Introduction
**Origin of blood cells**

All blood cells, such as granulocytes, neutrophils, eosinophils, monocytes, erythrocytes, macrophages, B and T lymphocytes, platelets and natural killer cells originate from a lymphohemopoietic stem cell in the bone marrow. This process is known as hematopoiesis. During normal hematopoiesis, bone marrow stem cells differentiate into specific cell types; however, injury to the stem cell can result in several of the most serious hematological disorders. As a result, a progressive expansion of a population of immature cells occur, this expanding clonal (or homogenous) population of cells may gradually replace the normal (heterogenous) cells in the marrow, which leads to severe hematologic disorder.

![Normal Hematopoiesis](https://example.com/hematopoiesis.png)

*Figure-4: Normal Hematopoiesis (Source: National cancer Institute, www.cancer.gov)*

**Hematologic disorders**

The hematologic disorders include the preleukemias, myeloproliferative disorders and acute leukemias. The chronic myeloproliferative disorders include polycythemia vera, primary thrombocytemia, idiopathic...
myelofibrosis, chronic monocytic leukemia, chronic neutrophilic leukemia and chronic myeloid leukemia (Lichtman, 1995). Leukemia arises when an immature white blood cell in the bone marrow (progenitor cell) develops uncontrollably and suppresses the production of healthy blood cells. The unregulated proliferating cells usually replace normal marrow, interfere with normal marrow function, may invade other organs and eventually cause death if untreated. Because of this, the condition was referred to as weisses blut (German for ‘white blood’). Later the word ‘leukemia’ was used for the disease (Greek leukos, ‘white’; haima ‘blood’).

**Leukemia**

The worldwide incidence of leukemia is about 1 per 100,000 per year (Cartwright, 1992). Previously the diagnosis of leukemia was fairly on cytomorphology reports. Today with the combination of morphological, cytogenetics, immuno phenotyping, cytochemistry, and molecular studies, the accuracy in the classification and diagnosis of leukemia have increased tremendously. Leukemia may be of two types chronic or acute and can be divided into those of lymphoid and myeloid origin. Chronic lymphocytic leukemia and CML. CML also called chronic granulocytic leukemia is hematopoietic stem cell disorder.

**Chronic Myeloid Leukemia (CML)**

CML was first described in 1845 (Bennet, 1845; Virchow, 1845). CML is a clonal disorder of hematopoietic stem cells (Fialkow, 1977). It is one of the best understood diseases from the aspects of its cytogenetic abnormalities and the molecular mechanisms involved. The disease arises as a consequence of a rare gene abnormality. CML was the first human disease in which a specific abnormality of the karyotype, the Ph chromosome, was reported to be linked to a malignant disease (Nowell and Hungerford, 1960). Later on, it was established that the Ph chromosome results from a reciprocal translocation between the long arms of chromosomes 9 and 22 (Rowley, 1973), which produces the BCR-ABL fusion oncogene [Figure: 5].
Introduction

The BCR-ABL oncoprotein, a constitutively activated tyrosine kinase, recruits and activates several pathways transducing intracellular signals, which ultimately lead to abnormal cellular adhesion, enhanced proliferation and inhibition of apoptosis (Deininger, 2000).

Etiology

As documented in latest edition of DeVita (Druker and Stephanie, 2008), the only known risk factor for development of CML is exposure to radiation in high doses. This is evident from two studies; one is survivors of the atom bomb explosions in Japan in 1945. The second is follow-up of patients treated with radiation for ankylosing spondylitis and cervical cancer. No known association has been found between CML and infectious agents or chemical exposures, and no familial predisposition has been implicated in CML; thus, patients can be counseled that CML is neither preventable nor heritable (Druker and Stephanie, 2008). However, various life style factors and molecular mechanisms might be the major contributing factors for rising trend of CML.

Epidemiology

CML accounts for ~ 15% of all leukemias (Reis et al, 2003). The incidence of CML is similar in all countries worldwide, per year, 1.6 to 2.0 cases per
100,000 persons are noted. The median age range is 30-60 yrs and sex ratio is 1.2:1 (M:F) (Turhan, 2008).

In the Asian population, the incidence is about 0.8 (women)-1.5 (men) per 100,000 of the population per annum (Farah and Kanjaksha, 2007). In most Indian studies, patients diagnosed on routine testing from 5-8% of CML cases as compared to 20% in Western studies (Farah and Kanjaksha, 2007).

The true incidence of the disease in India is not reported systematically; however, the data from various Indian cancer registries show a CML incidence of 0.8 to 2.2 per 100,000 populations for men and 0.6 to 1.6 per 100,000 for women. There is slight male predominance (Farah and Kanjaksha, 2007). Data from the Indian subcontinent shows that the disease is seen in a younger population. In India, the median age at onset is 38-40 years and this is consistently seen in all studies reported from India (Farah and Kanjaksha, 2007).

The average annual prevalence of CML in The Gujarat Cancer and Research Institute, India is 197 (29.3%) patients for the year 2002-2004 (Atul, 2007).

**Clinical characteristics of CML**

**Symptoms**

The typical symptoms at presentation are fatigue, anorexia, and weight loss. The most common abnormality on physical examination is splenomegaly, which is present in up to half of the patients. About 40 percent of the patients are asymptomatic. In these patients, the diagnosis is suspected because of accidental detection of abnormal blood counts (Sawyers, 1999).

**Peripheral Blood and Bone Marrow**

The diagnosis of CML is frequently suspected from examination of the peripheral blood and bone marrow. The white blood cell (WBC) count in the chronic phase of CML usually exceeds $50 \times 10^9$/L at the time of diagnosis and can range up to $800 \times 10^9$/L. During the chronic phase, leukemic cells retain the capacity to differentiate normally, and WBC function is normal. The
peripheral blood smear shows a full spectrum of myeloid cells from blasts to neutrophils, with blasts comprising less than 15% and usually less than 5% of the WBC differential count. Basophilia is invariably present, and its absence should prompt consideration of other myeloproliferative disorders. Eosinophilia is also commonly present. The majority of patients have thrombocytosis, and, on occasion, the platelet count may be more than 1000 x 10^9/L. Most patients with CML have a normochromic, normocytic anemia that is inversely proportional to the degree of leukocytosis. As in the peripheral blood, the myeloblast percentage in the marrow is less than 15% and most commonly less than 5%, and basophilia is also present. Megakaryocytes are usually increased in number and may form clusters. Occasional micromegakaryocytes may be present. Erythroid hypoplasia is frequently present and may seem exaggerated because of the increased myeloid-erythroid ratio. Erythroid precursors are otherwise morphologically unremarkable. Reticulin fibrosis is usually absent or mild but may become more prominent with disease progression.

