI dual color extrasignal BCR/ABL probe for detection of t(9;22) with other aberrations as; amplification, deletion or duplication.

- LSI dual color double fusion TCF2/PBX1 for detection of t(1;19)(q23;p13.3).

- LSI dual color breakapart rearrangement MLL probe for detection of 11q23 rearrangement.

II. Methods:

A- Samples collection:

2mL of BM aspirate and 4 mL of venous blood were collected from each patient before initiation of treatment under complete aseptic conditions. Samples were divided as follows:

2mL PB were collected in a tube containing EDTA solution used for CBC and Leishman stain (done for all cases) .

1mL BM were dispensed into EDTA containing sterile vaccutainer for performing the immunophenotyping by flow cytometry.

The next 1mL BM and/or 2mL PB were collected in sterile preservative free lithium heparin coated vacutainer tube for cytogenetic analysis and FISH.

B- Immunophenotyping:

1-Principle of FCM:

FCM is the measurement of numerous cell properties (cytometry) as the cells move in single file resulting in light scattering. Antibodies specific for various cellular antigens can be labeled with different fluorochromes that can absorb and emit light, allowing simultaneous flow cytometric analysis of two or more cell-associated antigens ***(Paraskevas F., 2004).***

2-Reagents:

1- A panel of fluorescein isothiocyanate (FITC)/ phycoerythrin (PE) conjugated monoclonal antibodies to B-lineage markers (CD19, CD10, CD97a), T-lineage markers (CD3, CD5, CD7), myeloid markers (CD13, CD33).

2- Phosphate buffered saline (PBS) NaCl 8.5mmol/L, $NaHP_{O4-}$ (anhydrous) 1.07mmol/L and NaH_2PO4. $2H_2O$ 0.39mmol/L D.W to 1 liter, pH 7.4, stored at 4˚C and used as long as there was no evidence of contamination.

3- Lysing solution: NH_4CL 1.5mmol/L, $KHCO_3$ 100 mmol/L and Tetrasodium EDTA 10 mmol/L, completed to 1 liter with D.W, pH adjusted at 7.2.

4- An isotype matched negative control (appropriate for the MoAbs used) was used in all cases to assess background fluorescence intensity (non specific binding of MoAbs).

3-Procedure:

Sample preparation:

The EDTA anticoagulated BM sample was diluted 1:3 or according to cellularity with PBS. The final cell count suspension was adjusted between $5:10\times10^9$/L.

Surface marker staining:

1. For each sample, a set of tubes was prepared for all the MoAbs to be used including one for the isotype control:
    - Fifty μL of the diluted marrow sample were dispensed into each tube.
    - Five μL of each MoAb were added to the test tube.
    - Five μL of the appropriate isotype matched negative control were added to the control tube.
2. The tubes were vortexed then incubated for 15 min in the dark at room temperature.
3. Two mL of PBS were added, as a wash buffer, to each tube and mixed well. The tubes were centrifuged at 3000 rpm for 5 min, and then the supernatant was discarded. The wash was repeated twice.

4- One and half mL of the lysing solution was added to each tube, mixed well by vortex, then the tubes were incubated for 10 min in the dark at room temperature.

5- The cells were washed once with 2 ml PBS, with centrifugation and discarding the supernatant.

6- Cells were suspended in 500μL PBS and processed by the FCM. If the tubes were not processed within 2 hours, 0.5mL of fixative (4gm paraformaldehyde in 100mL PBS with 0.1% Na azide, pH 7.4) was added and the tubes were kept at 4°C until analyzed within 24 hours.

Sample processing on FCM:

A minimum of 5000 events were studied. Gating was done on the blast cell population based on its forward and side scatter properties.

Data interpretation:

The positivity was expressed as a percentage with a cut off >20%

over the corresponding isotypic control .

C- Cytogenetic analysis:
Fluorescence in Situ Hybridization (FISH)

1-Principle:

The target DNA sequence in the chromosome to be analyzed is denaturated and hybridized to a single-stranded fluorophore-labeled complementary nucleic acid sequence (probe) that is detected using fluorescence microscopy **(Figure 12)** ***(Garimberti,2010)***

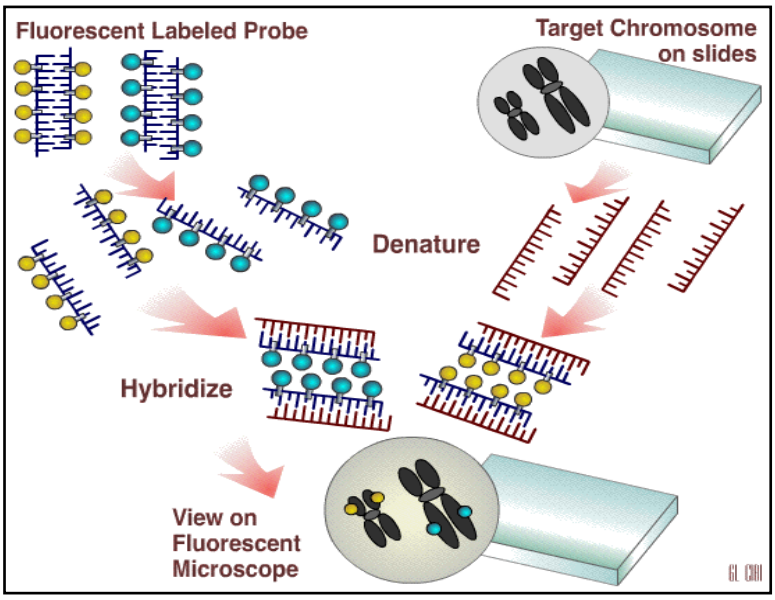


Figure (12): Nucleic acid hybridization

(***http://www.infobiogen.fr/services/chromcancer/ Deep/Compar Cancer Cytogenet. html***)

2-Reagents:

1. Tissue culture medium; RPMI1640[(1)] (100mLbottle) containing 2g/L $NaHCO_3$, 0.532g/L Na-acetyl-L-alanyl-L-glutamine, stored at 2-8°C.

- Fetal bovine serum (FBS) (20%,100mL bottle), stored at-20°C.

(1) – Biochrome, Berlin, Germany.

- Benzyl penicillin (10^6IU) and streptomycin (1g) powder▪ each dissolved in 5mL DW, stored at 2-8°C.
- Amphotricin (10mg/mL) in 5mL bottle, stored at 2-8°C.
- Colcemid solution bottle[(2)] (10μg/mL PBS, 25 mL bottle) , stored at 2-8°C or at-20°C.
- Phosphate buffer formed of 0.816g KH_2PO_4 and 0.816g Na_2HPO_4, each dissolved in 100 mL DW (solution A & solution B, respectively). Working solution was prepared by adding 102mL of solution A to 98 mL of solution B, titrated to pH 6.8 with 50% NaOH and stored at room temperature.
- Hypotonic KCl solution (0.56%) (0.56g KCl dissolved in 100 mL DW), stored at 37°C.
- Fixative: 3 parts absolute methanol + 1part glacial acetic acid, freshly prepared each time before use.

2. Vysis probes are direct-labeled DNA probes provided in a 20μ container together with hybridization buffer. Apply the probe on sample area of approximately 22x22 mm.

3-Procedure:

Culture and slide preparation of BM/PB sample

Preparation of culture media

- RPMI 1640 (100 mL bottle).
- 20 mL FBS.
- 400 μL penicillin.
- 200 μL streptomycin.

(2) −Al-Bardisi, Egypt, from GIBCO, Grand Island, New York, USA.

- 200 μL amphotrecin.

For each case, a sterile culture tube was set up under laminar airflow to avoid any contamination, containing:

- 5mL of the previous mixture.
- 500 μL of PB/BM sample or according to cellularity

Harvesting

- 50 μL of colcemid were added at the setup.
- The tube was mixed and incubated at 37°C for 24 hours.
- The tube was then centrifuged at 800-1000 rpm for 10 minutes.
- Most of the supernatant was discarded leaving only 0.5 mL to resuspend the cell pellet.
- Hypotonic treatment was done by adding pre-warmed 5-8mL of 0.56% KCl solution.
- The tube was then incubated at 37°C for 20 minutes.
- This was followed by centrifugation at 800-1000 rpm for 10 minutes and the supernatant was discarded.
- 10 mL of freshly prepared chilled fixative were added to the cell pellet by a clean glass Pasteur pipette together with gentle mixing.
- The tube was centrifuged at 800-1000 rpm for 10 minutes and the supernatant was discarded.
- The previous 2 steps were repeated until the supernatant became clear.
- The yielded cell pellet was suspended in 5mL fresh chilled fixative then stored at -20°C till the step of slide preparation.

Slide preparation

- The glass slides were washed with water and detergent, soaked in methanol, then placed in DW and kept in the refrigerator ready to use (clean, cold, wet slides).
- The tube containing the cell pellet was removed from-20°C and centrifuged at 800-1000 rpm for 10 minutes. The supernatant was discarded and the cells were suspended in 0.5 mL freshly prepared chilled fixative.
- 2 or 3 drops of cell suspension were added by a Pasteur pipette from a distance of one end of the slides. The slides were then tilted away from their ends at an angle.
- At least 2 slides were prepared for each tube.
- The slides were left to dry in air.
- Using a light microscope, the slides were examined by the low objective, and the area of maximum metaphase concentration was marked on each slide.

Probe preparation:

The probes and the hybridization buffer were brought to room temperature prior to use.

Application of LSI probe, denaturation and hybridization:

- The probe mixture (2µ distilled water, 1µ probe and 7µ hybridization buffer) was applied on each slide at the marked area and immediately covered with a glass cover slip to avoid dryness of the probe (during application of the cover slip, formation of air-bubbles was avoided to minimize weakness or absence of the hybridization signals).

- The perimeter of the glass coverslip was sealed to the slide by a thick layer of rubber cement.
- The slides were denaturated at 80°C in hybrite for 5 minutes, then, incubated overnight at 37°C in a dark pre-warmed humidified chamber, to allow probe hybridization.

Post hybridization wash (Rapid wash procedure):

- 70 mL of 0.4 XSSC/0.3%NP-40 were poured into a Coplin jar, and placed at 73°C for at least 30 minutes prior to use.
- 70 mL of 2 XSSC/0.1%NP-40 were poured into a Coplin jar, used at room temperature.
- The rubber cement was carefully removed with a forceps, and the slides were shaken sharply to slide the cover slip to the edge, this was then lifted gently.
- Each slide was immersed in 0.4 XSSC/0.3%NP-40 at 73°C, agitated for 1-3 seconds, and incubated for 2 minutes.
- Each slide was, then, immersed in 2 XSSC/0.1%NP-40 at room temperature, agitated for 1-3 seconds and incubated for 2 minutes.

Detection and counter staining:

- The slides were removed from the wash solution and excess fluid was blotted from the edge by a filter paper. The surface of the slides was not allowed to dry, as this would cause non-specific binding of the counter stain with high background fluorescence.

