Clinical Cytogenetics in the Diagnosis and Prognosis of Leukemias

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Introduction

Leukemias are a group of disorders characterized by accumulation of malignant white cells in the bone marrow and blood. They are neoplastic, clonal disorders of the hematopoietic stem cells. They can be broadly classified as acute and chronic leukemias. The term acute, historically referring to a rapid onset and usually fatal outcome, indicates the relatively undifferentiated nature of the leukemic cells. Acute leukemias usually present with bleeding manifestations, severe anemia and severe infections, where as many patients with chronic leukemias are asymptomatic or present with splenomegaly, fever, weight loss, malaise, frequent infections, bleeding, thrombosis, or lymphadenopathy. Based on the cell of origin they are classified as myeloid and lymphoid leukemias. In the WHO classification, the term "Myeloid" includes all cells belonging to the granulocytic (neutrophil, eosinophil, basophil), monocytic/macrophage, erythroid, megakaryocytic and mast cell lineages (Vardiman et al., 2009).

The overall annual incidence of these disorders in the general population is about 4 per 100,000 with approximately 70% of them being acute myeloid leukemia (AML). AML accounts for about 15% of childhood leukemias and for approximately 80% to 90% of acute leukemias in adults, with the median age at diagnosis of about 70 years. Acute lymphoblastic leukemia (ALL) is primarily a childhood disease, with the peak incidence between the ages of 2 to 3 years. Chronic leukemias predominantly occur in adults, the common ones being chronic myeloid leukemia comprising of 40% of all leukemias followed by chronic lymphoid leukemia.

Classification

Leukemias are broadly classified into myeloid or lymphoid, based on their cell of origin. Leukemias traditionally have been designated as acute or chronic, based on the percentage of blast cells and the course of the disease. According to the recently proposed classification system the blast cell count required to classify as acute leukemia is more than 20% either in bone marrow or peripheral blood, and does not require any minimum blast cell percentage when certain morphologic and cytogenetic features are present.

There have been several changes in the classification of the leukemias over the last few decades. Two widely used classification systems include: one devised by a group of French, American, British (FAB) hematologists and the other by the World Health Organization (WHO). The traditional classification of acute leukemia used criteria proposed by the French– American–British Cooperative Group (FAB), using the 30% bone marrow blast cell cutoff. This classification system originally distinguished different leukemia types by morphologic features and cytochemical studies (Hamid et al., 2011). The recent WHO 2008 classification takes 20% bone marrow blast cell cutoff and includes all the features like morphology, cytochemistry, immunophenotype and genetics (Vardiman et al., 2009; Yin et al., 2010) (Table-1 & Table-2).

Genetic Abnormalities in leukemias

Cytogenetic abnormalities seen in leukemias are mainly translocations, deletions, ploidy changes and mutations in specific genes. They differ based on the type of leukemia and are useful in classification and prognosis of the leukemia.

- Acute Myeloid Leukemia: Acute myeloid leukemia (AML) is a tumor of hematopoietic progenitors caused by acquired oncogenic mutations that impede differentiation, leading to the accumulation of immature myeloid blasts in the marrow. It is the common form of acute leukemia in adults and accounts for 25% of all leukemias diagnosed

Table 1 **WHO 2008 classification of Acute Myeloid Leukemia and related neoplasms.**

- **Acute myeloid leukemia with recurrent genetic abnormalities**
  - AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
  - AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
  - APL with t(15;17)(q22;q12); *PML-RARA*
  - AML with t(9;11)(p22;q23); *MLLT3-MLL*
  - AML with t(6;9)(p23;q34); *DEK-NUP214*
  - AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
  - AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
  - *Provisional entity: AML with mutated NPM1*
  - *Provisional entity: AML with mutated CEBPA*
- **Acute myeloid leukemia with myelodysplasia-related changes**
- **Therapy-related myeloid neoplasms**
- **Acute myeloid leukemia, not otherwise specified**
  - AML with minimal differentiation
  - AML without maturation
  - AML with maturation
  - Acute myelomonocytic leukemia
  - Acute monoblastic/monocytic leukemia
  - Acute erythroid leukemia
  - Acute megakaryoblastic leukemia
  - Acute basophilic leukemia
  - Acute panmyelosis with myelofibrosis
- **Myeloid sarcoma**
- **Myeloid proliferations related to Down syndrome**
- **Blastic plasmacytoid dendritic cell neoplasm**

in adults, and is increasingly common with age. Cytogenetic analyses and molecular analyses are currently used to risk-stratify AML. Cytogenetic abnormalities can be detected in approximately 50% to 60% of newly diagnosed AML (Kumar et al., 2011).