**Phases of CML**

*Figure-6: Disease progression in chronic myeloid leukaemia. (Source: Melo and Barnes, 2007)*
CML is a triphasic disease; the initial phase is chronic phase, most patients present in this phase. The bone marrow in patients with chronic-phase CML is markedly hypercellular, with a predominance of myeloid cells with full maturation. As disease progresses, patients enter ill-defined accelerated phase, often heralded by the appearance of increased number of immature myeloid cells in the bone marrow and peripheral blood, as well as new cytogenetic changes in addition to the Ph chromosome [Figure: 6].

Then progression proceeds to final blast crisis phase. The laboratory parameters differ in each phase. Values of WBC count, blasts, basophils, platelets, bone marrow picture, cytogenetic investigations and \( BCR/ABL \) status are the major determining factors for various phases of CML [Figure: 7] (Sawyers, 1999; Faderal, 1999).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chronic</th>
<th>Accelerated</th>
<th>Blast Crisis</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC count</td>
<td>≥20 x 10^9/L</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Blasts</td>
<td>1%–15%</td>
<td>≥15%</td>
<td>≥30%</td>
</tr>
<tr>
<td>Basophils</td>
<td>↑</td>
<td>≥20%</td>
<td>—</td>
</tr>
<tr>
<td>Platelets</td>
<td>↑ or normal</td>
<td>↓ or ↑</td>
<td>↓</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Myeloid hyperplasia</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cytogenetics</td>
<td>Ph+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>bcr-abl</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*WBC = white blood cell; Ph+ = Ph chromosome–positive.*

**Figure-7: Typical laboratory parameters by phase of CML.**

**Chronic phase (CP)**

It usually has an insidious onset, and the main clinical findings include enlarged spleen, fatigue and weight loss. The peripheral blood shows leukocytosis (~150x10^9/L-WBCs), predominantly owing to neutrophils in different stages of maturation as well as basophilia and eosinophilia. Blast usually represents <2% of the WBCs. The platelet count is normal or increased (Melo and Barnes, 2007).
**Accelerated Phase (AP)**

It is an intermediate stage of CML evolution. It is characterized by an increase in spleen size and total WBCs. The blast comprising 10-19% of the WBCs. Circulating basophils >20%, persistent thrombocytopenia and/or the appearance of new clonal cytogenetic abnormalities (Melo and Barnes, 2007).

**Blast Crisis (BC)**

This is a final stage of CML, which may or may not be preceded by an ‘accelerated phase’. Patients experience worsened performance status, and symptoms related to thrombocytopenia, anaemia and increased spleen enlargement. The World Health Organization criteria for the diagnosis of blast crisis include: blast in excess of 20% in the peripheral blood or bone marrow; and/or extramedullary blast proliferation; and/or large foci or clusters of blasts in bone marrow histological sections (Melo and Barnes, 2007).
Molecular pathogenesis of CML

The Ph chromosome

In 1960, a major clue to the pathogenesis of CML was provided by Nowell and Hungerford’s [Figure: 8a] landmark discovery of the Ph chromosome [Figure: 8b] and its association with the disease (Nowell and Hungerford, 1960).

After 13 years, using quinacrine fluorescence and Giemsa banding, Rowley [Figure: 9a] and colleagues showed that the Ph chromosome resulted from a reciprocal translocation between the long arms of chromosomes 9 and 22 [Figure: 9b]; t(9;22)(q34;q11) (Rowley, 1973).

Figure-8: a. Nowell and Hungerford, b. First metaphase with Ph published in which it was identified for the first time [Source: Nowell and Hungerford, 1960].

Figure-9: a. Dr. Rowley, b. First metaphase with t(9;22) published in which it was identified for the first time [Source: Rowley, 1973].
The Ph chromosome is a shortened chromosome 22 that results from the translocation of 3' (toward the telomere) \textit{ABL} segments on chromosome 9 to 5' \textit{BCR} segments on chromosome 22.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure10.png}
\caption{The translocation of 9 and 22 in CML (Source: Faderal et al, 1999).}
\end{figure}

**Normal ABL gene and protein product**

The name \textit{ABL} is derived from the Abelson virus, containing the viral oncogene v-abl. The human homologue of the oncogenic sequence of v-abl is called the c-abl gene. It comprises two alternative first exons, 1a and 1b, and the common exons 2 to 11. Due to alternative splicing, two transcripts can be formed called 1a and 1b of 6 kb and 7 kb, respectively. The c-abl gene is ubiquitously expressed with the highest mRNA levels in spleen, thymus and testis. The two mRNA molecules encode two proteins of about 145 kDa in size. These proteins belong to the family of non-receptor tyrosine kinases.

The \textit{ABL} protein has a function in cell cycle regulation. In G0 the \textit{ABL} protein is DNA bound in complex with the retinoblastoma (Rb) protein, one of the \textit{ABL} inhibiting proteins. During transition from G1 to S, the \textit{Rb} is phosphorylated and dissociates from \textit{ABL} giving rise to the activation of the
tyrosine kinase activity of the *ABL* protein. The activated *ABL* protein can subsequently alter transcription. *ABL* is phosphorylated itself by CDC2 kinases, after which the *ABL* protein dissociates from the DNA and the cell progresses to the S phase. Overexpression of the *c-abl* gene does not result in malignant transformation but in cell cycle arrest, very reminiscent of the behavior of tumour suppressor genes such as P53 and Rb. These results suggest a role for *ABL* as a negative regulator of cell growth. Interestingly, the growth suppressive potential of *BCR-ABL* seems to depend on p53 and Rb activity (Thijssen et al, 1999; Alfonso and Cortes, 2009).