- 10 μL of the DAPI (4',6-diamidino-2-phenylindole) counter stain were added to each slide, covered and viewed under the fluorescent microscope. **(Figure 13).**

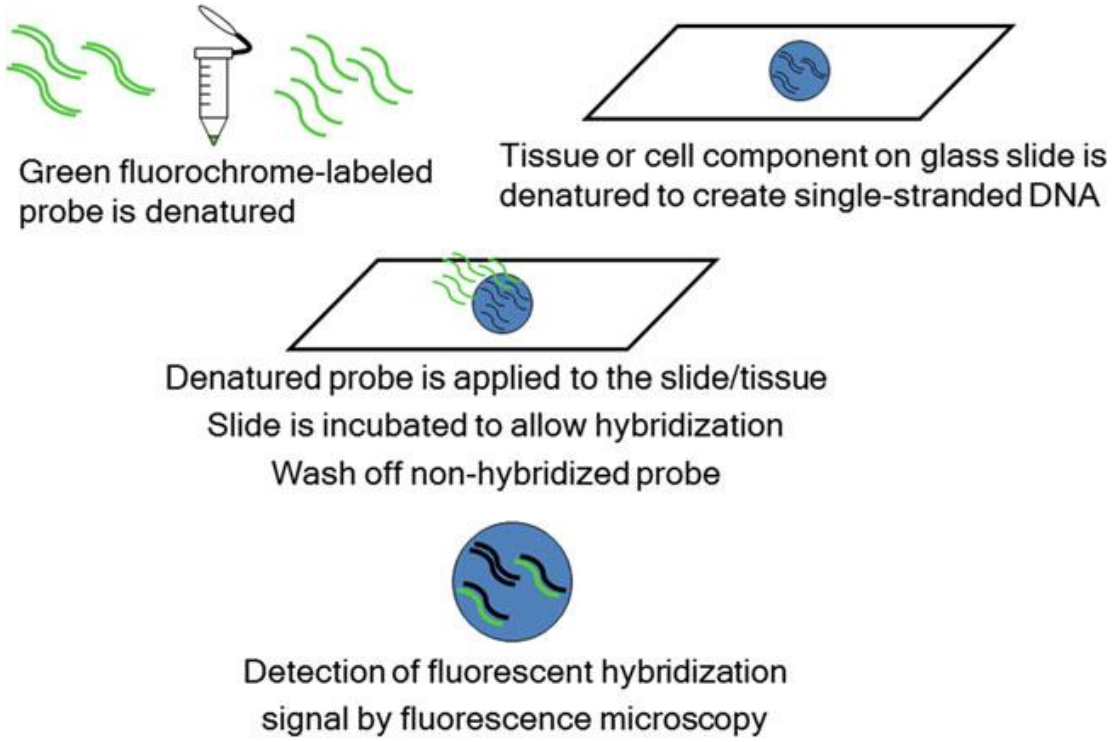


Figure (13): Schematic representation of the basic steps of the FISH procedure ***(Tansatit, 2017).***

Equipments:

- Laminar air flow.
- Sterile graduated plastic Falcon tubes (15mL).
- Fine bore Pasteur pipettes with rubber teats.
- Clean microscopic glass slides kept in cold sterile water.
- Coplin jars for staining and washing of slides.
- Incubat or set at 37°C.
- Low speed centrifuge (800-1000 rpm).
- Hot air oven adjusted to 90°C.
- Refrigerator for storage at 2-8°C.
- Freezer for storage at -20°C.
- Light microscope.

- Chromoscan analysis system♦ (camera system and fluorescence microscope).

4-Interpretation of the results:

At least 100 interphase nuclei and/or 20 metaphases were scanned in every case under the chromoscan for scanning using low power objective, followed by capture using oil immersion objective for the detection of the signals.

Interpretation and signals'patterns :

I) Dual color single fusion LSI BCR-ABL probe:

Negative (normal pattern): 2 red signals (2R) and 2 green signals (2G).

Positive : 1 yellow signal (1Y), 1 red (1R) and 1 green (1G) signals.

Cut off : 10%.

II) Dual color dual fusion LSI BCR-ABL probe:

Negative (normal pattern) : 2 red signals and 2 green signals.

Positive : 2 yellow, 1 red and 1 green signals.

Cut off : 1.3% .

III) LSI BCR/ABL ES dual Color translocation probe:

Negative (normal pattern) : 2 red signals and 2 green signals.

MBCR : 1 yellow, 2 red and 1 green signals.

mBCR : 2 yellow, 1 red and 1 green signals.

ABL deletion : 1 yellow, 1 red and 1 green signals.

Cut off : 10%.

♦Cytovision, Genus Application Software, Version 2.7, UK.

IV) Dual color dual fusion LSI TCF2-PBX1 probe:

Negative (normal pattern) : 2 red signals and 2 green signals.

Positive : 2 yellow, 1 red and 1 green signals.

Cut off : 1.3% .

V) Dual color LSI breakapart MLL probe:

Negative (normal pattern) : 2 yellow signals.

Positive : 1 yellow, 1 red and 1 green signals.

Cut off : 1.3%

5-Storage of the hybridized slides :

After interpretation of the results the hybridized slides were stored at -20°C, in a dark tight dry slide box.

Statistical analysis methods *:*

IBM SPSS statistics (V. 22.0, IBM Corp., USA, 2013) was used for data analysis. Data were expressed as both number and percentage for categorized data. Chi-square test to study the association between each 2 variables or comparison between 2 independent groups as regards the categorized data. The probability of error at 0.05 was considered significant, while at 0.01 and 0.001 are highly significant.

Results

The results of this study are summarized in Tables 13 to 18 and Figures 14 to 31.

Clinical findings:

The current study was carried out on 39 newly diagnosed adult ALL patients.

Out of all patients, 24 (61.5%) were males and 15 (38.5%) were females with male to female ratio of (1.6:1). Their age ranged from 19 to 71 years, with mean age of 45 ± 26 years. **(Table 13)**.

Out of the 39 patients, 18 patients (46.1%) had hepatosplenomegaly while 1 patient (2.6%) had hepatomegaly and 2 patients (5.1%) had splenomegaly, 24 (61.5%) patients presented with lymphadenopathy and 2 (5.1%) patients presented with CNS infiltration. **(Table13 & 14).**

Laboratory findings:

Complete blood picture:

Hemoglobin level (Hb):

In the current study the Hb level ranged from 4.6 to 10.1g/dl with a mean value of (7.35±1.6) g/dl. Thirty six patients (92.3%) had initial hemoglobin level <10g/dl and 3 (7.7%) had hemoglobin level >10g/dl.

Total leucocytic count (TLC):

The TLC ranged from 2.6 to $101x10^9$/L with a mean value of $(51.8±30.2)x10^9$/L. Thirty five (89.7%) patients presented with leukocytosis with 12 patients (30.7%) of them presented with TLC $>50x10^9$/L. The remaining 4 (10.2%) patients were leucopenic (TLC $<4x10^9$/L).

Platelets count:

The platelets count ranged from 33 to $128x10^9$/L with a mean value of $(80.5\pm26.7)x10^9$/L. Thirty three (84.6%) patients had platelets count $<100x10_9$/L, while 6 patients (15.4%) had platelets count $\geq100x10^9$/L.

Absolute peripheral blood blasts:

The absolute PB blasts ranged from 2 to $61x10^9$/L with a mean value of $(31.5)x10^9$/L.

Bone marrow examination:

According to WHO classification, The absolute BM blasts ranged from 24 to $98x10^9$/L with a mean value of $(66\pm21)x10^9$/L. **(Table13 & 14).**

Immunophenotyping (IPT):

Thirty five patients were expressing CD10 while 4 patients were negative to CD10 but positive to other B-cell markers (CD19, CD97a). Among these patients CD13 and CD33 were positive in 6 patients (Cases No.: 1,3,23,25,35 and 38).

Fluorescence in Situ Hybridization analysis:

Metaphase and/or interphase FISH analysis were successfully performed on 39 BM and/or PB samples and revealed the following:

Structural aberrations:

Positive results for t(9;22) was encountered in 11 (28.2%) patients, 11(q23) was detected in 2 (5.1%) patients, t(1;19) was detected in 1 (2.56%) patient.

In this study, ph^1 associated with other aberrations were present in 8 of 11 patients with a percentage of 20.5% of the total 39 patients in the form of; ABL amplifications in 3 patients (7.6%), derivative chromosome 9q34 deletion in 3 patients (7.6%), duplication in 1 patient (2.56%) and 1 patient showed combination of amplification and deletion (2.56%) in ALL patients.

Follow up & clinical outcome of studied all patients:

Follow up was done at day 14 of chemotherapy. Out of the 39 newly diagnosed patients, 16 (41%) patients achieved complete remission; while 23 (59%) patients showed incomplete remission.

Prognostic markers in association to patients outcome :

Statistical analysis of patients' outcome with prognostic markers revealed significant association ($p<0.05$) of CR with TLC $<50x10^9/L$ ($p=0.003$) , age <35 yrs ($p=0.000$) and t(9;22) with ($p=0.05$) **(Table 16).** On the other hand, gender, hepatosplenomegaly, Hb and platlet count showed non-significant statistical difference between the patients who achieved complete remission and those with incomplete remission ($p>0.05$) **(Table 16).**

Results of ALL patients't(9;22) in relation to different prognostic factors:

Showed significant association ($p<0.05$) of ph[1] +ve patients with age >35 years, hepatosplenomegaly, absence of lymphadenopathy, TLC $>50X10^9/L$ and absolute PB blasts $\geq 4.4X10^9/L$, immunophenotyping and other aberrations. On the other hand, gender, CNS infilteration, Hb and platelet count showed non significant statistical difference ($p>0.05$) **(Table 17).**

Results of patients't(9;22) with other aberrations in relation to different prognostic factors:

Showed a significant positive association between other aberrations and age >35 years and also with absolute-P.Bl blasts $\geq 4.4X10^9/L$ with ($p<0.05$). With no significance to other prognostic factors ($p>0.05$) **(Table 18).**

Table (13): Clinical & laboratory findings, IPT and out come of newly diagnosed ALL patients:

Case No	Age (ys)	Sex	Hepatomegaly	Splenomegaly	LN	CNS infilteration	Hb g/dl	TLC x10^9/L	Plat x10^9/L	Absolute PB blast x10^9/L	BM Blast%	IPT	Outcome
1	71	F	P	P	N	N	6	81	39	17.82	90	CD10/13	IR
2	62	M	P	P	N	P	5	76	45	12.16	60	CD10	CR
3	55	M	P	P	P	N	4.9	59	41	14.16	67	CD10/13	IR
4	32	F	P	N	P	N	8.2	3.3	75	0.32	39	CD10	CR
5	29	F	N	N	P	N	9	40	84	4.4	52	CD10	CR
6	41	M	N	N	N	N	8.6	43	58	9	82	CD10	CR
7	20	F	N	N	N	N	7.8	31.6	71	5	86	CD10	CR
8	59	F	P	P	P	N	10	101	33	61.6	98	CD10	IR
9	62	M	N	N	N	N	9.8	84	40	34.4	93	CD10	IR
10	54	M	N	N	P	N	8	33.8	111	2.03	41	N	IR
11	62	M	N	N	P	N	7	41	90	5.74	39	N	IR
12	33	M	N	N	N	N	6	2.6	88	0.23	48	CD10	CR
13	28	F	P	P	P	N	7	27	103	2.16	24	CD10	CR
14	19	F	N	N	P	N	8	39	90	5.5	44	CD10	CR
15	30	M	P	P	P	N	8	26	98	2.86	37	CD10	CR
16	21	M	N	N	P	N	7	28	100	1.68	39	CD10	CR
17	61	M	N	N	N	N	8.9	67.3	87	14.13	25	CD10	IR
18	59	M	P	P	P	N	7	2.6	128	0.312	32	N	IR
19	32	F	N	N	P	N	8	32.4	78	2.9	44	CD10	CR
20	23	F	N	N	P	N	7	32.6	98	2.28	39	CD10	CR
21	34	M	N	N	P	N	7	38.7	69	5.8	52	CD10	CR
22	36	F	P	P	N	N	4.6	44.6	39	4.9	98	CD10	IR
23	55	M	P	P	N	N	10.1	71.6	42	30	92	CD10/13	IR
24	52	M	P	P	N	N	8.6	60.2	61	15.05	67	CD10	IR
25	33	F	P	P	N	N	8.2	28.9	85	9	43	CD10/33	CR
26	19	F	N	P	P	N	7	31	76	3.4	39	CD10	CR
27	24	M	N	N	P	N	8	33.8	89	2.03	44	CD10	IR
28	29	M	N	N	P	N	7	56.1	91	9.5	52	CD10	IR
29	38	M	N	N	P	N	7	28.2	112	3.38	33	CD10	IR
30	45	M	N	P	P	N	7	22	128	0.44	32	N	IR
31	33	M	P	P	P	N	8	32.4	78	1.9	44	CD10	CR
32	34	F	N	N	P	N	7	26.5	98	1.06	39	CD10	IR

Continuous of Table (13): Clinical & laboratory findings, IPT and out come of newly diagnosed ALL patients:

Case No	Age (ys)	Sex	Hepatomegaly	Splenomegaly	LN	CNS infilteration	Hb g/dl	TLC x10^9/L	Plat x10^9/L	Absolute PB Blast x10^9/L	BM Blast%	IPT	Outcome
33	21	F	N	N	P	N	7	38.7	69	0.77	52	CD10	IR
34	25	F	P	P	N	N	5	3.6	39	0.4	98	CD10	IR
35	42	M	P	P	N	P	10.1	71.6	42	22.2	92	CD10/13	IR
36	37	M	P	P	N	N	8.6	51.3	61	10.26	67	CD10	IR
37	45	M	P	P	N	N	8.2	58.9	85	9.4	43	CD10	IR
38	28	M	P	P	P	N	7	31	76	2.8	39	CD10/33	IR
39	31	M	P	P	P	N	8	33.8	89	3.38	44	CD10	IR

CR: Complete Remission, IR: Incomplete Remission, P;Positive, N;Negative, M: Male, F: Female, Hb: hemoglobin, TLC: total leucocytic count, Plat: platelet count, IPT;Immunophenotyping.

Table (14): Discription of clinical and laboratory data of the 39 newly diagnosed ALL patients:

Parameter	No. of patients	Percentage
Gender		
Male	24	61.5%
Female	15	38.5%
Hepatomegaly		
Positive	19	48.7%
Negative	20	51.3%
Splenomegaly		
Positive	20	51.3%
Negative	19	48.7%
Lymphadenopathy		
Positive	24	61.5%
Negative	15	38.5%
CNS infilteration		
Positive	2	5%
Negative	37	95%
Hb		
<10g/dl	36	92.3%
≥10g/dl	3	7.7%
TLC		
<50x10^9/L	27	69.2%
≥50x10^9/L	12	30.8%
Platelet count		
<100x10^9/L	33	84.6%
≥100x10^9/L	6	15.4%
IPT		
CD10		
Positive	35	89.7%
Negative	4	10.3%
CD13/33		
Positive	6	15.4%
Negative	33	84.6%
Clinical outcome		
CR	16	41%
IR	23	59%

CR: Complete Remission, IR: Incomplete Remission, Hb: hemoglobin, TLC: total leucocytic count, IPT;Immunophenotyping.

Table (15): FISH pattern of newly diagnosed ALL patients

Case No.	FISH				Outcome
	Result	**Involved genes**	**Involved chromosomal bands and regions**	**%**	
4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,26,27,30,31,32,,33,39	***Normal FISH***			64.1%	14patients(56 %):CR 11patients(44 %):IR
1,2,3,22,23,24,25,34,35,36,37	t(9;22)	ABL,BCR	*(9q34;22q11)*	28.2%	2patients(18 %):CR 9patients(82 %):IR
28	t(1;19)	PBX1, TCF2	*(1q23;19p13.3)*	2.56%	IR
29,38	11q23 *Rearrangement*	MLL	*(q23)*	5.1%	IR

Table (16): ALL patients' outcome in relation to different prognostic factors

Parameter	Groups	No.	%	CR		IR		P	Significance
				No.	%	No.	%		
Age (years)	≥35	18	46.2	2	12.5	16	69.6	0.000	**HS**
	<35	21	53.8	14	87.5	7	30.4		
Gender M:F 1.6:1	Male	24	61.5	7	43.8	17	73.9	0.057	NS
	Female	15	38.5	9	56.3	6	26.1		
Hepatomegaly	Yes	19	48.7	6	37.5	13	56.5	0.242	NS
	No	20	51.3	10	62.5	10	43.5		
Splenomegaly	Yes	20	51.3	6	37.5	14	60.9	0.151	NS
	No	19	48.7	10	62.5	9	39.1		
Lymphadenopathy	Yes	24	61.5	11	68.8	13	56.5	0.440	NS
	No	15	38.5	5	31.2	10	43.5		
CNS infilteration	Yes	2	5.1	1	6.3	1	4.3	0.791	NS
	No	37	94.9	15	93.7	22	95.7		
Hb	<10g/dl	36	92.3	16	100	20	87	0.133	NS
	≥10g/dl	3	7.7	0	0	3	13		
TLC ($x10^9$/L)	<50	26	66.7	15	93.8	11	47.8	0.003	**HS**
	≥50	13	33.3	1	6.3	12	52.2		
Platelet count ($x10^9$/L)	<100	33	84.6	14	87.5	19	82.6	0.677	NS
	≥100	6	15.4	2	12.5	4	17.4		
Absolute PB blasts	<4.4	19	48.7	9	56.3	10	43.5	0.433	NS
	≥4.4	20	51.3	7	43.8	13	56.5		
IPT	**CD10:**								
	Positive	35	89.7	16	45.7	19	54.3	0.107	NS
	Negative	4	10.3	0	0	4	100		
	CD13/33:								
	Positive	6	15.4	1	16.7	5	83.3	0.306	
	Negative	33	84.6	15	45.5	18	54.5		
t(9;22)	Positive	11	28.2	2	12.5	9	39.1	0.05	**S**
	Negative	28	71.8	14	87.2	14	60.9		
Aberrations type	None	31	79.5	16	100	15	65.2	0.136	NS
	Ampl	3	7.7	0	0	3	13		
	Ampl,Del	1	2.6	0	0	1	4.3		
	Del	3	7.7	0	0	3	13		
	Dupl	1	2.6	0	0	1	4.3		

CR: Complete Remission, IR: Incomplete Remission, P: Prevelance HS: Highly Significant, S: Significant, NS: None Significant, Hb: hemoglobin, TLC: total leucocytic count, IPT; Immunophenotyping, Ampl: Amplification, Del: Deletion, Dupl: Duplication.

Table (17): ALL patients't(9;22) in relation to different prognostic factors:

Parameter	Groups	No.	%	t(9;22) (total No.=11)				P	Significance
				+ve		-ve			
				No.	%	No.	%		
Age(years)	≥35 <35	18 21	46.2 53.8	9 2	81.8 18.2	9 19	32.1 67.9	0.005	HS
Gender M:F 1.6:1	Male Female	24 15	61.5 38.5	7 4	63.6 36.4	17 11	60.7 39.3	0.866	NS
Hepatomegaly	Yes No	19 20	48.7 51.3	11 0	100 0	8 20	28.6 71.4	0.000	HS
Splenomegaly	Yes No	20 19	51.3 48.7	11 0	100 0	9 19	32.1 67.9	0.000	HS
Lymphadenopathy	Yes No	24 15	61.5 38.5	1 10	9.1 90.9	23 5	82.1 17.9	0.000	HS
CNS infilteration	Yes No	2 37	5.1 94.9	1 10	9.1 90.9	1 27	3.6 96.4	0.482	NS
Hb	<10g/dl ≥10g/dl	36 3	92.3 7.7	9 2	81.8 18.2	27 1	96.4 3.6	0.123	NS
TLC ($x10^9$/L)	<50 ≥50	26 13	66.7 33.3	3 8	27.3 72.7	23 5	82.1 17.9	0.001	HS
Platelet count ($x10^9$/L)	<100 ≥100	33 6	84.6 15.4	11 0	100 0	22 6	78.6 21.4	0.095	NS
Absolute PB blasts	<4.4 ≥4.4	19 20	48.7 51.3	1 10	9.1 90.9	18 10	64.3 35.7	0.002	HS
IPT	**CD10:** Positive Negative **CD13/33:** Positive Negative	 35 4 6 33	 89.7 10.3 15.4 84.6	 11 0 5 6	 100 0 45.5 54.5	 24 4 1 27	 85.7 14.3 3.6 96.4	0.314 0.000	NS HS
Other aberrations	**Yes** **No**	11 28	28.2 71.8	11 0	100 0	0 28	0 100	0.000	HS
Aberrations type	None Ampl Ampl,Del Del Dupl	31 3 1 3 1	79.5 7.7 2.6 7.7 2.6	3 3 1 3 1	27.3 27.3 9.1 27.3 9.1	28 0 0 0 0	100 0 0 0 0	0.000	HS
Del	Positive Negative	4 35	10.3 89.7	4 7	36.4 63.6	0 28	0 100	0.001	HS
Ampl	Positive Negative	4 35	10.3 89.7	4 7	36.4 63.6	0 28	0 100	0.001	HS
Dup	Positive Negative	1 38	2.6 97.4	1 10	9.1 90.9	0 28	0 100	0.106	NS

P: Prevelance HS: Highly Significant, S: Significant, NS: None Significant, Hb: hemoglobin, TLC: total leucocytic count, IPT; Immunophenotyping, Ampl: Amplification, Del: Deletion, Dupl: Duplication.