Genetic abnormalities seen in AML are:

- Structural rearrangements
  - Balanced chromosomal translocations, particularly t(8;21), inv(16), and t(15;17).
  - Translocations involving 3q21 and 3q26 and t(1;7)
- Gain or loss of whole or part of chromosome
  - -7/del(7q), -5/del(5q), +8, +9, +11, del (11q), del(12p), -18, +19, del(20q) +21,
- Mutations involving *FLT3, KIT, NPM1, CEPBA* genes.

The t(8;21) is more frequent in the young and is rare beyond the age of 50 years. The translocation results in the generation on the derivative chromosome 8 of a consistent hybrid gene, ETOAML- 1 that encodes a novel message for haematopoietic cell proliferation (Rueda et al., 2004).

Most common chromosomal abnormalities t(8;21) and inv(16), disrupt the *CBF1α* and *CBF1β* genes, respectively. These two genes encode polypeptides that bind one another to form

Table 2 **WHO 2008 classification of Acute Lymphoid Leukemia.**

- **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;q11.2);*BCR-ABL 1*
    - * B lymphoblastic leukemia/lymphoma with t(v;11q23);*MLL* rearranged
    - * B lymphoblastic leukemia/lymphoma with t(12;21)(p13;q22) *TEL-AML1 (ETV6-RUNX1)*
    - * B lymphoblastic leukemia/lymphoma with hyperdiploidy
    - * B lymphoblastic leukemia/lymphoma with hypodiploidy
    - * B lymphoblastic leukemia/lymphoma with t(5;14)(q31;q32) *IL3-IGH*
    - * B lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3);*TCF3-PBX1*
- **T lymphoblastic leukemia/lymphoma**

a CBF1α/CBF1β transcription factor that is required for normal hematopoiesis. The t(8;21) and the inv(16) create chimeric genes encoding fusion proteins that interfere with the function of CBF1α/CBF1β and block the maturation of myeloid cells.

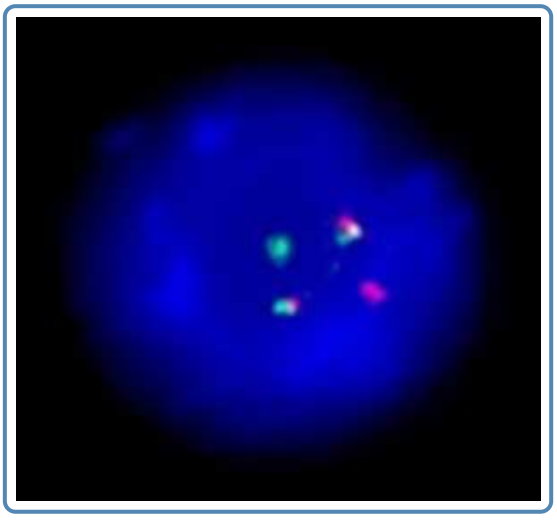

Figure 1 Fluorescent In Situ Hybridization showing the translocation in a patient with Acute Promyelocytic leukaemia. The cell shows 2 fusion signals and 1 independent red and 1 independent green signal (Vysis Dual color dual fusion probe).

The t(15;17) translocation is found in approximately 95% of (Acute Promyelocytic leukemia) APLs, a specific subtype of AML (Figure 1). The t(15;17) translocation is always associated with APL and leads to the expression of PML-RARα onco-fusion gene in hematopoietic myeloid cells. The PML-RARα onco-fusion protein acts as a transcriptional repressor that interferes with gene expression programs involved in differentiation, apoptosis, and self-renewal. Generally, patients with APL t(15;17) phenotype represent a unique group characterized by distinct biological features and good prognosis, particularly when all-trans retinoic acid (ATRA) is used as part of remission induction (Kumar et al., 2011).

