3. MATERIAL AND METHODS:

Present study has been done on six hundred fifty patients of leukemia from the Department of Pathology, Pt. B.D. Sharma University of Health Sciences, Rohtak. This study was done from the year 2008-12. All the leukemia patients were subjected to detailed morphogenetic, hematological and cytochemical analysis. In the present study detailed history of acute myeloid leukemia patients especially regarding family history, age at diagnosis, chief complaints like duration of fever, paleness, generalized weakness, risk factors, occupation, associated disorders and anemic condition were recorded. Two hundred twenty patients of acute myeloid leukemia were subjected to cytogenetic and molecular analysis. Karyotyping of AML patients was done to detect the ploidy levels and chromosomal abnormality. These patients were also evaluated for molecular analysis using RT-PCR for the detection of mutation specific mutation i.e. translocations t(8;21), t(15;17) and inversion inv(16). To find out the prognostics significance of the study cytogenetic anomalies, immunophenotypic markers, time taken in therapy of patient were compared using Kaplan-Meier survival analysis. Acute myeloid leukemia patients were followed up for 2.5 years to find out complete remission, remission duration and survival. Complete remission, remission duration and survival were further categorized in various chromosomal groups for survival analysis. Blood specimens were taken with due ethical committee permissions and personal consent of the patients or guardians. The following criteria were followed to study AML patients.

Inclusion criteria:

- The study included only primary acute myeloid leukemia cases which were clinically examined and confirmed by the co-supervisor Dr. Sunita Singh, Prof. Deptt. of Pathology, Pt. B.D. Sharma University of Health Sciences, Rohtak.
- The study subjects included patients of age groups and gender for hematological and risk factors study.

Exclusion criteria:

- Relapse cases of acute myeloid leukemia which occurred after treatment were excluded from the study.
- Leukemia patients from states other then Haryana were excluded from the study.
3.1. Questionnaire and Consent form:

All patients were classified into four main type of leukemia i.e., acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL) and chronic lymphoblastic leukemia (CLL) (Table-2). A specialized questionnaire has been framed keeping in mind the required data for hematological study, the prenatal-postnatal risk factors, other associated disorders, pathological characters and other information. The information was recorded carefully after the consent of the patients/parents of the patients/guardians (Appendix –I).

Table-2. Classification of leukemia.

<table>
<thead>
<tr>
<th>Leukemia</th>
<th>Acute</th>
<th>Chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloid</td>
<td>Acute Myeloid Leukemia (AML)</td>
<td>Chronic Myeloid Leukemia (CML)</td>
</tr>
<tr>
<td>Lymphoid</td>
<td>Acute Lymphoid Leukemia (ALL)</td>
<td>Chronic Lymphoid Leukemia (CLL)</td>
</tr>
</tbody>
</table>

3.2. Risk Factors:

In the present study detailed information of patients including prenatal, postnatal history and occupation of the patients and their parents was taken. Clinical information was noted down. Certain risk factors occurring significantly in the literature were considered in the patients of acute myeloid leukemia. The risk factors were divided in to three categories i.e. occupation of the patients, prenatal and postnatal risk factors (Table-3). The main risk factors subjected to study as prenatal risk factors in mother of the patients were drug intake, fetal loss, pesticides exposure, infection, chemicals and ionizing radiation including EMFs and X-rays. The postnatal risk factors considered were smoking, alcohol consumption, pesticides exposure, ionizing radiation, infection and chemicals. Occupation of the patients and their parents were categories as industrial workers, agriculture workers, self business may be on small scale or large scale and unrecognized sectors. Evaluation of risk factors and to estimate the strength of association between prevalence of risk factors in AML adjusted Odds Ratio (ORs) at 95% confidence interval (CIs) was computed. The data was statistically analyzed using Odd’s ratio in Medcalc software (version12.7.7). Each risk factor was compared with all possible combinations to estimate the most strong and least associated factors. Odds Ratio within the risk factors revealed the relative association of each factor in occurrence of acute myeloid leukemia. An Odd’s Ratio greater than 1 indicates that AML is more likely to occur in the numerators risk factor and an Odd’s Ratio less than 1 indicates
that the condition or event is less likely to occur in the numerators risk factor and more in denominator risk factor.