Table (18): ALL patients't(9;22) with other aberrations in relation to different prognostic factors:

Parameter	Groups	NO.	%	other aberrations								P	Significance
				Ampl		Ampl, Del		Del		Dupl			
				No.	%	No.	%	No.	%	No.	%		
Age(years)	≥35	7	87.5	3	100	1	100	3	100	0	0	0.046	S
	<35	1	12.5	0	0	0	0	0	0	1	100		
Gender M:F 1.6:1	Male	5	62.5	2	66.7	1	100	2	66.7	0	0	0.510	NS
	Female	3	37.5	1	33.3	0	0	1	33.3	1	100		
Hepatomegaly	Yes	8	100	3	100	1	100	3	100	1	100	*	*
Splenomegaly	Yes	8	100	3	100	1	100	3	100	1	100	*	*
Lymphadenopathy	Yes	1	12.5	0	0	0	0	1	33.3	0	0	0.592	NS
	No	7	87.5	3	100	1	100	2	66.7	1	100		
CNS infilteration	NO	8	100	3	100	1	100	3	100	1	100	*	*
Hb	<10g/dl	6	75	2	66.7	0	0	3	100	1	100	0.217	NS
	≥10g/dl	2	25	1	33.3	1	100	0	0	0	0		
TLC (x109/L)	<50	2	25	0	0	0	0	1	33.3	1	100	0.217	NS
	≥50	6	75	3	100	1	100	2	66.7	0	0		
Platelet count (x109/L)	<100	8	100	3	100	1	100	3	100	1	100	*	*
Absolute PB blasts	<4.4	1	12.5	0	0	0	0	0	0	1	100	0.046	S
	≥4.4	7	87.5	3	100	1	100	3	100	0	0		
IPT	CD10: Positive	8	100	3	100	1	100	3	100	1	100	0.446	NS
	Negative	0	0	0	0	0	0	0	0	0	0		
	CD13/33: Positive	4	50	2	66.7	1	100	1	33.3	0	0	0.446	NS
	Negative	4	50	1	33.3	0	0	2	66.7	1	100		

P: Prevelance HS: Highly Significant, S: Significant, NS: None Significant, Hb: hemoglobin, TLC: total leucocytic count, IPT; Immunophenotyping, Ampl: Amplification, Del: Deletion, Dupl: Duplication.

*No statistical comparison could be done as all cases had hepatosplenomegaly, Plts<100 x109/L and no CNS infilteration.

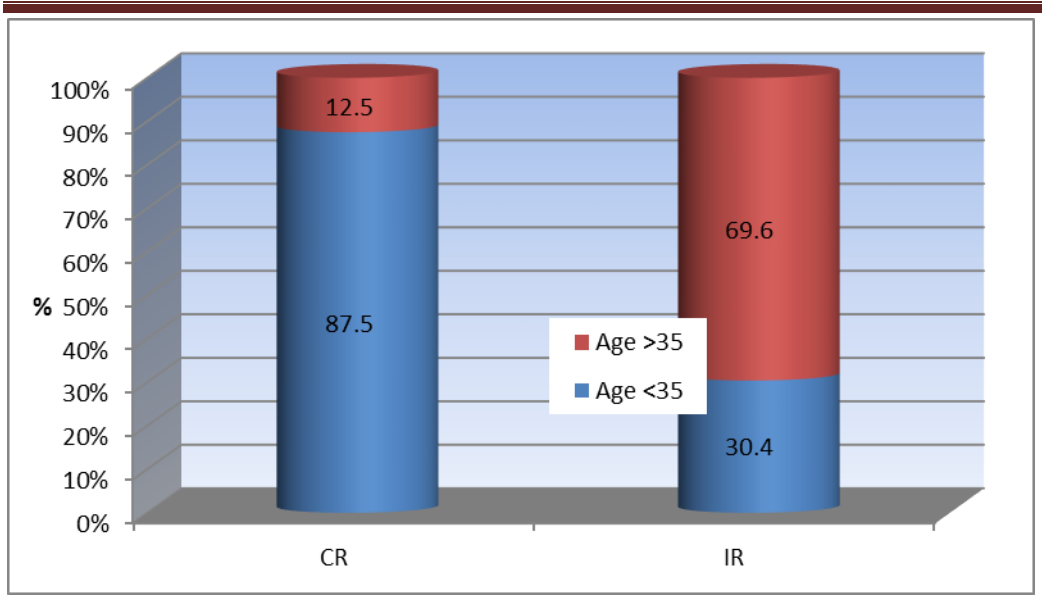


Figure (14): Bar chart of outcome according to age .

- 69.6% of patients with IR had age> 35 yrs old.

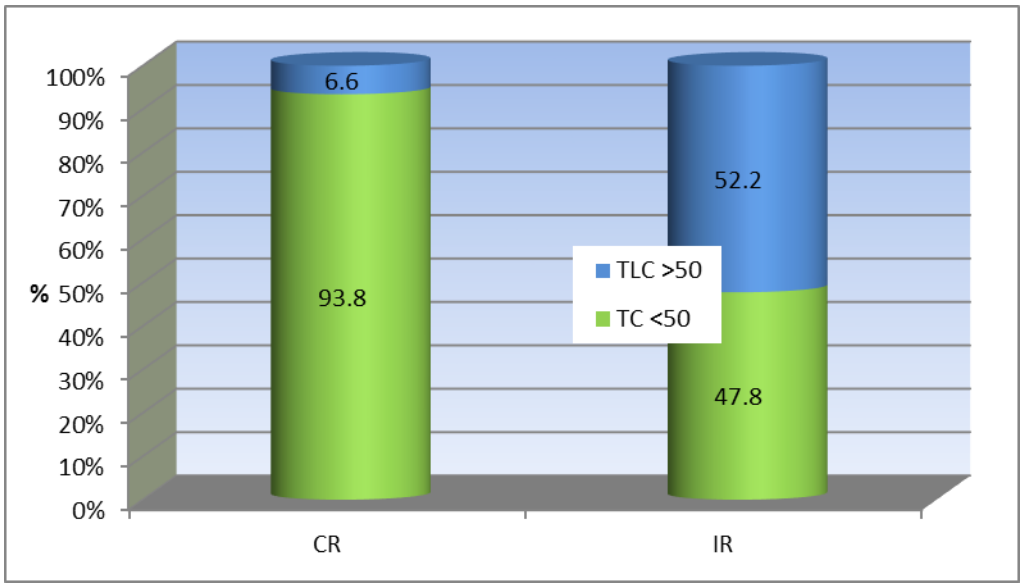


Figure (15): Bar chart of outcome according to TLC.

- 52.2% of patients with IR had TLC > $50X10^9$/L.

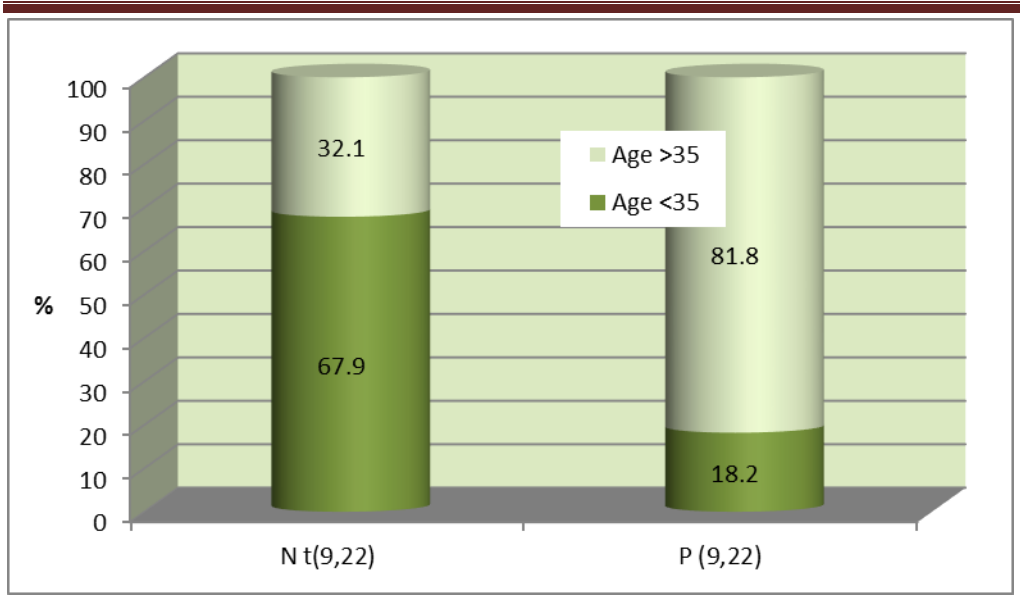


Figure (16): Bar chart of age in relation to t(9;22) positive ALL patients.

- 81.8% of patients with t(9;22) had age> 35 yrs old.

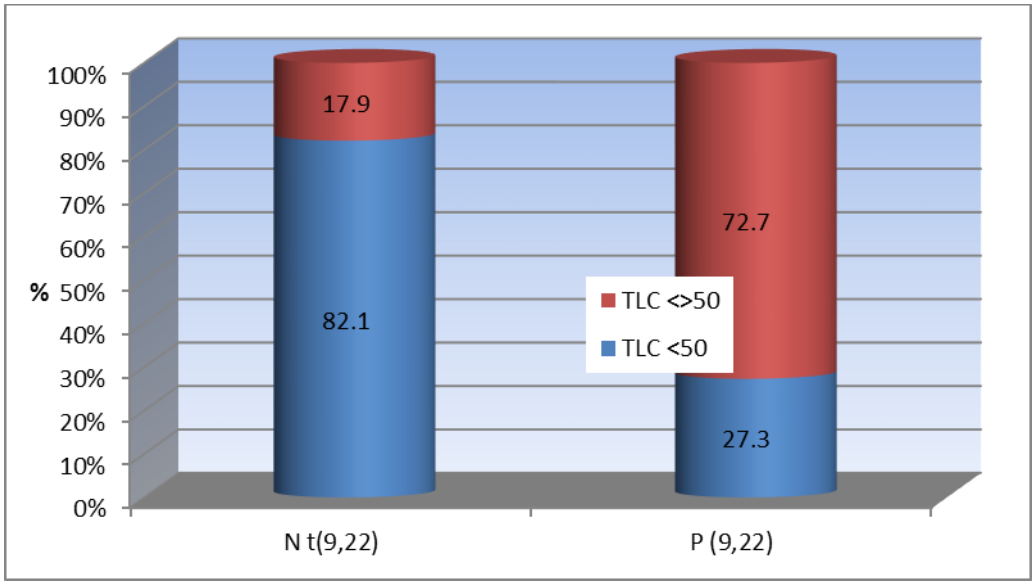


Figure (17): Bar chart of TLC in relation to t(9;22) positive ALL patients.

- 72.7% of patients with t(9;22) had TLC > $50X10^9$/L.