**Normal BCR gene and protein product**

The *BCR* was first identified as a region on chromosome 22 involved in the *BCR-ABL* translocation. Subsequently, it was demonstrated that the *BCR* region is part of a gene later called the *BCR* gene. It spans a region of 135 kb and comprises 23 exons. Gene expressions give rise to two mRNA types of 4.5 and 6.7kb, both translates into 160kDa protein. The *BCR* gene belongs to a family of genes with unclear function. *BCR* protein is localized in the cytoplasm in noncycling cells, while in mitosis the *BCR* protein can be detected perichromosomal, suggesting a role in cell cycle regulation. The *BCR* gene is ubiquitously expressed in all human tissues (SFT Thijssen et al, 1999; Alfonso and Cortes, 2009).

**The BCR-ABL fusion gene and their respective protein product**

In most cases; breakpoints ([Figure: 10](#)) in the *ABL* gene are located in the 5' end (toward the centromere) of exon a2. Various breakpoint locations have been identified along the *BCR* gene on chromosome 22. Depending on which breakpoints are involved, differently sized segments from *BCR* are fused with the 3' sequences of the *ABL* gene. This results in fusion messenger RNA molecules (e1a2, b2a2, b3a2, and e19a2) of different lengths that are translated into different chimeric protein products (p190, p210 and p230) with variable molecular weights including  m-bcr: minor breakpoint cluster region, M-bcr: major breakpoint cluster region, and μ-bcr: micro breakpoint cluster region ([Figure: 10](#)). The b3a2 is more prevalent than the
b2a2. Both mRNA molecules are translated into a protein of 210kDa. However, the b3a2 variant is 25 amino acids larger than the b2a2 variant. The clinical features, response to treatment and prognosis are similar in patients with b2a2 and b3a2 transcripts, except for a higher platelet count in patients with b3a2 transcripts. P190^{BCR-ABL} is rare in CML and is mainly seen in adults and children with Ph-positive ALL. p230^{BCR-ABL} has been identified to associate with neutropenic CML as well as some rare cases of CML (Faderal et al, 1999; Alfonso and Cortes, 2009).

At first, the true nature of Ph chromosome was unknown. With the advent of various conventional cytogenetic and molecular studies, present understanding of the clinical, biological, biochemical, molecular features and invention of targeted therapy of CML was established. Hence, cytogenetic studies provide the platform for precise understanding of the molecular pathogenesis of the disease.

**Importance of Cytogenetic studies in hematologic malignancies**

*Cytogenetics* is the study of chromosome structure and number. Recent improvements in cell culture and processing techniques have allowed the identification of numerous cytogenetic abnormalities in different malignancies (Mitelman, 2010). Cytogenetic analysis has become increasingly important in the diagnosis, classification, management, and scientific investigation of hematopoietic and lymphoid disorders. Cytogenetic studies have become an essential aspect of routine clinical work-up for diagnosis and management of abnormal hematopoiesis and lymphopoiesis. Thus, it plays a prominent role in the clinical practice of hematology and oncology. The use of cytogenetics in the diagnosis and management of each disease depends on selection of the appropriate tissue and cell type for analysis, availability of cells for karyotyping, and proper interpretation of cytogenetic findings.

Classic cytogenetic analysis studies cells in metaphase, or dividing cells. However, with the advent of molecular cytogenetic techniques such as
fluorescence in situ hybridization (FISH), cytogenetic analysis now includes the study of interphase or nondividing cells also (Montogomery et al, 1997).

The cytogenetic in leukemia serves mainly three functions.

- The first of these is to assist in more accurate diagnosis, thereby providing prognostic information and allowing the more rational selection of therapy for a particular patient.
- The second is to identify the sites of consistent rearrangements, providing the precise localization required for the isolation and cloning of DNA from these regions. Using molecular techniques, identification of the function of the genes located in these regions and to determine the mechanisms by which their altered function is involved in leukemogenesis. These findings may show the way for invention of novel targeted therapies.
- The third is to find of rare and novel unknown mutations playing role in leukemogenesis. Nonrandomness of these findings may serve a different disease subgroup and also therapeutic regimens.

**Revolutions in cytogenetic Technique**

Cytogenetic studies of human cells can be divided into three phases. The first phase involved the development of methods to obtain a good metaphase spreads. In 1952, hypotonic solution was discovered by three different scientists [Figure: 11a] independently by Hughes, Makino and T. C. Hsu in 1952 (Hsu, 1979). In 1958 [Figure: 11b] correct concentration of hypotonic was confirmed (Hungerford and Diberardino, 1958).

![Figure-11: a. Invention of hypotonic solution. b. Confirmation of correct chromosome number.](image-url)
In 1922 Thomas Painter reported chromosome count at 48 for 30 years and after invention of hypotonic solution, 30 years later, in 1956 Tijo and Levan identified the correct number of chromosomes in man. Four years later phytohemagglutinin (PHA) was discovered to stimulate T-lymphocytes in the peripheral blood to undergo mitosis. This transformed cytogenetic analysis from a research tool to a diagnostic tool. Cytogenetic studies from 1956 to 1969 were performed using Giemsa stain resulting in the chromosomes staining uniformly along their length. The development of chromosome banding techniques from 1969 to 1971 led to the second phase of cancer cytogenetics [Table: 1] (Gustashaw, 1997).

The third phase was advancement to revolutionize cytogenetics with FISH [Figure: 12]. FISH provided a direct link between microscope and sequence, which involves the use of specific DNA probes labeled with fluorochromes to identify genes or chromosome regions. The next transformation of cytogenetics came with the realization that genome-wide scan for the loss or gain of chromosomal material with the technique, Comparative genomic hybridization (CGH) (Kallionemi, 1992).

All chromosomal abnormalities can be identified by spectral karyotyping (SKY) or multicolor FISH (M-FISH) (Speicher et al., 1996). However, this analysis identifies only the chromosome involved but not a specific region of the chromosome. M-band technique provides the precise information on intra-chromosomal rearrangements and exact break-point mapping (Choudoba et al, 1999).