**Table-3. Risk factors for acute myeloid leukemia patients.**

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Prenatal Factors</th>
<th>Risk Factors</th>
<th>Postnatal Factors</th>
<th>Risk Factors</th>
<th>Occupation as Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Drug intake</td>
<td>Smoking</td>
<td></td>
<td>Industry</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Fetal loss</td>
<td>Alcohol consumption</td>
<td>Agriculture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Pesticides exposure</td>
<td>Pesticides exposure</td>
<td>Self business</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Ionizing radiation</td>
<td>Ionizing radiation</td>
<td>Unorganized sector</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Infection</td>
<td>Infection</td>
<td></td>
<td>Brick making</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Chemicals</td>
<td>Chemicals</td>
<td></td>
<td>others</td>
<td></td>
</tr>
</tbody>
</table>

**3.3. Clinical Features:**

Clinical features like low grade fever, progressive pallor, generalized weakness, bodyaches, weight loss, bleeding, lymphadenopathy, anemia, hepatomegaly, splenomegaly, bone tenderness, jaundice, purpura, retinal haemorrhages of leukemia patients were noted from patient’s clinical history proforma with the help of clinician (Table-4). Percentage frequency of these clinical features was calculated in all subtypes of acute myeloid leukemia. Factors affecting leukemia like age, gender types and subtypes of leukemia were studied in detailed to know the interaction of various factors in diagnosis of leukemia. In the present study, p-values were calculated by subjected to ANOVA between age, sex and type of leukemia. Correlation and regression between clinical variable like age, WBC count, blast cell percentage, hemoglobin concentration, platelet count, complete remission and disease free survival was subjected to analysis with each other. Pearson’s correlation coefficients for the correlations between clinical variables in AML was calculated in age with WBC count, blast cell percentage, hemoglobin concentration, platelet count, complete remission and disease free survival. Likewise all factors were correlated and r (person correlation coefficient) values were calculated. In the regression analysis, regression analysis relationship between the clinical variables among AML cases and dependent variable and the predictor variables were analyzed. Complete remission (CR) categorical variable and disease free survival (DFS) were dependent variable while age with WBC count, blast cell percentage, hemoglobin concentration, platelet count and disease free survival analyzed as predictor variables.
Table-4. Clinical features of acute myeloid leukemia patients.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Low grade fever</td>
</tr>
<tr>
<td>2.</td>
<td>Progressive Pallor</td>
</tr>
<tr>
<td>3.</td>
<td>Generalized weakness</td>
</tr>
<tr>
<td>4.</td>
<td>Bodyaches</td>
</tr>
<tr>
<td>5.</td>
<td>Weight Loss</td>
</tr>
<tr>
<td>6.</td>
<td>Bleeding</td>
</tr>
<tr>
<td>6.</td>
<td>Lymphadenopathy</td>
</tr>
<tr>
<td>7.</td>
<td>Aneamia</td>
</tr>
<tr>
<td>8.</td>
<td>Hepatomegaly</td>
</tr>
<tr>
<td>9.</td>
<td>Splenomegaly</td>
</tr>
<tr>
<td>10.</td>
<td>Bone Tenderness</td>
</tr>
<tr>
<td>11.</td>
<td>Jaundice</td>
</tr>
<tr>
<td>12.</td>
<td>Purpura</td>
</tr>
<tr>
<td>13.</td>
<td>Retinal hemorrhages</td>
</tr>
</tbody>
</table>

3.3.1. Hematological analysis:

Six hundred fifty patients of leukemia were included for hematological analysis. The blood samples of patients were analyzed for hemoglobin concentration, T.L.C. (Total Leucocytes Count), D.L.C. (Diffential Leucocytes Count), blast cell percentage and platelets count. The number of W.B.C. was calculated using hemocytometer, hemoglobin by hemometer and blast cell percentage was calculated by using standardizes staining methods. Material required and precaution were given in the last in appendixes for all experiments (Appendix –II and III).