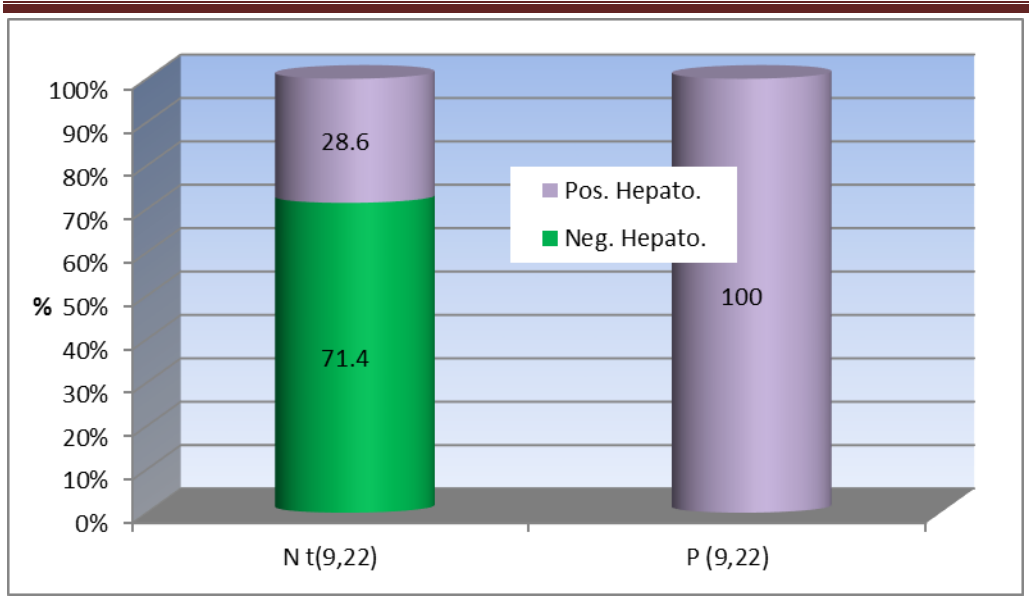


Figure (18): Bar chart of hepatomegaly in relation to t(9;22) positive ALL patients.

- 100% of patients with t(9;22) had hepatomegaly.

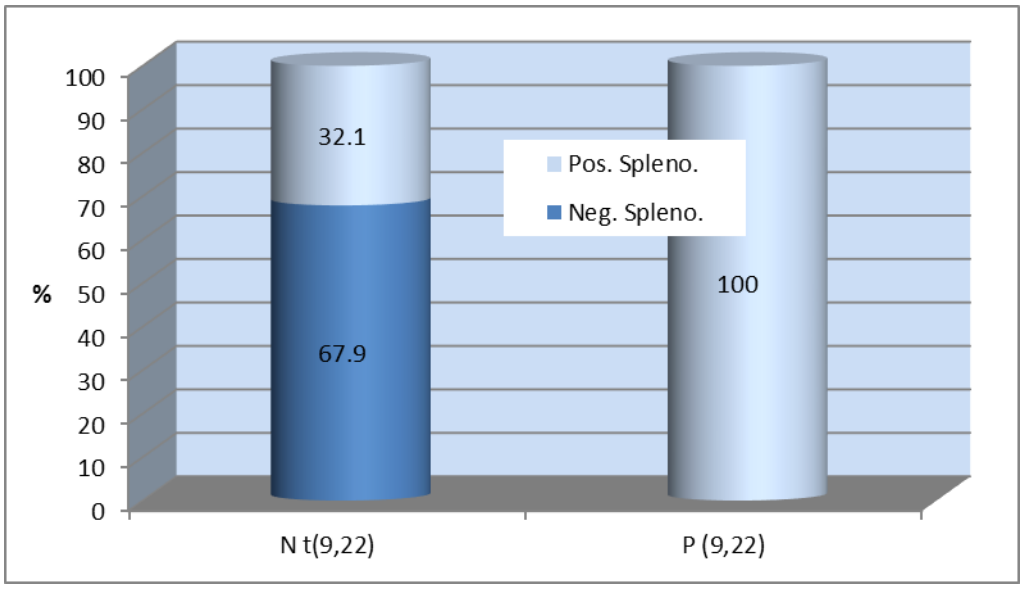


Figure (19): Bar chart of splenomegaly in relation to t(9;22) positive ALL patients.

- 100% of patients with t(9;22) had splenomegaly.

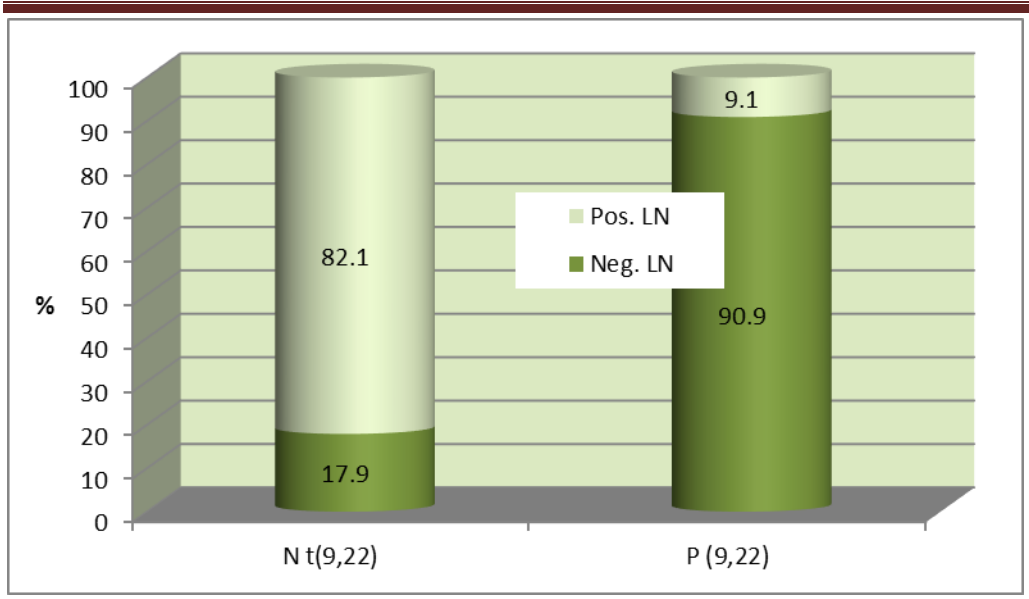


Figure (20): Bar chart of lymphadenopathy in relation to t(9;22) positive ALL patients.

- 90.9% of patients with t(9;22) had no lymphadenopathy.

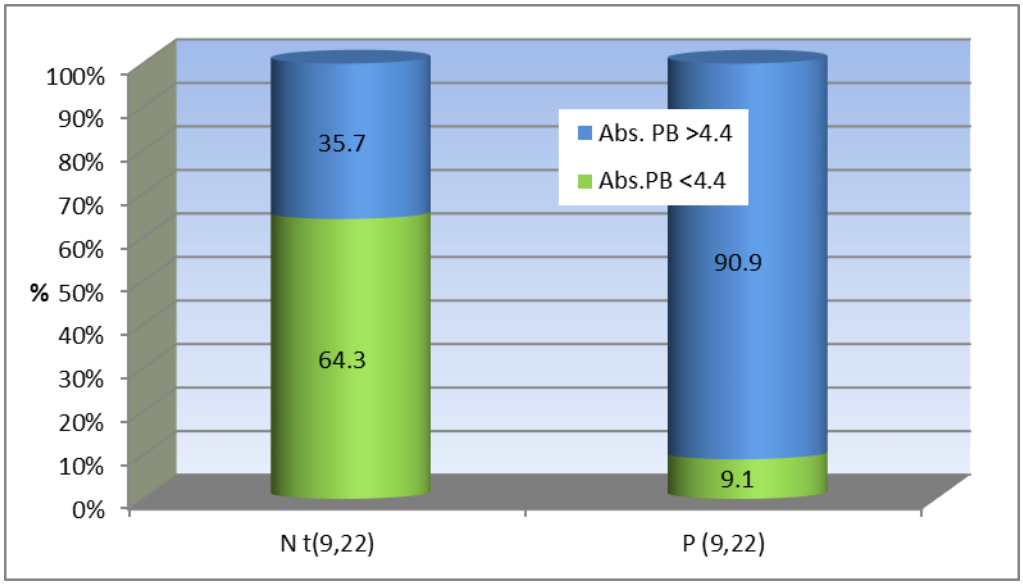


Figure (21): Bar chart of absolute PB blasts in relation to t(9;22) positive ALL patients.

- 90.9% of patients with t(9;22) had absolute PB blasts >4.4X10^9/L.

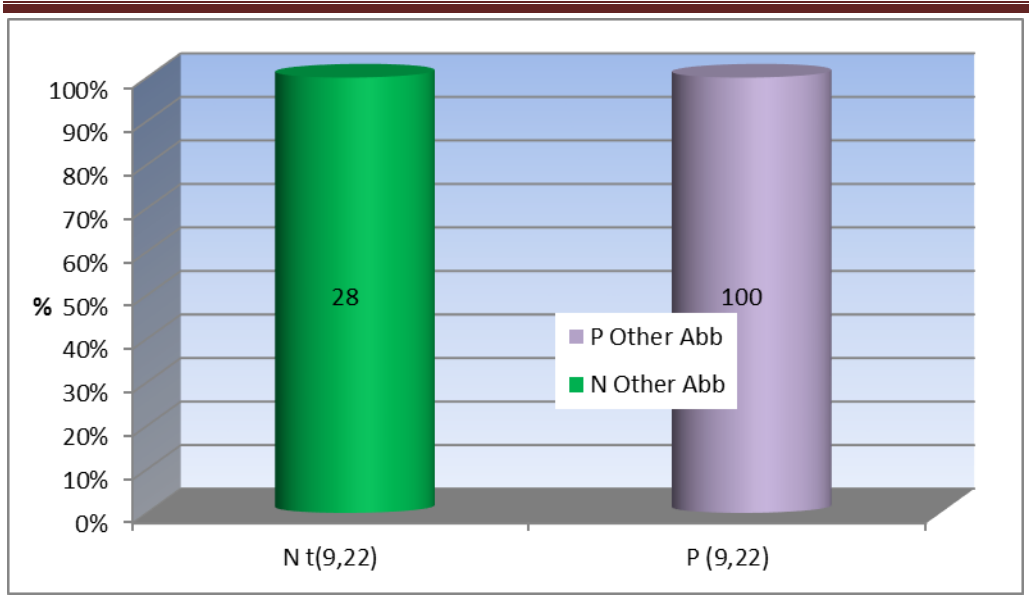


Figure (22): Bar chart of other aberrations in relation to ph[1] positive ALL.

- 100% of patients with t(9;22) had other aberrations.

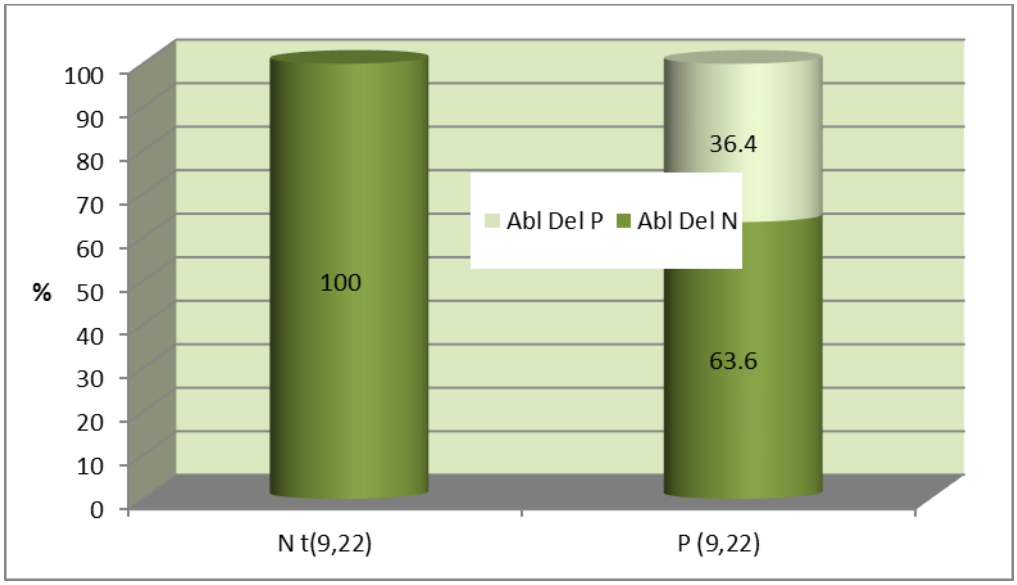


Figure (23): Bar chart of ABL deletion in relation to ph[1] positive ALL.