Among the mutated genes *FLT3 (ITD)* is the most common, seen in one third of AML cases. FLT3 is a receptor tyrosine kinase that is expressed on hematopoietic progenitor cells and indicative of poor prognosis. Other mutated genes are *NPM1, c-KIT, CEBPA*. *NPM* mutations seen in 30% of cases, it is a protein which normally acts as a nucleocytoplasmic shuttling protein and regulates the p53 pathway (Verhaak et al., 2005; Stirewalt et al., 2008). *NPM1* mutations often coincide with mutations in *FLT3*, particularly with the ITD-type mutations. The *NPM1* mutations in AML are associated frequently with normal karyotypes. The *CEBPA* gene provides instructions for making a protein called CCAAT/enhancer-binding protein alpha. This protein is a transcription factor, which means that it binds to specific regions of DNA and helps control the activity of certain genes. It is believed to act as a tumor suppressor, helping to prevent cells from growing and dividing too rapidly or in an uncontrolled way. *CEBPA* are detected in 15% of

patients and associated with favorable response to therapy (Verhaak et al., 2005). Other molecular markers, such as *IDH1, IDH2,* and *DNMT3A* have been suggested to be predictive of risk and response to treatment.

- Acute Lymphoblastic Leukemia (ALL): ALLs are neoplasms composed of the immature T or B cell, called lymphoblasts. ALL is a malignant clonal proliferation of lymphoid progenitor cells, most commonly of the B-cell lineage (B-ALL). In the pediatric population, ALL accounts for 81% of childhood leukemias; leukemia overall accounts for one third of cancers diagnosed in children between ages 0–14 years. Overall, T-ALL has a bad prognosis when compared to B-ALL. Approximately 90% of ALLs have numerical or structural chromosomal changes.

Genetic abnormalities seen in B-ALL are:

- Hyperdiploidy
- Hypodiploidy
- Balanced chromosomal translocations t(12;21), t(1;19), t(9;22), t(4;11).

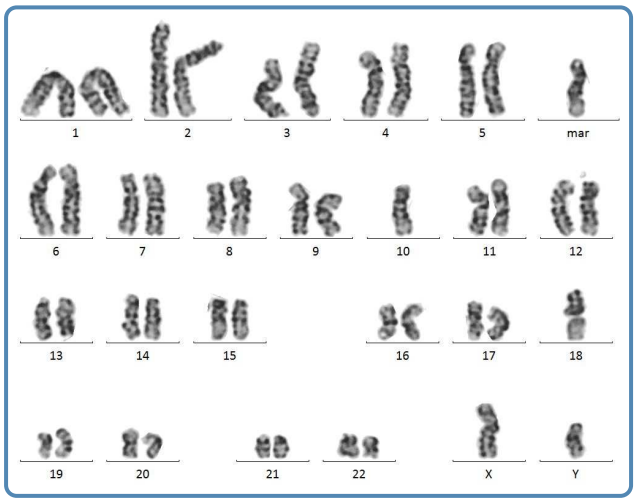

Figure 2 Karyotype of a patient with Acute lymphoblastic leukaemia showing hypodiploidy for chromosome 10 and 18 and a marker chromosome.

High hyperdiploidy (51–65 chromosomes) is one of the most common cytogenetic abnormalities observed in childhood B-ALL. It is seen in 25-30% of total childhood B-ALL cases, with the highest frequency in the 1 to 4 year age range. High hyperdiploidy is characterized by a nonrandom gain of chromosomes, including +X, +4, +6, +10, +14, +17, +18, and +21. This diagnosis confers a good prognosis in childhood B-ALL (Woo et al., 2014; Ruth et al., 2013). Approximately 20% of hyperdiploid ALL have activating mutations in the receptor tyrosine kinase *FLT3*. Hypodiploidy is characterized by fewer than 46 chromosomes and is seen in 5-8% of total B-ALL cases (Figure 2). The majority of hypodiploid B-ALL contain 45 chromosomes. The remainder of hypodiploid cases is much rarer and includes high-hypodiploid (40–44 chromosomes).

The t(12;21) is the most common chromosomal rearrangement in ALL. It occurs in 25% of children with B-ALL and confers an excellent prognosis. This translocation joins the *TEL* (or *ETV6*) gene on chromosome 12p with the *AML1* (or *CBFA2*) gene on chromosome 21 and is associated with an early pre-B immunophenotype, a younger childhood population, and non hyperdiploidy. The ETV6-RUNX1 fusion protein is thought to disrupt the normal expression of RUNX1-regulated genes by converting RUNX1 to a transcriptional repressor.