3.3.2. Hemoglobin concentration:

1. Cleaned and dried the graduated tube of hemometer with distilled water.
2. N/10 HCl was added into graduate tube up to 2 ml mark.
3. The blood was sucked into pipette up to 0.2 marks and transferred into tube.
4. Mixed the blood and N/10 HCl properly.
5. N/10 HCl was added drop by drop into tube till the color matches with standard color of hemometer.
6. Hemoglobin was measured in gm/dl (Figure-11).
3.3.3. White blood cell count:

1. Well-mixed blood was drawn exactly to the 0.5 mark in a white blood cell diluting pipette.
2. The excess blood was wiped from the outside of the pipette to avoid transfer of cells to the diluting fluid.
3. Immediately draw diluting fluid to the ‘11’ mark while rotating the pipette between the thumb and forefinger to mix the specimen and diluents.
4. Mixed the contents of the pipette for 3-5 minutes to ensure even distribution of cells. Expel unmixed and relatively cell-free fluid from the capillary portion of the pipette (usually 4 drops).
5. Placed the forefinger over the top (short end) of the pipette, hold the pipette at a 45° angle and touch the pipette tip to the junction of the cover glass and the counting chamber.
6. Allowed the mixture to flow under the cover glass until the chamber is completely charged. Similarly, fill the opposite chamber of the hemocytometer.
7. Allowed the cells to settle for about 3 minutes. Under low-power magnification and reduced light, focus on the ruled area and observe for even distribution.
8. Counted the white cells in the four 1 sq mm corner areas corresponding to those marked A, B, C and D in each of two chambers.
9. Counted all the white cells lying within the square and those touching the upper and right-hand center lines. The white cells that touch the left-hand and bottom lines were not counted. In each of the four areas, conduct the count as indicated by the ‘snake-like’ line (Figure-12).
10. The white blood cells were calculated per liter of blood (Figure-13).

Figure-11. Apparatus for Hemoglobin count.
Figure 12. W.B.C count of leukemia patients.

Figure 13. White blood cells of human blood.

Cell count (D): = N x (D/A) x 10 x 10^6

- N = total number of cells counted,
- D = dilution of blood,
- A = total area counted (in mm²),
- 10 = factor to convert area to volume (in μl), assuming a chamber of 0.1 mm depth
- 10^6 = factor to convert count per μl to count per liter

W.B.C count: Number of W.B.C X10^6/L
3.4. FAB classification:

In present study, French-American-British (FAB) was done for acute myeloid leukemia patients (Table-5). In most patients, it was relatively simple to distinguish between AML and ALL on morphologic grounds. The blasts from patients with AML were larger, with more abundant cytoplasm and more prominent, multiple nucleoli. The definitive diagnosis was done on the presence of Auer rods, concentrations of myeloid-containing granules, or demonstration of at least 3% granulated precursors in AML, usually visible on Leishman’s stain and confirmed by staining with Sudan black B. The FAB classification was done on the basis of 20% circulating white blood cells, evaluated on Leshman’s stained smears. The classification was done on the basis of morphology, cytogenetic and immunophenotyping and molecular analysis of acute myeloid leukemia. This classification was found to be more clinically useful and produced more meaningful prognostic information.

Table-5. Classification of AML patients as FAB subtype.

<table>
<thead>
<tr>
<th>FAB subtype</th>
<th>Classification criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>Undifferentiated acute myeloblastic leukemia</td>
</tr>
<tr>
<td>M1</td>
<td>Acute myeloblastic leukemia with minimal maturation</td>
</tr>
<tr>
<td>M2</td>
<td>Acute myeloblastic leukemia with maturation</td>
</tr>
<tr>
<td>M3</td>
<td>Acute promyelocytic leukemia</td>
</tr>
<tr>
<td>M4</td>
<td>Acute myelomonocytic leukemia</td>
</tr>
<tr>
<td>M4eos</td>
<td>Acute myelomonocytic leukemia with eosinophilia</td>
</tr>
<tr>
<td>M5</td>
<td>Acute monocytic leukemia</td>
</tr>
<tr>
<td>M6</td>
<td>Acute erythroid leukemia</td>
</tr>
<tr>
<td>M7</td>
<td>Acute megakaryocytic leukemia</td>
</tr>
</tbody>
</table>
3.4.1. Leishman’s staining:
1. Clean and dried slides of thin blood smear were taken.
2. These slides were fixed with methanol for 10 minutes.
3. Slides were stained in working stain solution for 3 to 5 minutes.
4. Then slides were diluted with Buffer pH-6.8 or double distilled water.
5. Rinsed well in buffer. If stain was too intense, washed longer in buffer.
6. If it was too weak, restrained the slides (Figure-14 and 15).
7. Completely air dried slides were observed under microscope.