- 36.4 % of patients with t(9;22) had ABL deletion.

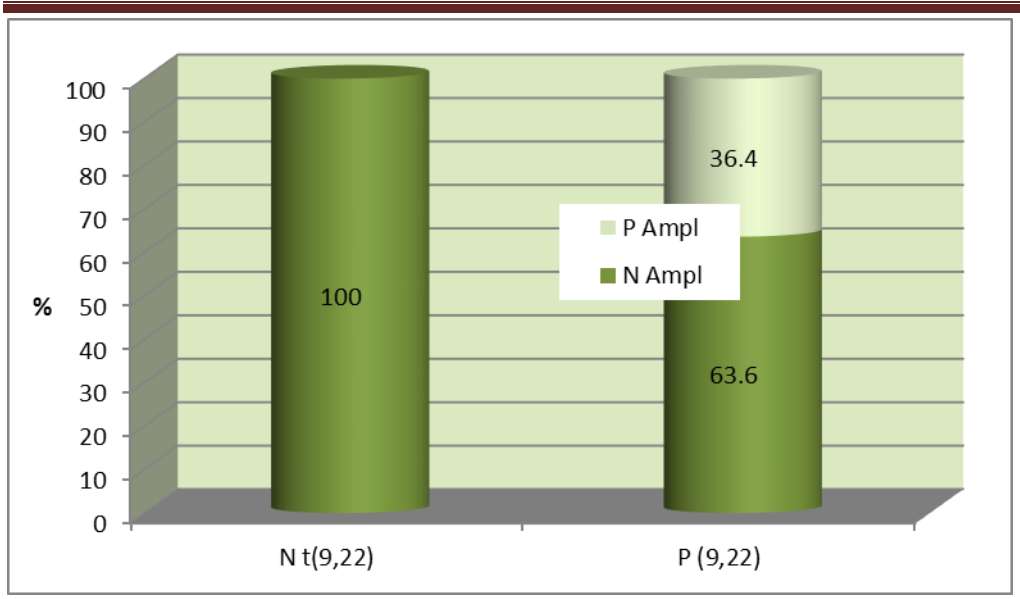


Figure (24): Bar chart of amplification in relation to ph[1] positive ALL.

- 36.4 % of patients with t(9;22) had amplification.

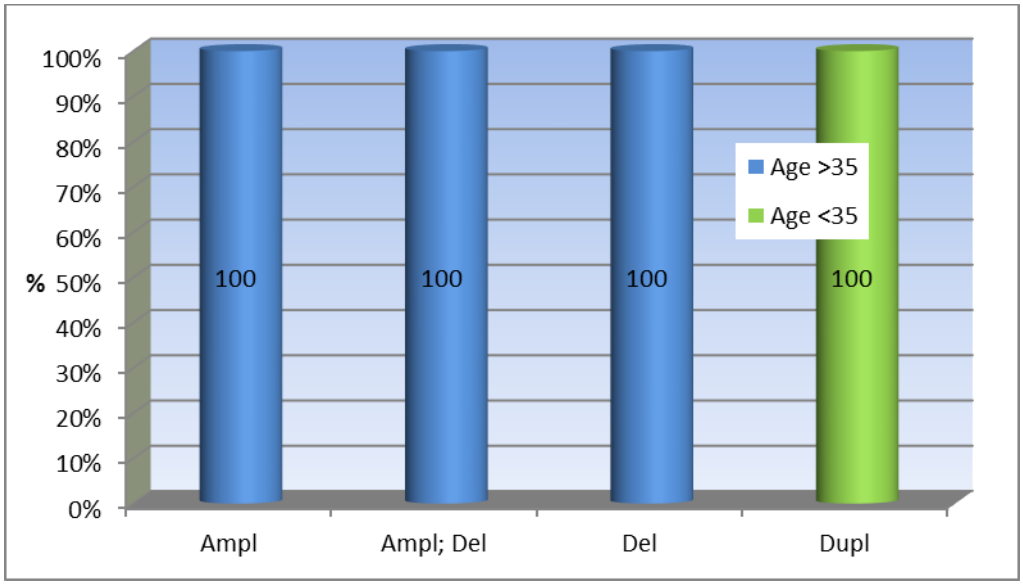


Figure (25): Bar chart of age in relation to ph[1] positive ALL associated with other aberrations

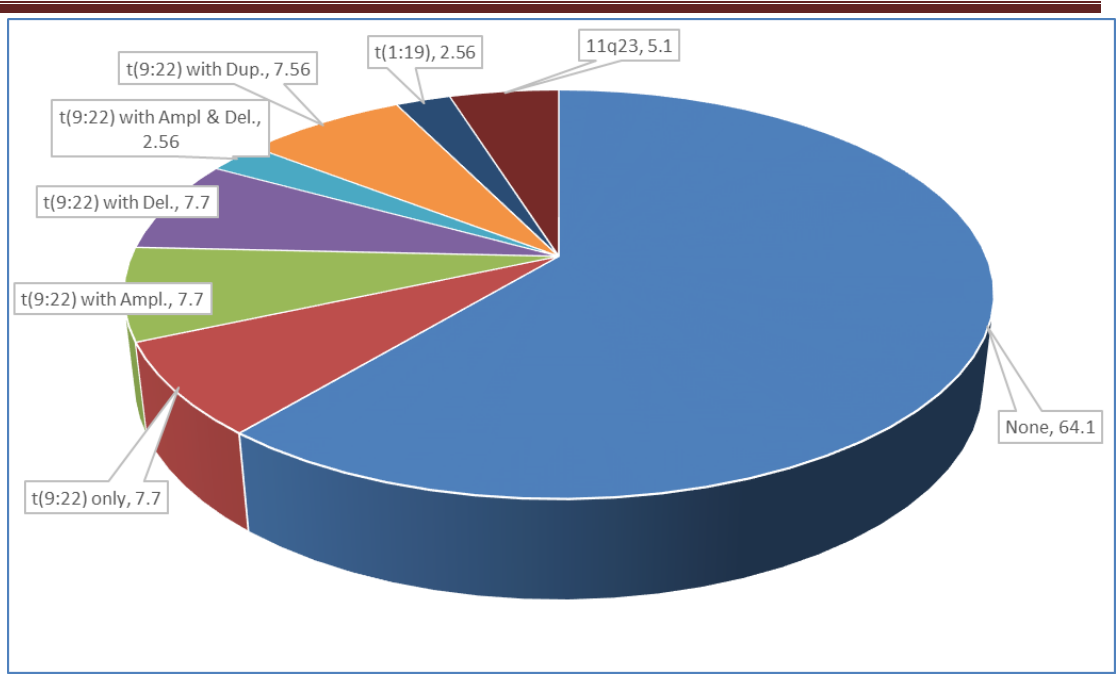


Figure (26): A pie chart results of structural aberrations in all patients. structural aberrations are detected in 14/39(35.9%), among them t(9;22) is the most common (78.5%).

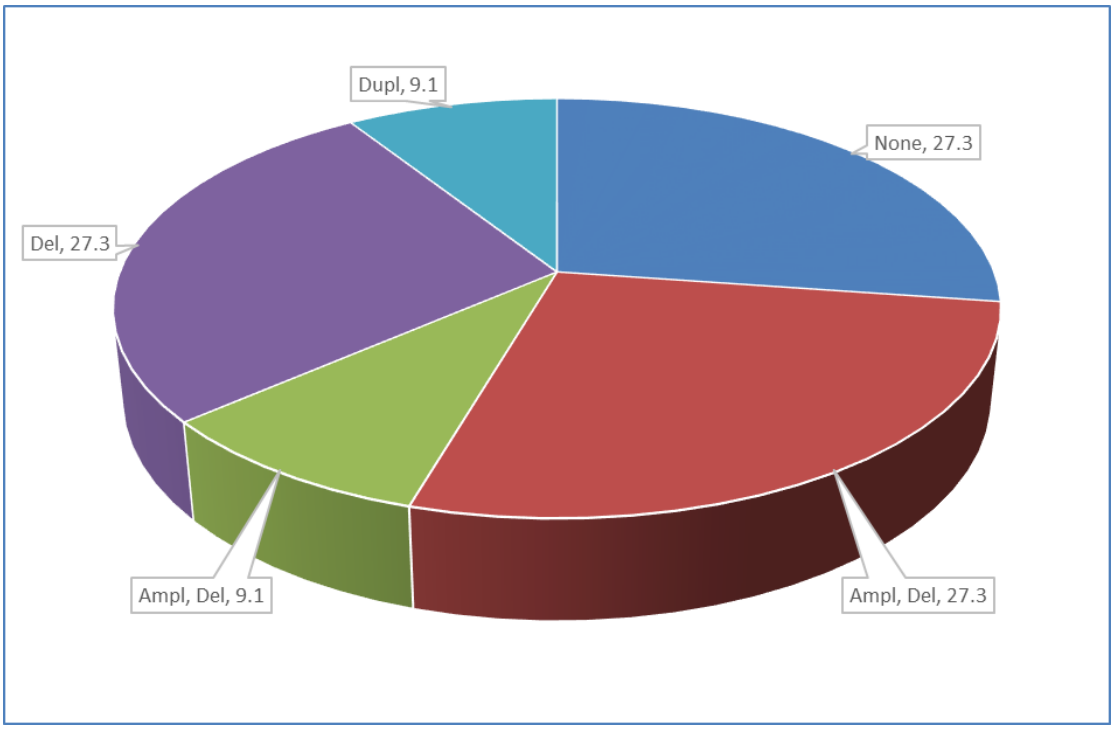


Figure (27): A pie chart results of other aberrations detected in ph[1] positive ALL.