The current excitement in cytogenetics revolves around the promise of Micro-Array techniques (Array-CGH, Single Nucleotide Polymorphism-Array, Copy Number Variation-Array), which allows the thousands of FISH experiments at once. In this technique, metaphase chromosomes are replaced by an array of thousands of Bacterial Artificial Chromosome clones, each of which contains a ~150-kb segment of the human genome.
<table>
<thead>
<tr>
<th>Stain or Banding Techniques</th>
<th>Investigators</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTG (Giemsa band by Trypsin and Giemsa stain)</td>
<td>Seabright (<em>Lancet</em>:2;971-972)</td>
<td>1971</td>
</tr>
<tr>
<td>GAG (Giemsa band by acetic saline and Giemsa stain)</td>
<td>Sumner, Evans, Buckland (<em>Nature New Biol</em>:232;31-31)</td>
<td>1971</td>
</tr>
<tr>
<td>CBG (Centromeric bands using barium hydroxide/Saline/ Giemsa)</td>
<td>Arrighi, Hsu (<em>Chromosoma</em>:34;243-253)</td>
<td>1971</td>
</tr>
<tr>
<td>T-Banding</td>
<td>Dutrillaux (<em>Humangenetik</em>:30;297-306)</td>
<td>1975</td>
</tr>
<tr>
<td>Ag-NOR Banding (Silver staining for Nucleolar Organizer Regions)</td>
<td>Howell, Denton, Diamond (<em>Experientia</em>:31;260-262)</td>
<td>1975</td>
</tr>
<tr>
<td>High resolution human chromosomes</td>
<td>Yunis JJ (<em>Science</em>:191;1268-1270)</td>
<td>1976</td>
</tr>
<tr>
<td>M-Band</td>
<td>Chudoba et al (<em>Cytogenet Genome Res</em>:104;390-3)</td>
<td>2004</td>
</tr>
</tbody>
</table>

Table-1: Development in cytogenetic techniques.

**The Chromosome Nomenclature:**

Identification of chromosomes was difficult especially when chromosomes were of similar size and shape. A nomenclature to group chromosomes according to size was accepted at the Denver Conference in 1960, and this system was modified at the Chicago Conference in 1967. The Paris Conference in 1971 established the conventional use for numbering of each chromosome and chromosome band (ISCN, 2009). Nomenclature of chromosome was subsequently updated to describe the chromosomal anomalies and FISH results. The most recent guidelines for cytogenetic nomenclature were published in 2009 (ISCN, 2009). The nomenclature mainly involves the description of chromosomal landmarks.
The signatory landmarks of chromosomes

The term *landmark* is used to designate distinct morphologic features important in identifying chromosomes [*Figure: 12*]. Landmarks include the centromere, the ends of the chromosomes (*telomeres*), and prominent chromosome bands. *Chromosome regions* are defined as areas lying between adjacent landmarks. The number of regions on the short and long arms of individual chromosomes ranges between one and four. Regions of each chromosome arm are numbered sequentially, moving outward from the centromere toward the telomere. Band is the part of chromosome clearly distinguishable from its adjacent segments by appearing darker or lighter following staining procedure. The unique light and dark banding pattern of each chromosome is a signature for identification. The bands within regions are also numbered sequentially, also moving outward on each arm from the centromere toward the telomere.
To designate chromosome bands, one specifies the chromosome number, the chromosome arm, the region number, and the band number within the region, given in order, without spacing or punctuation. For example, 7q22 (chromosome 7, long arm, region 2, band 2) designates the second band of the second region of the long arm of chromosome 7.
**Karyotype:**
Chromosomes are counted in each of the chosen metaphase spreads to determine whether they are present in normal numbers. Chromosomes are then identified as per the size and banding pattern and characterized as structurally normal or abnormal. To facilitate analysis, chromosomes from each metaphase spread are arranged in a prescribed order, called a *karyotype*. Karyotype description is done with standard nomenclature. Normal karyotype for female is 46,XX [**Figure: 14a**] and for male is 46,XY [**Figure: 14b**].

![Figure-14a: Representative image of normal female karyotype.](image)

![Figure-14b: Representative image of normal male karyotype.](image)
**Types of chromosomal abnormalities**

**Constitutional chromosomal abnormalities** are abnormalities that are present in all or almost all cells in the body and exist at the earliest stage of embryogenesis.

**Acquired chromosomal abnormalities** are abnormalities that develop in somatic cells, usually in association with malignant transformation.

**Primary versus Secondary Acquired chromosomal abnormalities**

Acquired chromosomal abnormalities may be either primary or secondary. *Primary cytogenetic abnormalities* are aberrations that are often found as sole chromosomal changes in malignancies. Moreover, they are often associated with specific tumor types. For example, t(9;22)(q34;q11) in CML. *Secondary chromosomal abnormalities* are changes that generally occur not by themselves but in addition to primary changes. For example, +8, i(17)(q10), +Ph and +19 occurring in addition to t(9;22)(q34;q11). Primary cytogenetic abnormalities are changes that are thought to be associated with the pathogenesis of malignant transformation, whereas secondary chromosomal changes likely correlate with tumor progression and may worsens the prognosis (Salt and Baer, 2003).

**Numerical Chromosomal Abnormalities**

A cell with a normal complement of chromosomes is called *diploid*. A cell with 46 chromosomes, but with numerical chromosomal abnormalities is called *pseudodiploid*. The presence of an abnormal number of chromosomes is called *aneuploidy*. Cells with more than 46 chromosomes are called *hyperdiploid*, whereas the presence of fewer than 46 chromosomes is called *hypodiploidy*. Loss of one copy of a chromosome results in monosomy for that chromosome; loss of both copies results in nullisomy. Gain of an additional copy of a chromosome results in *trisomy* for the chromosome that has been gained. When two additional copies of a chromosome are gained, it is referred to as *tetrasomy*. 
Chromosome loss is indicated in the karyotype by a minus sign followed by the number of the missing chromosome, whereas chromosome gain is indicated by a plus sign followed by the number of the chromosome that has been gained. The number of chromosomes in the cell, given in the karyotype, reflects the chromosomes that have been lost or gained.

**Structural Chromosomal Abnormalities**

Different types of structural rearrangements are described below.

**Deletion** (*del*) is loss of a chromosome segment.

**Isochromosome** (*i*) consists of two chromosome arms positioned as mirror images of each other. Isochromosome formation leads to both loss and gain of genetic material.

**Inversion** (*inv*) is a structural chromosome change consisting of a 180-degree rotation of a chromosome segment. Inversions may be pericentric or paracentric.

**Ring Chromosome** (*r*) is a closed circle, or ring. Breaks occur in both the short and the long chromosome arms, and the breakpoints in the short and long arms have joined together and creates ring.