3.4.2. Sudan Black B Staining:
1. Clean and dried slides of thin blood smear were taken.
2. Petriplate with absolute formalin was taken and added pieces of filter paper into it.
3. Petriplate was covered with slides of thin peripheral blood smear.
4. Incubated the petriplate with slides at 55°C for 1 hour.
5. Slides were air dries completely and incubated in ‘Sudan Black B’ solution for 1 hour at room temperature.
6. Then the slides were dipped 3-4 times in 70% alcohol and again washed with tap water and dried.
7. The slides were stained with Leishman’s Stain for 2 minutes and diluted with Buffer pH-6.8 or double distilled water.
8. Then washed with distilled water and watched under compound microscope.
9. Sudan Black B was negative for ALL & positive for AML (Figure-16).

Figure-14. Normal human PBF smear stained with Leishman’s stain.
Immunophenotyping was done by using flow cytometry (FCM) in collaboration from private laboratory. The samples of patients were taken in 5ml purple capped EDTA vacutainers for hematological studies. The AML diagnosis was based on peripheral blood (PB) smears stained with Leishman’s and Sudan Black B in 220 patients. All patients had $\geq 20\%$ blasts in peripheral blood. In 188 patients, peripheral blood was taken for immunophenotyping. The diagnosis was confirmed in most cases by appropriate
cytochemical staining and by immunophenotyping using flowcytometry (Figure-17). All patients were subtyped according to FAB (French-American-British) criteria (Bennett et al; 1991).

Figure-17. Manual analysis of flow cytometrical data’s of control sample.

3.5.1. CD Markers used for primary Diagnosis:

Leukemic blasts were obtained from peripheral blood of leukemia patients. Cell surface antigens were detected with flow cytometry (Becton Dickinson FAC Scan, Lysis 1 Program) and Fluorescein-isothiocyanate (FITC) conjugated antibodies. Antigens with MoAb percentage of over 20% were considered positive. B cell antigens were considered as CD19, CD10, CD20, CD24 and surface (SIg) and cytoplasmic (CyIg) immunoglobulins; T cell antigens as CD2, CD7, surface and cytoplasmic CD3 and CD5; myeloid antigens as CD13, CD14, CD33, CD65w, CD11b MPO and CD15; lineage-nonspecific markers as CD34 and HLA-DR (Table-6).
Table-6. Immunological characteristics in acute myeloid leukemia.

<table>
<thead>
<tr>
<th>FAB Subtypes</th>
<th>M0</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
<th>M7</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-/+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD11b</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD13</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
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<tr>
<td>CD14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD15</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
<td>-/+</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CD19</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
<td>-/+</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CD33</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>CD34</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>-/+</td>
<td>-/+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD56</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CD61</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CD64</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-/+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD65w</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>CD117</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>-/+</td>
<td>-/+</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
</tbody>
</table>

- Antigen not expressed, +/- Antigen expressed in less than 50%, +/- Antigen expressed in majority of patients, + Antigen expressed, blank represented partial expression without specificity for diagnosis or lack of reliable data.

3.6. Cytogenetic Analysis (Rooney and Czepulkowski; 1994):

The patients of newly diagnosed AML in the year 2008-2009 were considered for cytogenetic analysis. Two hundred twenty patients of acute myeloid leukemia were subjected to cytogenetic analysis however a successful cytogenetic preparation could be obtained in 188 patients due to inadequate samples in remaining cases for cytogenetic analysis. The whole blood sample was subjected to culture the lymphocytes. Chromosome harvesting was done after proper incubation of 72 hours at 37°C. Structural and numerical anomalies of the patients were noted down. Chromosomes are dynamic entities, continuously changing in appearance and carrying out inherent activities. For their physical examination, the highly dividing cells were arrested at metaphase stage. Different steps of culturing were as follows:
Step-1. Cleaning of Glassware:

All the glasswares were washed properly with the detergent before the use for culture techniques. These were soaked in acidic solution (K$_2$Cr$_2$O$_7$ + Conc. H$_2$SO$_4$ + D.W.) for one day and in running water for two days. After washing with hot distilled water, glasswares were kept in oven.