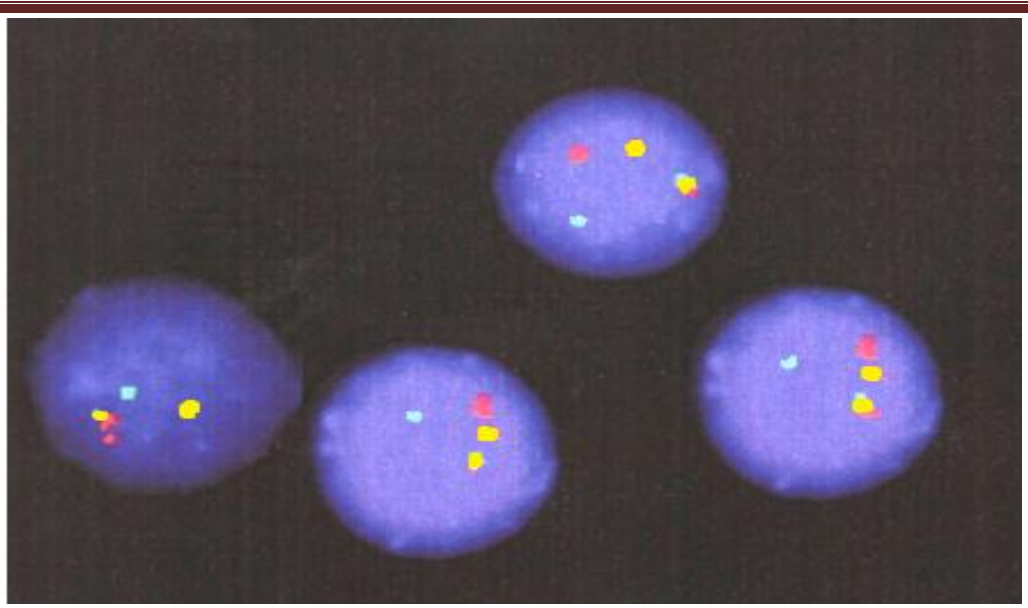


Figure (28): mBCR / ABL1 positivity by ES-FISH in 63 % of interphase cells showed a signal pattern of 2Y1G1R .

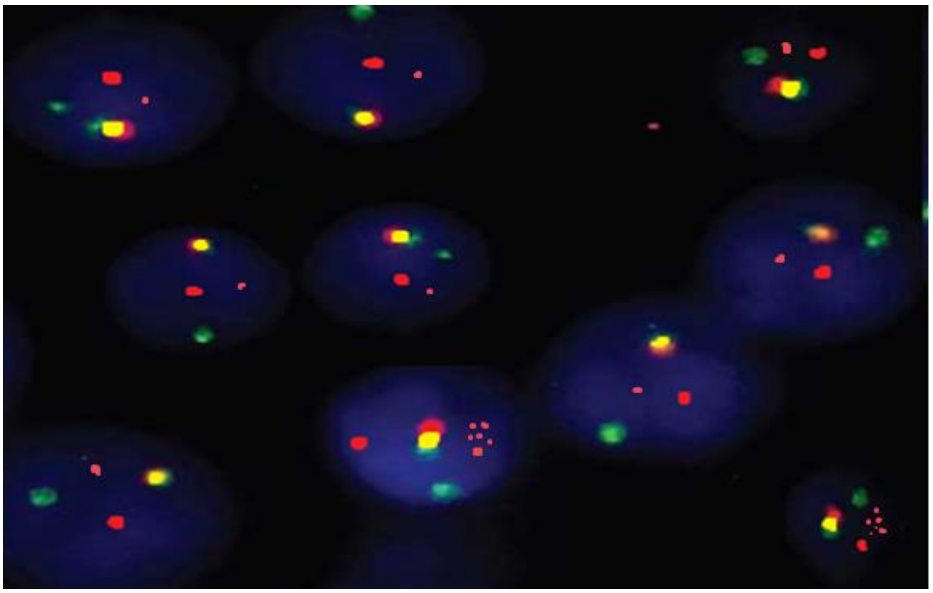


Figure (29): Ph-positive ALL by ES-FISH in 93% of interphase cells showed a signal pattern of 2R1G1Y in 78% of cells with identified MBCR / ABL1, and a signal pattern of 1R1G2Y in 15% of cells identified mBCR / ABL1 associated with amplification of ABL gene (mutiple copies of ABL gene in red) in 16% of cells.

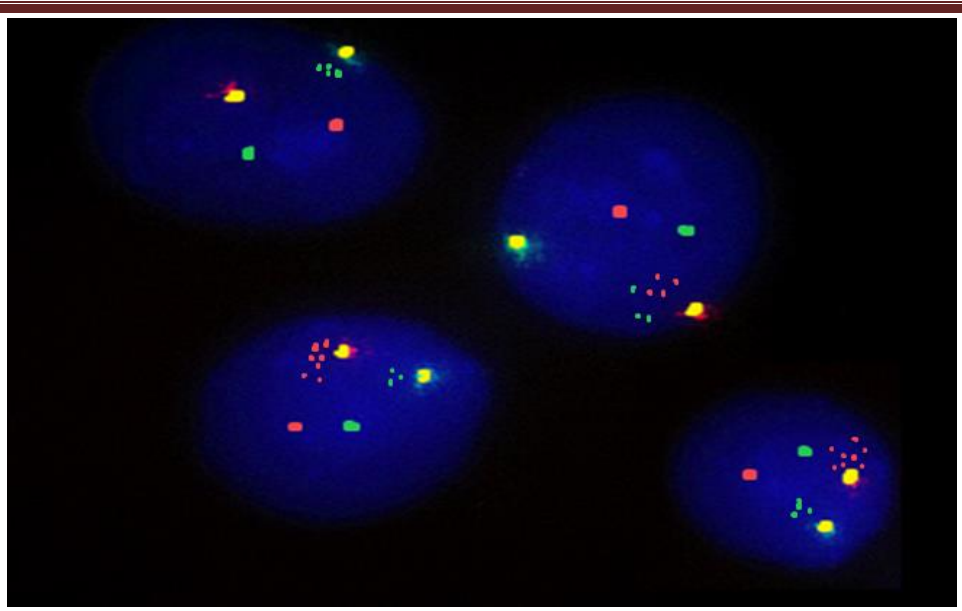


Figure (30): mBCR / ABL1 by ES-FISH in interphase cells showed a signal pattern of 2Y1G1R associated with amplification of both ABL gene (mutiple copies of ABL gene in red) and BCR gene (mutiple copies of BCR gene in green) in 22% of cells.

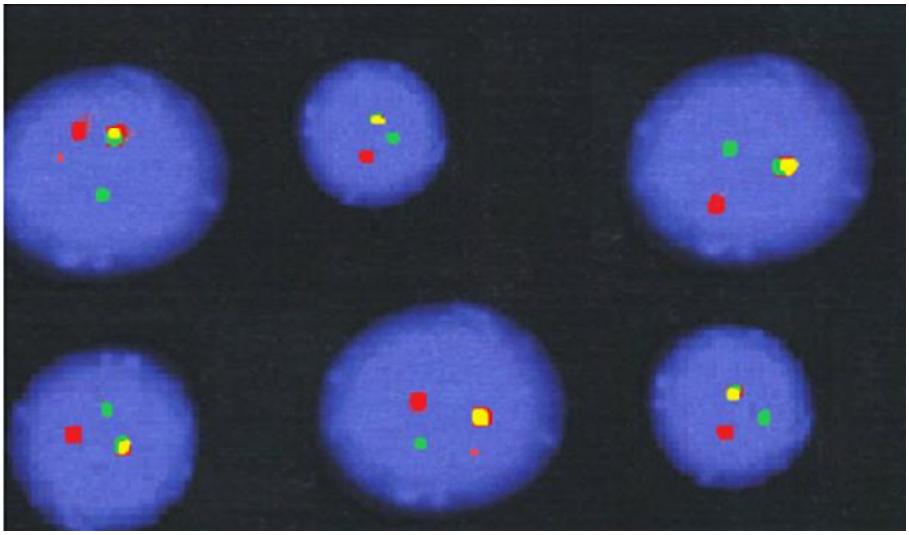


Figure (31): Deletion of ABL gene by ES-FISH in interphase cells with signal pattern of 1Y1G1R in 59% of cells.

Discussion

Acute lymphoblastic leukemia (ALL) is a hematologic malignancy propagated by impaired differentiation, proliferation, and accumulation of lymphoid progenitor cells in the bone marrow and/or extramedullary sites. **(Paul Shilpa et al., 2016).**

Cytogenetic abnormalities are independent prognostic variables for predicting the outcome of ALL. Recent genomic studies have analysed various cytogenetic abnormalities of ALL and increased the number of potential prognostic markers ***(Moorman et al., 2014).***

Adult and childhood ALL differ markedly in the prevalence of various cytogenetic abnormalities. Philadelphia chromosome (Ph^1) positive ALL, a high-risk cytogenetic subset, accounts for 25-30% of adult ALL cases but occurs in less than 5% of children ***(Ghazavi F et al.,2015).***

Extra-signal FISH is a fast and cost effective technique not only to evaluate BCR/ABL fusion in ALL and CML but moreover able to discriminate various rearrangement of BCR/ABL into major & minor fusions, ABL & BCR deletion, duplication and amplification ***(Lee et al.,2011).***

In the light of this, the present work aims to detect BCR-ABL genes fusion, amplification and deletion in acute lymphoblastic leukemia patients, using extra signal fluorescence in situ hybridization (ES-FISH), and to assess their relation with other standard prognostic factors and therapeutic response.

The current study was carried out on thirty nine newly diagnosed patients suffering from acute lymphoblastic leukemia.

Follow up was done at day 14 of chemotherapy. Out of the 39 newly diagnosed patients; 16 (41%) patients achieved complete remission while 23 (59%) patients showed incomplete remission, this result is in concordance with ***Paul Shilpa et al.,2016*** who stated that the rate of CR in adult ALL represent about 40%.

The age of ALL patients in this study ranged from 19 to 71 years with a mean of (45 ± 26) years. 21 (53.8%) out of them were less than 35 years old . High significant relation (p=0.000) was detected between the patients age less then 35 years and good patients outcome. Similarly to ***Hoelzer et al., 1988 and Greer et al.,2014*** who found a better prognosis in patients younger than 35 years.

In the current work, male to female ratio was (1.6:1) with slight male predominance, Similar observation was reported by ***Ilana de Franc et al.,2014*** who noticed a male predominance in ALL adults patients. In the present study, no statistical association was encountered between gender and outcome (p>0.05) with agreement to; ***Bassan et al.,2009 and Ilana de Franc et al.,2014*** who recorded no significant difference between gender and patients succeeded to achieve complete remission and those with incomplete remission.

As regards clinical findings in this work, 18 patients (46.1%) had hepatosplenomegaly while 1 patient (2.6%) had hepatomegaly and 2 patients (5.1%) had splenomegaly, 24 patients (61.5%) had lymphadenopathy and 2 patients (5.1%) had CNS infilteration . All showed no significant association (p>0.05) with patients outcome. These previous findings are in concordance with ***Lu et al., 2008*** who observed no relation between clinical data and patients outcome.

As regards TLC four (10.2%) patients were presented with leucopenia, while all the rest of thirty-nine (89.7%) presented with

leucocytosis, 12 (30.7%) out of them were ≥50X10^9/L. High significant association (p=0.003) was detected between the initial TLC ≥50X10^9/L and poor patients outcome (IR). These results showed agreement with ***Paul Shilpa et al., 2016*** where high TLC was highly significantly correlated with poor prognosis. All patients were anemic and thrombocytopenic. No significant statistical relation was detected between Hb level<10g/dl (p=0.133) and platelets <100x10^9/L (p=0.677) and patients outcome.

Immunophenotypic patterns of the 39 ALL patients reveled precursor B-ALL in all of them. CD10 was positively expressed in 35 (89.7%) patients, 16 of them achieved CR; no significant (P=0.107) statistical association was detected between CD10 positivity and favourable outcome. Similary ***Vitale et al.,2006*** reported that CD10 expression had no statistical relationship with the outcome. Also, there was no significant association (p=0.306) between patients' outcome and CD13 or 33 positive aberrant expression.