**Translocation** (*t*) is a relocation of material from one chromosome to a different chromosome. Translocations are usually reciprocal in which an exchange of material between different chromosomes occur. *Robertsonian translocations* are whole arm transfers, almost always between acrocentric chromosomes.

**Dicentric Chromosome** (*dic*) has two centromeres.

**Addition** (*add*) is a gain of chromosomal material of unknown origin.
**Insertion** (ins) is the presence of a chromosome segment in a new position within the same or another chromosome.

**Duplication** (dup) is the presence of an extra copy of a segment of a chromosome.

**Double Minutes** (dmin) are marker chromosomes without centromeres or banding patterns, which usually result from gene amplification.

**Marker Chromosomes** (mar) is a structurally abnormal of unknown origin.

**Cytogenetic Definition of Clonality**
Cytogenetically, a *clone* is defined by a minimum of two mitotic cells with the gain of the same chromosome or with the same structural abnormality, or three mitotic cells with the loss of the same chromosome. Cytogenetic changes occurring in an insufficient number of cells to define a clone are considered to be random changes. Alternatively, if apparently random changes are ones that can be detected by FISH, FISH may be used to determine their frequency in larger numbers of metaphase spreads and in interphase cells (ISCN, 2009).

**Formation of clones**
The clonal patterns of malignancies have been demonstrated with cytogenetic studies, molecular genetic method, immunocytochemical techniques, enzyme markers, and many other procedures. Cytogenetic studies have suggested that nearly all malignant disorders are associated with chromosomally abnormal clones. Numerous cytogenetic reports have been published in recent years recording the presence of chromosomally abnormal clones in a wide variety of hematologic disorders and solid tumor [Mitelman, 2010].

In the early stages, the cells of a chromosomally abnormal clone may die as a consequence of genetic imbalance or may be destroyed by the immune
system. If not, the clone may proliferate and form a malignant tumor, and subclones may form as a result of chromosome evolution [Figure: 15].

![Figure-15: Formation of clone.](image)

The formation of structural abnormalities of chromosomes may be relatively random with respect to the site of breakpoints. Theoretically, a break could affect any site of any chromosome. Considering that the human diploid genome has ~ 6 billion base pairs distributed among 46 chromosomes, about $36 \times 10^{18}$ different chromosome rearrangements are possible. The probability of developing a common chromosome abnormality by accident seems so remote that chance alone would not explain the relatively high frequency of hematologic disorder (Dewald et al, 2002).

**Significance of Cytogenetic studies in CML**

Cytogenetic analysis detects the Ph chromosome [Figure: 16] in approximately 95% of patients with CML at the time of diagnosis. The G-banding karyotyping is utilized for cytogenetic analysis and usually 25-30 metaphase cells are examined.

![Figure-16: Partial karyotype showing Ph.](image)
The rest CML cases carry masked translocations that can be detected only by molecular techniques, such as fluorescence in situ hybridization (FISH) or reverse transcriptase-polymerase chain reaction (RT-PCR) for the \textit{BCR-ABL} fusion (Cortes et al, 1995; Aurich et al, 1998).

\textbf{Secondary chromosomal changes in CML}

Secondary chromosomal changes in CML may be present at diagnosis (in 10\%, possibly with unfavorable significance), or may appear during course of the disease. Chromosomal changes in addition to the Ph are the norm in blast crisis. A number of these chromosomal abnormalities are relatively common, though it is unclear if this is because their occurrence drives progression. They may have a selective advantage in the progression environment, or if they are simply more frequent because of structural elements making their recombination more common.

\begin{center}
\begin{tabular}{cccccccc}
8 & 22 & 17 & 19 & y & 21 & 17 & 7 \\
34\% & 30\% & 20\% & 13\% & 8\% & 7\% & 5\% & 5\%
\end{tabular}
\end{center}

\textit{Figure-17: Representative image shows the most common secondary chromosomal changes in CML and frequency of each anomaly reported in the literature [Ref. No: Johansson B, 2002].}

Major route cytogenetic changes include trisomy 8, isochromosome i(17q10), trisomy 19 and an extra Ph chromosome (double Ph or +Ph). Minor route cytogenetic changes include monosomies of chromosomes 7, 17, and loss of Y; trisomies of chromosomes 17, 21; and translocation t(3;21)(q26.2;q22) [\textbf{Figure: 17}] (Johansson, 2002; Reid et al, 2008)

\textbf{Most common secondary aberrations in CML (Major route changes)}

\textbf{Chromosome 8 trisomy}

Trisomy 8 is common cytogenetic change in blast crisis, since \textit{MYC} is located at 8q24, it is tempting to speculate that here \textit{MYC} is driving progression.
There are several lines of evidence linking *MYC* to progression. *MYC* is often overexpressed in blast crisis compared with chronic phase. Curiously, trisomy 8 is a common feature of cases of clonal evolution in patients with CML treated with Imatinib who are in cytogenetic remission (thus, these clones have trisomy 8, but not the Ph). These cases with trisomy 8 seem to have a benign course, suggesting that trisomy 8 in and of itself may not be leukemogenic (Radich, 2007).

**Double Ph chromosome**
The role of the double Ph chromosome in disease progression is also unclear. Perhaps the presence of this chromosomal abnormality leads to increased expression of *BCR/ABL*, which has also been reported in advanced disease stages. However, the relationship between *BCR/ABL* levels and presence of the second Ph chromosome has not been formally tested. Whether the increased expression of *BCR/ABL*, per se, is sufficient to induce the phenotype of CML-blast crisis cells is uncertain (Calabretta and Perrotti, 2004; Radich, 2007).

**Isochromosome i(17q10)**
i(17q10) causes the loss of a copy of the p53 tumor suppressor gene. However, the remaining p53 allele does not seem to be mutated in these cases, so a direct link of p53 inactivation and progress to blast crisis is not so clear. However, reduction of the total p53 level may upset the complex integration of genetic repair and apoptosis and contribute to progression (Radich, 2007).