Step-2. Sterilization:

Glass wares (Petri plates, test tubes, flasks, culture bottles, measuring cylinder) were sterilized in oven at 175°C for about 2 hours. Plastic petriplates were steam sterilized in autoclave at 15 lb/in$^2$ for 20 minutes. Sterile double distilled water (Millipore filter assembly) was used throughout the culture procedure. For maintaining aseptic conditions culturing was done in laminar air flow cabinet. The platform of laminar air flow was properly cleaned using absolute alcohol then UV irradiation for 1 hour and blower on for 30 minutes. Hands were properly washed and rinsed with absolute alcohol to prevent any contamination. Needles and forceps used for culturing or sub culturing were wiped clean with alcohol. Whole work was carried out in front of a flame in the laminar air flow to avoid contamination.

Step-3. Sample Collection:

Cytogenetic analysis was done on peripheral blood samples of patients with AML. The blood sample of AML patients was collected in heparinised vacutainer before initiation of treatment.

Step-4. Setting up of culture:

1. The whole blood sample (0.5ml) was cultured in (5ml) RPMI-1640 media supplemented with fetal calf serum, antibiotics and phytohemagglutinin (PHA) (Himedia).
   2. The culture was incubated at 37.5 ± 0.5°C for 24 hours in some cases 48 hours.
   3. The shaking of culture was done after every 2 hours.

Step-5. Arresting the cells:

After proper incubation, colchicine was added in the sample (Himedia) final concentration 0.012 mg/ml) and incubated at 37°C for 30 minutes.

Step-6. Harvesting:

1. The culture was centrifuged at 1000 rpm for 15 minutes.
   2. The supernatant was discarded and 7-8ml (0.075 KCl) hypotonic salt solution was added to bottom solution.
   3. The resulting solution was kept in water bath at 37°C for 8-10 minutes.
   4. It was centrifuged at 1000 rpm for 15 minutes.
5. Supernatant was again decanted off and 5ml of cold fresh fixative (1 part glacial acetic acid: 3 parts methanol) was added without disturbing the cell pellet.
6. The solution was centrifuged at 1000 rpm for 15 minutes.
7. The above step was repeated three times by adding 5ml fresh fixative every time.

**Step-7. Slide preparation:**

1. Slides were prepared by soaking in cleansing solution (95% alcohol+5ml) from the beginning of harvesting in plankton.
2. The slides were rinsed in DW and dried.
3. Heavy (turbid) cell suspension was used for chromosome preparation.
4. Pasteur pipette was used to drop the cell suspension on slides.
5. Slides were held at 60° to 80° angles down from horizontal position.
6. The drop of cell suspension was allowed to run down the slide quickly.
7. Excess liquid was removed from the end of the slide by touching quickly with blotting paper and slides were allowed to dry completely in the slanted position.

**Step-8. Banding and Staining (G-banding) (Sumner; 1982):**

Giemsa staining of chromosome preparation after proteolytic enzyme (Trypsin) treatment revealed G-banding. The slides were matured at 37°C for three days. The three day old unstained matured slides were flooded with 0.25% Trypsin for 10-15 seconds, then the slides were given two washes in phosphate buffer saline (PBS) and were stained in 2% Giemsa stain for 5-7 minutes, thereafter, they were washed in distilled water. Metaphases were analyzed using Cytovision 3.7 software provided by Applied Imaging (ZEISS Microscope) classified according to ISCN 1995 (Mitelman; 1995). At least 20 metaphases were karyotyped and analyzed (Figure-18).