In the present work BCR/ABL fusion was detected in 11 patients (28.2%). This is in concordance with ***Ghazavi F et al., 2015*** who reported that BCR/ABL fusion gene is presented with an incidence about 30% in adult but slightly higher than ***Noreen et al.,2012*** who reported that BCR/ABL fusion gene is detected with an incidence 20.3% **.**

Moreover *MLL* (11q23) gene rearrangements were presented in 2 patients (5.1%) which lesser than **Schafer et al., 2015** who reported *MLL* gene rearrangement with 10% in adult ALL and 8% of pediatric ALL with about 80% of them in infants. The t(1;19) was encountered in one patient (2.56%) which is in concordance with ***Al Ustwania et al., 2016*** who reported t(1;19) 3% in adult ALL.

Using ES-FISH probe, interphase analysis showed 28.2% positivity for the *BCR-ABL* fusion gene in the form of ; minor pattern in 4 cases (36.4%), major pattern in 1 case (9.1%) and mixed pattern in 6 cases (54.5%) which differ from ***Ilana de Franc et al.,2014*** who observed a 32.2% positivity for the *BCR-ABL* fusion gene in 31 B-cell adult ALL patients including the minor (40%), major (30%) and both forms (30%) and differ from ***Steven et al., 2016*** who observed half of ALL patients with *BCR-ABL* fusion gene had major pattern and another half had minor pattern.

Other aberrations associated with ph^1 positive patients were presented in 8 cases (72.7%). This is in concordance with ***Chang Ahn et al.,2017*** who reported the presence of other aberrations associated with ph^1 with an incidence (73%). On the other hand, out of the total 39 patients in our study other aberrations associated with ph^1 represented (20.5%). Derivative chromosome 9q34 deletion was observed in 3 patients (7.7%) and the forth showed deletion associated with amplification (2.56%). Duplication was observed in 1 patient (2.56%) while ABL amplifications was observed in 3 patients (7.7%) and the forth was presented above as deletion with amplification. These results is higher than ***Harrison, 2009*** who reported amplification with a frequency of 5.8%.

There was high significant, negative association between outcome and positive philidelphia chromosome, among the 11 ph^1 positive patients; nine patients had IR and only two patients had CR. these results are in agreement with ***(Aldoss et al.,2015).***

However when the patients were divided according to t(9;22) in relation to different prognostic factors. It showed that most of ph^1 positive ALL patients were presented with age > 35 years and a high significant relation (p=0.005) was detected between the patients age and ph^1 positive

ALL. As regards clinical findings in this work, all of ph^1 positive ALL patients had hepatosplenomegaly with high significant relation (p=0.000) between them. While 9.1% of ph^1 positive ALL patients had lymphadenopathy with high significant, negative relation (p=0.000) between lymphadenopathy and ph^1 ALL. The CNS infilteration in 9.1% showed no significant association (p=0.482) to ph^1, but these results differ from ***Ilana de Franc et al.,2014*** who stated no statistically significant differences between BCR-ABL positive and negative patients in respect to the clinical variables.

As regards the hematological findings, there was high significant statistical association between t(9;22) and TLC $\geq 50X10^9$/L where 72.7% of ph^1 positive ALL patients had TLC $\geq 50X10^9$/L with p=0.001 and with absolute PB blasts $\geq 4.4X10^9$/L with p=0.001. These findings are concordant with the previously published reports by ***Cetin et al.,2012.***

No significant statistical association was detected between t(9;22) and Hb level<10g/dl (p=0.123) and platelets $<100x10^9$/L (p=0.095). These finding are concordant with the previously published reports by ***Cetin et al.,2012.***

All the ph^1 positive ALL patients in this work showed CD10 +ve (11 patients) with aberrant expression of CD13 or 33 in 5 patients, with no significant (P=0.314) statistical association between CD10 and t(9;22). Similary ***Sanam et al.,2015*** reported that CD10 expression had no statistical relationship with t(9;22). On the other hand, there was high negative significant association (p=0.000) between t(9;22) and CD13 or 33 positive aberrant expression.

As regard the 8 patients with ph^1 associated with other aberrations, there was significant statistical association between other aberrations (deletions, amplifications and duplication) and patients age ≥35 years

(p=0.046) and also with absolute-PB blasts ≥4.4X10^9/L with (p=0.001) while no significant statistical association could be detected with any of the following; gender, hepatomegaly, splenomegaly, lymphadenopathy, CNS infilteration, TLC ≥50X10^9/L, Hb level<10g/dl, platelets <100x10^9/L and IPT of CD10 & CD13 or 33. According to our best knowledge, no previous studies had analysed statistical relation between BCR/ABL gene deletion, amplification or duplication and standard prognostic factors.

Summary and Conclusion

Acute lymphoblastic leukemia (ALL) is a malignant disorder of lymphoid progenitor cells that proliferate and replace the normal hematopoietic cells of the bone marrow. These lymphoblasts replace the normal bone marrow elements that result in a marked decrease in the production of normal blood cells.

There are many prognostic factors in ALL such as age, sex, leukemic burden, laboratory criteria (initial TLC, hemoglobin level and platelet count), immunophenotyping, cytogenetic profile, duration of induction of remission, drug resistance profiles, and minimal residual disease. Assessment of these factors is mandatory for therapeutic assignment.

Cytogenetic abnormalities are independent prognostic variables that predict the outcome of adult ALL patients. Recent genomic studies have provided a refined genetic map of ALL and increased the number of potential prognostic markers.

The Philadelphia chromosome (ph^1) results from a translocation involving the break-point cluster region of the BCR gene on chromosome 22 and the ABL gene on chromosome 9. Ph^1 positive ALL represent a high-risk cytogenetic subset, accounts for 25-30% of adult ALL cases and this chromosome is known to be associated with the worst prognosis among patients with ALL.

The use of ES-FISH probes in interphase nuclei of a large series of BCR/ABL+ve leukemias is associated with the observation of a variable number of different interphase FISH patterns. The most frequently detected patterns corresponded to typical BCR/ABL gene

rearrangements involving the MBCR and the mBCR breakpoints. Discrimination between these two breakpoint regions could not be achieved with the single fusion or double fusion D-FISH probe. Interestingly, additional chromosomal abnormalities (eg; supernumerary Ph, gain or loss of chromosomes 9 and 22, as well as deletions of 9q and 22q) can occur in BCR/ABL+ve ALL patients.

The present study aimed to detect BCR/ABL genes fusion, amplification, deletion and/or other aberrations in acute lymphoblastic leukemic patients, using extra signal fluorescence in situ hybridization (ES-FISH), and to assess their relation with other standard prognostic factors and therapeutic response.

The current study was carried out on 39 newly diagnosed adults ALL patients. Informed consent was obtained from patients to use their samples in this study. Patients were evaluated at day 14 of therapy to assess therapeutic response. All patients were subjected to the following:

A. History and clinical examination laying stress on the presence of hepatosplenomegaly, lymphadenopathy and CNS infelteration.

B. Laboratory investigations, which included:

1. Complete blood count using Sysmex XN-1000 & SA-01.

2. Examination of Leishman stained PB smears laying stress on differential leucocytic count , assessment of blast cell number and morphology.

3. Bone marrow aspiration and examination of Leishman stained smears.

4. Immunophenotyping on BM or PB samples, using acute leukemia panel performed on EPICS XL Coulter Flow Cytometer, USA.

5. Fluorescence in situ hybridization using the following probes:

-LSI dual color single fusion and double fusion BCR/ABL probes for detection of t(9;22)(q34; q11).

-LSI dual color extrasignal BCR/ABL probe for detection of t(9;22) with other aberrations as; amplification, deletion or duplication.

-LSI dual color double fusion TCF2/PBX1 for detection of t(1;19)(q23;p13.3).

- LSI dual color breakapart rearrangement MLL probe for detection of 11q23 rearrangement.

In this work, BCR/ABL fusion observed with a percentage of 28.2% (11/39 cases) in adult ALL patients. The ph[1] associated with other aberrations were presented in 8 of 11 patients with a frequency of (72.7%) and represented (20.5%) of total 39 cases of ALL adults patients. The most common two atypical FISH signal patterns were amplifications and deletions. ABL amplifications were observed in three cases with a frequency of 7.7% in ALL patients while derivative chromosome 9q34 deletion was observed in three patients with a frequency of 7.7% in ALL patients the same as amplifications. Duplication was observed in one patient with a frequency of 2.56% in ALL patients and one patient show combination of amplification and deletion with a frequency of 2.56%.

Follow up was done at day 14 of chemotherapy. Out of the 39 newly diagnosed patients; 16 (41%) patients achieved complete remission while 23 (59%) patients showed incomplete remission.

In the present study there was high significant, negative relation between outcome and positive philidelphia chromosome t(9;22). Among

11 cases with ph^1 +ve, nine patients had IR and only two patients had CR.

Statistical analysis of patients'outcome with prognostic markers revealed significant association ($p<0.05$) of CR with age <35 years and TLC $<50x10^9/L$**.** On the other hand, gender, hepatosplenomegaly, lymphadenopathy, CNS infilteration, Hb, platelet count and immunophenotyping showed non significant statistical difference between the patients who achieved complete remission and those with incomplete remission ($p>0.05$).

Analyzing the relationship of t(9;22) with various studied standard prognostic factors, revealed significant association ($p<0.05$) of ph^1 +ve patients with age >35 years, hepatosplenomegaly, absence of lymphadenopathy, TLC $\geq 50X10^9/L$ and absolute-PB blasts $\geq 4.4X10^9/L$, immunophenotyping and other aberrations. On the other hand, gender, CNS infilteration, Hb and platelet count showed non significant statistical difference ($p>0.05$).

Regarding the presence of other genetic aberrations associated ph^1 in relation to different prognostic factors. The present study showed a significant positive association between their presence and age ≥35 years and also with absolute-PB blasts $\geq 4.4X10^9/L$ with ($p<0.05$). With no significance to other prognostic factors.

In conclusion, BCR/ABL fusion gene analysis by ES-FISH may serve as a powerful prognostic marker in adulthood ALL. The age, TLC and t(9;22) represent the significant standard prognostic factors in relation to patients' outcome. Moreover, philidelphia chromosome with additional chromosomal abnormalities and gene amplification affecting BCR/ABL are efficiently detected by ES-FISH and show significant

association with patients' outcome that may be used as prognostic indicators for therapeutic response.

RECOMMENDATIONS

In view of the present study, we recommend the following:

- Further studies on a wide scale of ALL patients for accurate assessment of incidence and prognostic value of BCR/ABL genes fusion with amplification and/or deletion or duplication.
- Further studies are needed to decide an optimal approach to analyze BCR/ABL genes expression in clinical samples.
- Integration of morphology, IPT, cytogenetic and molecular analysis is mandatory for accurate diagnosis of ALL patients.
- Screening for BCR/ABL kinase domain mutations on wide scale ALL patients is highly recommended if minimal residual diseases is suspected, especially for T3151, E255K and Y253H mutations.