The t(1;19)(q23;p13) translocation is found in one fourth of patients with the pre-B immunophenotype. This translocation represents fusion of the *E2A* and *PBX1* genes on chromosomes 1 and 19, respectively. The t(9;22)(q32;q11), or Philadelphia (Ph) chromosome, is present in 20 to 30% of adults and 1 to 5% of children with ALL at the cytogenetic level and is associated with older age, higher leukocyte count, and more frequent CNS involvement at the time of diagnosis (Woo et al., 2014; Ruth et al., 2013).

MLL (mixed-lineage-leukemia) gene rearrangements at 11q23 are present in 80% of all infant B-ALL cases and 10% of all childhood B-ALL. The most common gene rearrangements include t(4;11)(q21;q23) encoding *MLL-AF4*, t(9;11)(p22;q23) encoding *MLL-AF9*, t(11;19)(q23;p13.3) encoding *MLL-ENL*, t(10;11)(p13-14;q14-21) encoding *MLL-AF10* and t(6;11)(q27;q23) encoding *MLL-AF6* (Woo et al., 2014; Ruth et al., 2013). The t(4;11)(q21;q23) is also associated with high-risk features, most notably a high WBC count and age of onset < 1 year (Hamerschlak et al., 2008).

Conventional karyotyping identifies structural chromosomal aberrations in 50% of T-ALL. Numerical changes are rare, except for tetraploidy which is seen in approximately 5% of cases. The common abnormalities involve the rearrangements involving T-cell receptor genes like deregulation of homeobox genes *TLX1* (*HOX11*) t(10;14)(q24;q11), *TLX3* (*HOX11L2*) t(5;14)(q35;q32), and *TAL1*

(*SCL,TCL5*) t(1;14)(p32;q11), t(1;14)(p34;q11) and t(1;7)(p32;q34); deregulation of *MYB* gene-duplication t(6;7)(q23;q34); and fusion gene rearrangements like *PICALM-MLLT10* (*CALM-AF10*) t(10;11)(p13;q14) and *MLL*-fusions. Favorable prognosis is associated with subtypes *HOX11* or *MLL-ENL* (Ruth et al., 2013; Hamerschlak et al., 2008).

Up to 70% of T-ALLs have gain-of-function mutations in *NOTCH1*, a gene that is essential for T-cell development. A high fraction of B-ALLs have loss-of-function mutations in genes that are required for B-cell such as *PAX5*, *E2A* and *EBF*.

- Chronic Myeloid Leukemia (CML): It is a clonal stem cell disorder characterized by the acquisition of an oncogenic BCR/ABL fusion protein, usually the result of a reciprocal translocation t(9;22)(q34;q11) and by proliferation of granulocytic elements at all stages of differentiation. The majority of CML cases are in adults. The frequency of this type of leukemia is 1 per 1 million children up to the age of 10 years. Among adults, the frequency is around 1 in 100,000 individuals (Hamerschlak et al., 2008). CML can be divided into three clinically distinct phases: an initial chronic phase followed by an accelerated phase which subsequently leads to blast crisis.

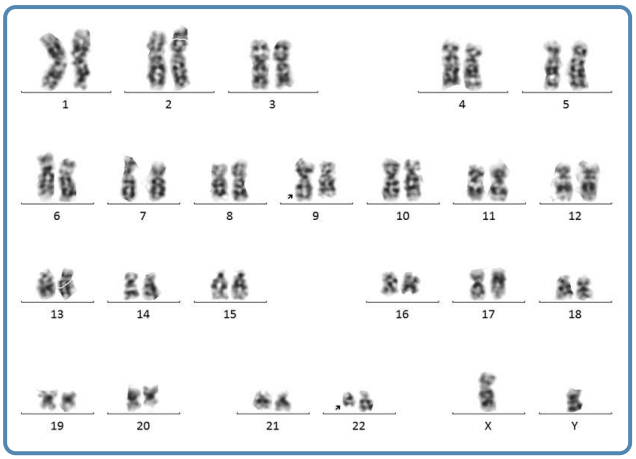

Figure 3 Karyotype of a patient with Chronic myeloid leukaemia showing t(9;22) (Philadelphia translocation).