**Step-9. Cytogenetic Category (WHO classification) (Gustashaw; 1991):**

Patients were divided into groups based on chromosome finding. Patients were first classified according to presence or absence of a chromosomal abnormality and then by modal number of chromosomes. The results of cytogenetic analysis were correlated with patient’s clinical and hematological parameters for risk stratification.
3.7. Molecular analysis:

Patients of acute myeloid leukemia were subjected to molecular analysis to find out the mutations in these patients. Patient acute myeloid leukemia who were FAB subtype, immunophenotyped and identified for particular translocation for specific mutation were further subjected to confirm the translocation.

3.7.1. Trizol-method of RNA extraction (Chomczynski; 1993):

Selected AML patients were considered for RNA isolation. To confirm the mutation RNA from acute myeloid leukemia was extracted cDNA was synthesized by reverse transcriptase polymerase chain reaction. The product was visualized by agarose gel
electrophoresis. The samples of patients were taken in full Yellow-Top BD Vacutainer tube of human blood (equals roughly 8 ml) to yield approximately 30 μg of RNA.

1. Contents of vacutainer tube were transferred into a 50 ml polypropylene conical centrifuge tube.
2. Volume was taken to 50 ml with RBC Lysis Buffer and left at room temperature for 10 to 15 minutes or for 20 to 30 minutes at 4°C.
3. Cells were pelleted at 1,400 rpm for 10 minutes in a 4°C centrifuge.
4. Supernatant was carefully discarded.
5. The pellet was gently resuspended in 1 ml of RBC Lysis Buffer and transferred to a 1.5 ml microcentrifuge tube and left for 2 to 5 minutes.
6. Cells were pelleted for 2 minutes by centrifuging in a microfuge at 3000 rpm. This does not need to be cold and the supernatant was carefully aspirated.
7. Resuspend the pellet in 1 ml of sterile PBS and repeated the above step.
8. 800 μl of TRIzol solution was added to each tube and resuspend the cells.
9. 0.2 ml of Chloroform (CHCl3) was added and vortex each tube for 15 seconds.
10. Samples were left stand on ice for 5 to 10 minutes.
11. The samples were centrifuged at 14,000 rpm for 15 minutes at 4°C.
12. Removed the upper phase and transfered to a cleaned microcentrifuge tube to which an equivalent volume of ice-cold isopropanol was added. Immediately vortex and place on ice for 15 minutes.
13. Samples were centrifuged at 14,000 rpm for 15 minutes at 4°C.
14. Carefully decanted the supernatant, and rinsed the pellet with 0.5 ml of ice-cold 75% ethanol.
15. The samples were centrifuged at 7500 rpm for 8 minutes at 4°C and the supernatant was decanted.
16. The samples were quick-spanned (14,000 rpm, 30 seconds, 4°C) to drive the remaining fluid to the bottom of the tube.
17. Using a P10 pipettor, carefully removed all of the remaining liquid in the bottom of the tube.
18. The pellet was allowed to dry for 5 to 10 minutes to remove any remaining ethanol.
19. The RNA pellet was dissolved by adding 30 to 50 μl of RNase-free H2O to each sample.
20. Purity of RNA sample was checked by OD at OD260/OD280. All the samples were found to be in desirable reference ratio of 2.0.
RNA was isolated from whole blood using Trizol-method of RNA extraction. Purity of RNA was checked at the OD 260/OD 280 (Figure-19a and 19b). All the samples were found to be in desirable reference ratio 1.9 to 2.0. The purified RNA samples were stored in elution buffer at -80°C.

![Figure-19(a). Estimation and quantification of RNA sample of FAB-M2 subtype purity by using Nanodrop method.](image)

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>User</th>
<th>Sample Type</th>
<th>Timestamp</th>
<th>Conc.</th>
<th>Unit</th>
<th>A (260 nm)</th>
<th>A (280 nm)</th>
<th>260 / 280</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(8;21) M2</td>
<td>Radha Rathee</td>
<td>RNA</td>
<td>6/19/2014 12:44:38 PM</td>
<td>49.665</td>
<td>ng/μl</td>
<td>0.992</td>
<td>0.480</td>
<td>2.064</td>
</tr>
</tbody>
</table>