**Trisomy of chromosome 19**
As a secondary or additional abnormality, +19 is frequently encountered CML, though not as common as other secondary changes. +19 is seen in up to 13-15% of CML patients with additional abnormalities and may play a role in the pathogenesis of megakaryoblastic leukaemia (Ma ESK and Wan TSK, 2003).
Less common chromosomal abnormalities in CML (Minor route changes)

Loss of chromosome Y
It is generally considered a secondary event of no added clinical significance. Genes involved, if any, are unknown. Likewise, it is not known whether the Y chromosome loss is a secondary genetic change, or if the critical (submicroscopic) genetic change simply occurs by chance in a -Y cell. Speculatively, loss of the Y could provide a proliferative advantage simply because it tends to replicate late in S-phase. Its loss might therefore shorten the cell cycle slightly (Van Dyke DL, 2001; Zhang et al, 2007). Recent data indicate that loss of the Y chromosome should be taken into account in the prognostic evaluation of CML patients (Lippert et al, 2010).

Trisomy of chromosome 21
The second most common is trisomy 21, which is seen in 7% of blast crisis, but mostly in combination with one or more major route anomalies (Sandberg, 1991). It may contribute to clonal evolution and Imatinib resistance in CML (Roche-Lestienne, 2008).

Monosomy of chromosome 7
-7/del(7q) is a frequent cytogenetic change in secondary MDS, ANLL and leukemias occurring in individuals with constitutional syndromes including predisposition to myeloid disorders. These findings suggest the presence of a putative myeloid leukemia suppressor gene in the commonly deleted genomic segment 7q22. Multiple genes in 7q22 -31.1 are playing a role in leukemogenesis (Desangles F, 1999).

Trisomy of chromosome 17
Trisomy 17 is found about 4% of the total and 1% of cases that have no major route changes (Heim and Mitelman, 1991).

The (3;21)(q26;q22) and (7;11)(p15;p15) translocations
Translocations of known oncogenes in blast crisis occur rarely (<5%). The most notable of these recurrent translocations are t(3;21) and t(7;11),
involving the *AML-1/EVI-1* and *NUP98/HOXA9* genes, respectively. *EVI-1* and *HOXA9* are both transcription factors, and their aberrant expression in the context of these fusion proteins causes differentiation arrest in the case of *AML-1/EVI-1*, and increased proliferation in the case of *NUP98/HOXA9* (Radich, 2007).

**Significance of Fluorescence in situ hybridization (FISH) studies in CML**

![Fluorescence In Situ Hybridization](image)

*Figure-18: Schematic presentation of FISH principle*

FISH analysis is typically performed by denaturation and co-hybridization of a probe DNA to denatured metaphase chromosomes or interphase nuclei [Figure: 18]. Traditional FISH is a two-color technique in which fluorescent probes are utilized with contrasting colors to detect the position of the respective genes. The random superimposition of fluorescent probes in normal interphase nuclei can lead to false-positive results. The frequency of false positivity can be as high as 3-10%, making quantification below 10% is unreliable (Dewald 1993; Garcia-Isidoro, 1997; Werner, 1997). Now a days three color and four color FISH strategies are also available.
In addition, a FISH study usually analyzes 200 to 500 nuclei. Thus, quantification generated by FISH might be more accurate than conventional cytogenetics. FISH can also be performed on interphase cells from both peripheral blood and bone marrow. Therefore, FISH is another tool that can be routinely used for quantification of residual leukemic cells (Bennour et al, 2009).

**Different aspects of FISH studies in CML**

Dual color FISH is an effective tool, not only for the characterization and interpretation of variant translocations through the localization of the different signals on the partner chromosomes, but also for the deletions of *ABL* and *BCR* adjacent to the translocation junction on der(9).

1. **Deletion on der(9)**

FISH also detects deletion on both the chromosomes, three types of deletions can be observed on der(9); *ABL* deletion, *BCR* deletion, and *ABL/BCR* deletion. About 15% cases have a large deletion adjacent to the translocation breakpoint on the derivative chromosome 9, which represents an independent prognostic factor associated with shorter survival time but with the newer treatment strategies, no such association observed (Lee et al, 2006, Bennour et al, 2009).

2. **Variant Ph in CML**

5-10% of CML patients carry variant Ph translocation in which the Ph chromosome is derived through rearrangements other than the classic t(9;22) (Bennour et al, 2009). How the variant translocations form is controversial (Gorusu, 2007; Richebourg, 2008; Bennour, 2009). There are two types of mechanisms described as one-step mechanism and two-step mechanism.

![Figure-19a: schematic representation of one-step mechanism.](image-url)
**One-step mechanism**, in this type chromosome breakage occurs on three different chromosomes simultaneously in a three-way translocation, for instance and reciprocally rejoin at the same time [Figure: 19a].

![Two-step mechanism](image)

*Figure-19b: schematic representation of two-step mechanism.*

**Two-step mechanism**, in this type standard two way t(9;22) is followed by subsequent translocations involving additional chromosome. The two-step mechanism suggests that the formation of a variant translocation similar to or is in essence a clonal evolution and might be associated with poorer prognosis [Figure: 19b].

- Gorusu et al, 2007 have reported, variant CML cases regarding the genesis and prognosis and found less favorable therapeutic response, whereas, no association was observed between type of variant mechanism and the cytogenetic response to Imatinib therapy. Such analysis improves the knowledge of the genesis and the outcomes of CML. Bennour et al, also showed that with use of simple tools such as, Locus Specific Identifier (LSI) and Whole Chromosome Paint (WCP)-FISH mechanism of genesis of variants can be confirmed (Richebourg, 2008; Bennour, 2009).

3. **Place variation of and masked Ph in CML**

In the great majority of cases, rearrangement of chromosome 9 and 22 is cytogenetically visualized on Ph chromosome, harboring the chimeric gene. In about 2-4% patients have a cryptic or masked rearrangement with no cytogenetic evidence for the Ph chromosome but are positive by FISH and/or RT-PCR (Verma and Chandra, 2000). These latter patients are described as CML Ph-ve BCR/ABL+ve with the chimeric gene present on the derivative
chromosome 22, as in most cases, or alternatively on the derivative 9 and rarely on other chromosome.