The t(9;22) translocation leads to derivative chromosome 22 also referred to as the Philadelphia chromosome (Ph) (Figures 3 and 4) seen in 95% of AML as well as in some acute lymphoblastic leukemias (ALL) (Pokharel, 2012). The ABL gene located on chromosome 9 is the human homologue of the v-*abl* oncogene carried by the Abelson murine leukemia virus and it encodes a nonreceptor tyrosine kinase. Human Abl is a ubiquitously expressed 145-kd protein with 2 isoforms arising from alternative splicing of the first exon. The activity of the c-Abl tyrosine kinase domain is regulated by the SH3 and SH2 regions. The SH3 domain is a negative regulator of c-Abl activity, as a deletion mutant of this domain activates the kinase, whereas the SH2 domain is a positive regulator. The BCR gene (Breakpoint cluster region) located on chromosome 22 encodes a 160-kd Bcr protein which, like Abl, is ubiquitously expressed. The first N-terminal exon encodes a serine threonine kinase. As a result of the t(9;22)(q34;q11) Bcr-Abl fusion, the N-terminus of Bcr binds to the SH2 domain of Abl in a phosphotyrosine independent manner, constitutively activating the tyrosine kinase. This oncoprotein is associated with enhanced expression of several major downstream effectors such as Ras, phosphoinositide 3-kinase (PI-3K) and protein kinase B (Deininger et al., 2000; Di Bacco et al., 2000).

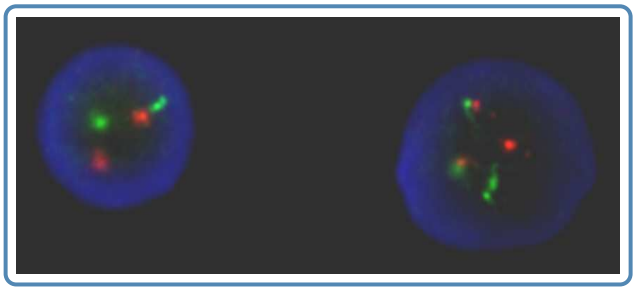

Figure 4 Fluorescent In Situ Hybridization showing the t(9;22) translocation in a patient with chronic myeloid leukaemia. The left cell shows two green and two red independent signals suggestive of normal FISH for t(9;22)(bcr-abl) whereas the right side cell shows 2 Fusion signals (suggestive of presence of t(9;22)(bcr-abl)) and 1 independent red and 1 independent green signal each.

Approximately 5% of cases appear to be "Ph-negative" by conventional cytogenetics because of the presence of a cryptic or submicroscopic BCR-ABL rearrangement. These cases require FISH and/or molecular RT-PCR testing for further evaluation and documentation of a BCR-ABL transcript

(Hsiao et al., 2011). Based on the breakpoint region in *BCR* gene three transcripts are formed first, the major breakpoint cluster region (M-*bcr*) and chimeric protein derived from this mRNA is 210-kd. This is the most common breakpoint region. Second, the minor breakpoint cluster region (m-*bcr*) and resultant mRNA is translated into a 190-kd protein. Recently, a third breakpoint cluster region (µ-*bcr*) was identified, giving rise to a 230-kd fusion protein (Deininger et al., 2000). Identification of fusion transcript helps in targeted treatment by tyrosine kinase inhibitors and in prognosis by identifying minimal residual disease.

The *p53* and *Rb1* genes are most often implicated in the transformation. Alterations in the *p53* and *Rb1* genes occur in about 30% and 20% of blast crisis cases, respectively. Additional clonal cytogenetic abnormalities, seen are trisomy 8, isochromosome 17q, or duplication of the Ph chromosome. Recently, it has been observed that in greater than 85% of cases, CML is associated with the appearance of mutations that interfere with the activity of *Ikaros*, a transcription factor that regulates the differentiation of hematopoietic progenitor.

- Chronic Lymphocytic Leukemia: Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of mature appearing lymphocytes in the blood, marrow, lymph nodes, and spleen. They are characterized by the absolute lymphocyte count >4000/mm^3. Genetic abnormalities commonly encountered are deletions of 13q14.3, 11q, and 17p, and trisomy 12q; translocations are rare.