Figure-19(b). Estimation and quantification of RNA sample of FAB-M3 subtype purity by using Nanodrop method.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>User</th>
<th>Sample Type</th>
<th>Timestamp</th>
<th>Conc.</th>
<th>Unit</th>
<th>A (260 nm)</th>
<th>A (280 nm)</th>
<th>260 / 280</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(15;17) M3</td>
<td>Radha Rathee</td>
<td>RNA</td>
<td>6/19/2014 12:59:03 PM</td>
<td>46.265</td>
<td>ng/μl</td>
<td>1.157</td>
<td>0.604</td>
<td>1.916</td>
</tr>
</tbody>
</table>
3.7.2. Molecular analysis using RT-PCR (Krauter et al; 1998):

Patients of acute myeloid leukemia were subjected to molecular analysis to confirm the mutations in these patients. Total cellular RNA was extracted from peripheral blood cells using the Trizol-method (GibcoBRL) according to the manufacturer’s guidelines with some modifications. cDNA was synthesized for 1 h at 37°C using 2 µg RNA, random primers (1 µmol/l) and murine Moloney virus reverse transcriptase in a total volume of 20 µl. The primers for the amplification of the fusion transcripts AML1/MTG8, PML/RARα and CBFβ/MYH11 (Chomczynski; 1993, Krauter et al; 1998) are shown in (Table-7). The PCR amplification was carried out with 2 µl cDNA in a total volume of 50 µl PCR buffer with 0-5 µmol/l primers, nucleotides 100 nmol/l and Taqpolymerase (Perkin-Elmer) 0-02 U/µl. Thermal cycling was done at 94°C for 60 s, 57°C for 60 s and 72°C for 120 s, with cycles repeated 35 times. After the first 35 cycles with the outer primers, 2 µl of the reaction product was further amplified for another 35 cycles with the nested primers under the same conditions. The amplification of the c-abl transcript was used as a positive control. In negative controls the RNA template was omitted. The reaction products were migrated on 2% agarose gels and stained with ethidium bromide.

**Table-7. Oligonucleotide primers used in our study.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer AML1-primer</td>
<td>5′-AGCCATGAAGAACCCAGG-3′</td>
</tr>
<tr>
<td>Outer MTG8-primer</td>
<td>5′-AGGCTGTAGGAGAATGG-3′</td>
</tr>
<tr>
<td>Nested AML1-primer</td>
<td>5′-TACCACAGAGCCATCAA-3′</td>
</tr>
<tr>
<td>Nested MTG8-primer</td>
<td>5′-GTTGTGGTGAATGGAA-3′</td>
</tr>
<tr>
<td>Outer PML-primer</td>
<td>5′-AGCGCGACTACGAGGAGATG-3′</td>
</tr>
<tr>
<td>Outer RARα-primer</td>
<td>5′-CATGGTCTTCTGGATGCTGC-3′</td>
</tr>
<tr>
<td>Nested PML-primer</td>
<td>5′-CTGGTGACTACGAGGAGATG-3′</td>
</tr>
<tr>
<td>Nested RARα-primer</td>
<td>5′-CCATAGTGTTAGCCTGAGGA-3′</td>
</tr>
<tr>
<td>Outer CBFβ-primer</td>
<td>5′-CAGGCAAGGTATATTTGAAGG-3′</td>
</tr>
<tr>
<td>Outer MYH11-primer</td>
<td>5′-CTCCTCCTCCTCATTGCTG-3′</td>
</tr>
<tr>
<td>Nested CBFβ-primer</td>
<td>5′-GTCTGTGTATCTGGAAAGGCTG-3′</td>
</tr>
<tr>
<td>Nested MYH11-primer</td>
<td>5′-CATGTGGACTCTCCAGCCGAGTT-3′</td>
</tr>
</tbody>
</table>

3.7.3. Agarose gel electrophoresis:

- Prepared 2% agarose gel by dissolving 0.8 g of agarose in 40 ml 1XTBE buffer in a microwave oven.
• Added 1 µl ethidium bromide.
• Poured the melted agarose in a tray with a comb to make the wells (Easy-Cast)
• When the agarose has solidified, removed the comb and put the gel in a refrigerator at 4°C until further use. The gel was not kept for more than a day.
• Put 1 µl of the nested PCR product, 1 µl of loading dye and 6 µl distilled water on a piece of parafilm. Mixed and loaded onto a well in the agarose gel.
• For the molecular marker, added 1 µl of 1000bp ladder, 10 µl of distilled water and 2 µl of loading dye on a piece of parafilm. Mix and load 6 µl each at the opposite ends of the wells.
• Run the electrophoresis at 130V for an hour (or until the dye is about three-quarter way of the gel length).