![Possible mechanisms for BCR-ABL variant localization](image)

**Figure-20:** A schematic representation is showing possible mechanisms for BCR-ABL variant localization.

Two alternative mechanisms have been postulated to explain the *BCR/ABL* fusion gene in Ph-ve patients (Takahasi et al, 1996). First, a standard t(9;22) produces the der(9) and der(22) chromosomes, which then undergo a second translocation reconstituting both chromosomes to their original size or only slightly abnormal [Figure: 20]. The positioning of the breakpoints on the second translocation would determine whether the *BCR/ABL* fusion would be left behind on the der(22) or would move to the der(9). The second mechanism is insertion of 9q34 sequences including *ABL* onto the der(22) [Figure: 20]. Alternatively, insertion of 22q11.2 sequences comprising the 5’ portion of *BCR* onto *ABL* on 9q34 results in the chimeric gene located on the der(9). The first mechanism requires four breakpoints; the second requires three. At least 21 cases are described in the literature (Storlazzi et al, 2002; Wan et al, 2004; Fugazza et al, 2005) showed fusion gene located at 9q34.

- They seem to have the same clinical and molecular characteristics as Ph+ve patients, however some authors have noted a worse prognosis associated with location of *BCR/ABL* on the der(9) and some have found no correlation. (Michalova, 2002; Dufva, 2005). The few reported cases with unusual localization of the *BCR/ABL* fusion gene had a poor prognosis.
Despite the advances made in defining genes related with translocations in CML, the molecular mechanisms of leukemogenesis in the Philadelphia-negative BCR/ABL positive CML cases remain to be studied.

4. Location of the BCR/ABL fusion genes on both chromosomes 9 and masked Ph

Several investigators have reported localization of fusion gene on region 9q34, and different mechanisms of origin of this rearrangement have been suggested [Figure: 21].

![Figure-21: A schematic representation is showing possible mechanism for BCR-ABL variant localization on both 9.](image)

- Latest reports showed that, only six such cases are reported with BCR/ABL fusion genes on both chromosomes 9q34 and masked Ph (Michalova et al 2002; Dufva et al, 2005).
- The presence of two copies of BCR/ABL could be the sign of start of acceleration and/or blast crisis of CML. The chromosome 9 carrying the BCR/ABL chimeric gene may have been duplicated and a normal
chromosome 9 lost, this duplication is a similar event to an extra Ph chromosome. These cases suggest that the formation of BCR/ABL chimeric gene is a critical event in CML, no matter where it may be located (Michalova, 2002; IH Dufva, 2005).

**Significance of Multi-Color FISH (M-FISH) studies in CML with complex chromosomal rearrangements (CCR)**

During recent years, several molecular cytogenetic methods including M-FISH, Spectral karyotyping (SKY) and multicolor combined binary ratio labeling (COBRA) have been introduced. The advances have simultaneous visualization of all chromosomes in different colors (Babicka et al, 2006).

These methods have been used successfully to identify subtle or cryptic chromosomal abnormalities and to delineate further structurally altered chromosomes in hematologic malignancies (Barbouti, 2002; Speicher et al, 1996). There are two main groups targeted by M-FISH to identify, cryptic chromosome rearrangements not detectable by conventional cytogenetics: 1. those with an apparently normal karyotype which may harbor small rearrangements. 2. Those with a complex karyotype which are difficult to karyotype accurately due to the sheer number of aberrations.

- Complex chromosomal rearrangements are rare in CML, and their role in pathogenesis is still poorly understood; however, they seem to represent random aberrations as result of the genomic instability. Babicka et al (2006) have shown that, analysis of CCR in 16 CML patients with sensitive molecular methods is important, which could lead to detection of new or nonrandom complex rearrangements. Using SKY, Ikuta et al (2008) confirmed five-way translocation and showed that determination of the details of the complex translocations.

**M-FISH: technical aspects**

One of the most appealing aspects of FISH technology is the ability to identify several regions or genes simultaneously using different colours. There are two
methods for generating multicolour FISH probes using the small number of available spectrally separable fluorochromes. The first, combinatorial labeling generates probes labeled with a unique combination of fluorochromes in a 1:1 ratio (Nederlof et al., 1992). Combinatorial labeling is the most straightforward way to label probes in a multiplex way, because each fluorochrome is either present or absent. Using this strategy, the total number of colours achievable (N) is given by the equation \( N = 2^n - 1 \), where \( n \) = number of spectrally separate fluorochromes. Therefore, three fluorochromes can generate seven colors and only five fluorochromes are required to generate 31 colors [Figure: 22].

![Figure-22: M-FISH principle](image)

*Figure-22: M-FISH principle: The figure shows the combinatorial labeling of chromosomes 1-22, X and Y with five different fluorescent dyes.*

M-FISH uses specific narrow bandpass fluorescence filter sets and digital imaging equipment as part of a conventional epifluorescence microscope, with appropriate computer software. This software computes the combinatorial labeling algorithm and allows separation and identification of all chromosomes, generating a color karyotype in which each chromosome is given a characteristic pseudo color.

M-FISH and different FISH probes are commercially available however, for novel and rare breakpoint probes are not available commercially. In such cases home-brew or BAC FISH probes can be generate to map the
breakpoints and also to rule out the mechanism of genesis involved in variant formation.

**Significance of BACs or Homebrew FISH studies in CML**

BAC probe system is able to identify not only the t(9;22)(q34;q11.2) rearrangement and detect submicroscopic deletions on der(9) but also the mapping of different breakpoints involve in variant Ph.

- Recently, Malvestiti *et al* (2009) identified different breakpoints involved in complex rearrangements with Ph in CML with use of different BAC probes. Virgili *et al* (2008) have done FISH mapping with BAC clones and confirmed the presence of true breakpoint hot spots and assessed their implications in Ph negative *BCR/ABL* positive 9 CML patients.

**Therapeutic regimens for CML**

Historically, therapy for CML was empirically based. During the late 1800s, the mainstay of therapy for CML was Fowler solution, which was developed by Dr Thomas Fowler in the mid-1700s. The active ingredient in Fowler solution was probably potassium arsenite and there has been a resurgence of interest in the use of arsenic preparations for CML (Druker, 2008).

**Conventional treatment before the Imatinib era**

During the 1900s, radiation, busulfan, hydroxyurea, Interferon-α, and stem cell transplantation were developed for other indication, tried broadly, and found to have activity in CML (Geary, 2000). Allogenic stem cell transplantation is the only proven curative therapy, but is associated with significant morbidity and mortality (Baccarni, 2006).