The most frequent structural abnormality in CLL is a deletion at 13q14. Approximately one half of the abnormalities involves an interstitial deletion and almost invariably is associated with loss of the *Rb* gene. Translocations involving 13q are found in the remaining one half of cases, and most of these involve a breakpoint at 13q14, which is the site of the *Rb* gene, with translocations from a variety of other chromosomes. A deletion of 11q22–q23 which occurs in 20% of cases, is seen in younger patients, and is associated with marked lymphadenopathy, rapid disease progression, and poor survival. Trisomy 12q13 occurs as a result of duplication of one homolog and is seen in 10 to 20% of CLL patients. Trisomy 12 is frequently associated with "atypical" CLL and CLL/PLL. A deletion of 17p13 is usually associated with a mutation of p53, and these patients usually have aggressive and drug-resistant disease (Puiggros et al., 2014).

Diagnostic Workup

Laboratory work up in diagnosis of leukemias includes:

- Morphological examination of peripheral blood and bone marrow.
- Cytochemistry
- Immunophenotyping
- Cytogenetic analysis
- Molecular genetic studies

The diagnosis of a hematological neoplasm usually starts from a clinical suspicion, although for chronic leukemias the diagnosis is sometimes an incidental one. A blood count and blood film is an essential first step whenever leukemia, lymphoma or other hematological neoplasm is suspected. Diagnosis is based on peripheral smear and confirmation is done by bone marrow examination. The next step in the diagnostic process depends on the clinical features and the specific condition that is suspected. With advances in immunophenotyping and other techniques, the role of cytochemistry in hematological diagnosis has declined considerably. Further typing of leukemia into myeloid or lymphoid either B cell or T cell, requires the analysis of antigen expression by immunophenotyping.

Cytogenetic and molecular genetic studies are done for molecular classification of leukemia and also to know the bad and good prognostic features, which help in the treatment of the disease. Cytogenetic studies include karyotyping and Fluorescence In-Situ Hybridization (FISH) for detection of the deletions and translocations. Molecular analysis includes PCR, Real Time PCR and the mutational analysis for detection of the fusion transcript and mutation in specific genes. The major applications of each technique are:

- **Applications of Immunophenotyping**
  - Diagnosis and classification
  - Assessment of prognosis
  - Monitoring of minimum residual disease
  - To monitor effectiveness of treatment
- **Applications of Cytogenetic analysis**
  - Confirmation of diagnosis of specific subtype
  - Assessment of prognosis
  - Assessment of clonality
  - Detection of minimum residual disease
- **Applications of Molecular genetic studies**

- Diagnosis of specific subtype
- Early detection of minimum residual disease
- Assessment of prognosis
- Assessment of clonality

Prognostic Markers

Prognostic markers help in predicting the survival of the patients and for planning the appropriate treatment for the disease. The presence of favorable markers in a case means that the survival rate of the patient for that particular disease is more and unfavorable markers have less survival rates. Some of the markers are classified as intermediate markers based on their survival rate, which have less clinical implication when compared to bad and good markers and further evaluation is required (Table-3). Genetic prognostic markers for the chronic leukemias are not well established as in the case of acute leukemias. The major prognostic markers are:

Table 3 Prognostic genetic markers in Leukemias.

Type of Leukemia	Favorable	Unfavorable
ALL	Hyperdiploidy (>50) t(12;21)	Hypodiploidy t(9;22) t(4;11) t(1;19)
AML	t(8;21) t(15;17) inv(16)	-7/del(7q) -5/del(5q) +8 +9
CLL	del 13q Trisomy 12	del 17p del 11q
CML	Ph chromosome +	Trisomy 8 del 22q

Conclusions

Leukemias are among the commonest cancers worldwide. Their diagnosis depends mainly on the morphology, cytochemistry and immunophenotyping, but cytogenetic and molecular studies are essential for the molecular classification and predicting the prognosis of the disease. Common cytogenetic studies include karyotyping and FISH for finding the translocations and deletions, and molecular studies include PCR, Real Time PCR and mutation analysis of the relevant genes. Hence, for accurate early diagnosis and treatment, a stepwise evaluation of the case with the suitable diagnostic tests is employed for the favorable outcome.

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