**Imatinib Mesylate: A targeted gene therapy in CML**

The late 1990s saw the introduction into clinical practice of Imatinib mesylate (Glivec, Gleevec; Novartis, United States), the first true “magic bullet” in cancer therapy — that is, a treatment that specifically interacts with abnormal cellular processes in cancer cells, largely sparing normal cellular processes. Imatinib is a synthetic tyrosine kinase inhibitor (TKI) specifically designed to
inhibit the *BCR-ABL* fusion protein, by competitive binding at the ATP-binding site [**Figure: 23**].

![Mechanism of action of Imatinib](image)

**Figure-23: Mechanism of action of Imatinib.**

The antecessor of Imatinib was initially developed, by scientists at Ciba-Geigy (currently Novartis, Basel, Switzerland), as a specific platelet-derived growth factor receptor (PDGFR) inhibitor. It was also found to be a potent *ABL* tyrosine kinase inhibitor. Further optimized for v-*ABL* tyrosine kinase inhibition, Imatinib mesylate was generated. Imatinib selectively inhibits *ABL* tyrosine kinase, including *BCR-ABL* (Druker, 2008).

**Efficacy of Imatinib**

5-year follow-up results from the IRIS trial have allowed a mature assessment of Imatinib. A complete haematological response is seen in 97% of patients; and a complete cytogenetic response is seen in 82% of patients. The estimated 5-year progression-free survival is 84%. The estimated 5-year survival without progression to accelerated phase or blastic transformation is 93%. The overall survival on first-line Imatinib on an intention-to-treat analysis is 89.4%, rising to 95.4% if CML-unrelated deaths are excluded. Moreover, it is clear that patients who achieve major molecular response enjoy an extremely low risk of progression to accelerated or blastic phase CML, regardless of when the response is achieved. Another key finding is that this is maintained: there has been no increased incidence over time of
progression to accelerated phase or blastic transformation, unlike with earlier therapies (Druker, 2008). Recently, Holzerova et al. (2009) also showed in cohort of 72 previously treated patients, the Imatinib mesylate was successful in patients with specific additional chromosomal changes, apart from the patients with complex karyotype.

**Imatinib Resistance**

Although Imatinib induces a high frequency of satisfactory responses, primary and acquired resistance can be seen in all stages of CML. Resistance to Imatinib is multifaceted. Different mechanisms of resistance have been intensively studied in the recent five years. Among these mechanisms, the *BCR-ABL* kinase domain mutations are the most studied. The first reported mutation mediating resistance was T34I (T315I in the type 1a numbering scheme originally used). To date, more than 50 different *BCR-ABL* kinase domain mutations have been found to be associated with Imatinib resistance (Druker, 2008).

**The second generation of BCR-ABL inhibitors**

Much like the original paradigm, whereby an understanding of the molecular pathogenesis of CML led to the development to Imatinib, the understanding of the mechanism of resistance to Imatinib led to the rapid development of new drugs to circumvent resistance. Dasatinib and Nilotinib have progressed rapidly through clinical trials, and both are FDA-approved for patients with resistance or intolerance to Imatinib. Both showed 10- to 30-fold increased potency over Imatinib against the major resistant mutants, except T315I. These drugs also showed significant activity and good durability of responses in patients with relapsed, chronic-phase disease (Druker, 2008).

Treatment outcome in CML is generally evaluated by cytogenetic and hematologic response according to NCCN.V.2.2010 (National Comprehensive Cancer Network®) clinical practice guidelines in oncology™.
The assessment of therapeutic response of cytogenetic studies in CML

Conventional and molecular cytogenetics is a current standard tool for monitoring CML patients. For Cytogenetic response: Complete (0% Ph+), Partial (1-35% Ph+), Minor (36-65 % Ph+), Minimal (66-95% Ph+), and no response (>95% Ph+), in at least 20 metaphase in marrow samples, at least at every 6 months until complete response achieved and confirmed, hence at least every 12 months.

The therapeutic response of Hematologic response in CML management

For hematologic response (NCCN.V.2.2010), complete normalization of peripheral blood counts with white-cell count of <10X10^9/L, a platelet count of <450 X10^9/L, No immature cells (myelocytes, promyelocytes or blasts) in peripheral blood and nonpalpable spleen should be achieved. Partial response is same as complete response, except for presence of immature cells, platelet count <50% of the pretreatment count, but <450X10^9/L and persistent spleenomegaly, but <50% of the pretreatment extent.

Indian scenario of cytogenetic studies

In India, it is the commonest type of leukemia accounting for 30-45% of all leukemia cases. The most of the work regarding cytogenetic profile in CML and their significance in terms of diagnosis and prognosis has been reported from Western and European countries, while characterization of cytogenetic abnormalities in CML patients from India are poorly documented. The systematic studies are available from few centers in India, including our laboratory (Rajjapa, 2008).

The first cytogenetic data on CML patients from India was published by Das et al in 1978 and the study was based on chromosomal analysis from unbanded metaphases. Since then several centers started cytogenetic diagnosis of CML patients and other cancers. The systematic studies are available only from few centers in India. The major chromosome abnormality
t(9;22) is seen in 80-90% of CML cases as per the reports from India. Kadam et al (1991) from Tata memorial hospital, Mumbai reported cytogenetic data in 100 CML patients. 95% CML patients reported to be Ph+ve and the additional anomalies frequency in CML-BC was 66.6%. From Hyderabad, Jacob et al (2002) reported cytogenetic data in large group of CML patients and 86.3% were Ph+ve and common additional anomalies about 8%. The cytogenetic data are also available from AIIMS, New Delhi, the study was done with combination of conventional cytogenetic and FISH and patients were followed up during the treatment (Jobanputra et al, 1999). A study from a diagnostic center in Ahmedabad reported is 110 CML patients and with a rare complex translocation (Sheth et al, 2002). Recently, a study of 175 CML patients was reported from Ranbaxy laboratory, Mumbai. The Ph was detected in 86.5% cases and variant anomalies were detected in 8.10% cases. Among variant chromosome aberrations del(Xq), del(11q) were rare anomalies in Indian population (Chavan et al, 2006). Previous report from our laboratory, the cell biology division, The Gujarat Cancer and Research Institute, have reported, an interesting study of 169 CML patient data and deletion of 9q status and additional chromosomal abnormalities and also reported various novel findings with the help of molecular cytogenetic tools (Adhvaryu et al, 1990, Patel et al, 1998; Roy et al, 2002; Bakshi et al, 2006, 2007; Patel et al 2008; Brahmbhatt et al, 2010).