3.7.4. Photography:
  o Viewed the gel under UV light using Gel Documentation System.
  o Took a photograph of the gel using the camera system.
  o Results were interpreted according to the position of the bands with the 1000 bp ladder as a molecular marker.

3.7.5. Rapid Isolation of DNA:

DNA was extracted from peripheral blood by using rapid improvised isolation of mammalian DNA method (Sambrook; 2002). Its purity was checked spectrophotometrically as well electrophoretically. Purity of DNA sample was checked by OD at OD260/OD280. All the samples were found to be in desirable reference ratio of 1.65 to 1.85. The samples which were found to be fluctuating from the reference range were purified again by RNAse and proteinase treatment. The purified DNA was stored in the TE buffer (pH-7.6) at -20°C for further use. Mammalian DNA prepared from blood was 20-50Kb in size and suitable for use as a template in PCRs. The yield of DNA varies between 0.5 and 3.0 µg/mg tissue or 5-15 µg per 300 µl of whole blood. It was stored for further processing or DNA sequencing.

STEP-1. Transferred 300 µl aliquots of whole blood to each of the two microfuge tubes.
   Added 900 µl of 20Mm TRIS Cl (pH 7.6) to each tube and invert the capped tubes to mix the contents.

STEP-2. Incubated the solution at room temperature for 10 minutes, occasionally inverted the tubes.

STEP-3. Discarded all but kept 20 µl of each supernatant.
STEP-4. Resuspended the pellets of white cell in the small amount of supernatant left in each tube. Combined the resuspended cell pellet in a single tube.

STEP-5. Transferred the resuspended white blood cell pellet to microfuge tube containing 600 µl of ice cold lysis buffer. Homogenized the suspension quickly.

STEP-6. Added 3ul of proteinase K solution to the lysate to increase the yield of genomic DNA. Incubated the digest for at least 3 hours but no more than 16 hours at 55°C.

STEP-7. Allowed the digest to cool to room temperature and then added 200 µl of potassium acetate solution and mixed the content of the tube by vortexing vigorously for 20 seconds.

STEP-8. Pelleted the precipitated protein/SDS complex by centrifuging at maximum speed for 3 minutes at 4°C in a microfuge.

STEP-9. Transferred the supernatant to a fresh tube containing 600 µl of Isopropanol. Mixed the solution well and then recovered the precipitate of DNA by centrifuging the tube at maximum speed for 1 minute at room temperature in a microfuge.

STEP-10. Removed the supernatant by aspiration and add 600 µl of 70% ethanol to the DNA pellet. Inverted the tube several times and centrifuged the tube at maximum speed for 1 min. at room temperature in a microfuge.

STEP-11. Carefully removed the supernatant by aspiration and allow the DNA pellet to dry in air for 15 min.

STEP-12. Redissolved the pellet of DNA in 100 µl of TE (pH 7.6). Presence or absence of DNA was visualized by agrose gel electrophoresis.

3.7.5.1. Quantification of DNA (purification) for storage:

Purity of DNA was checked at the OD 260/OD 280. All the samples were found to be in desirable reference ratio 1.7 to1.8 (Figure-20a and 20b). The samples which were found to fluctuate from the reference range were purified again by RNAse and Proteinase K treatment. The purified DNA sample was stored in TE buffer (pH 7.6) at -20°C. DNA was quantified for purity. Wavelength of DNA was checked by Nanodrop at 260λ and 280λ and their ratio was checked.
Figure-20(a). Estimation of DNA sample of FAB-M2 subtype purity by using Nanodrop method.

Figure -20(b). Estimation of DNA sample of FAB-M3 subtype purity by using Nanodrop method.
3.8. Remission and Survival:

Patients were studied for complete remission, relapse, remission duration and overall survival. Relapse was defined by the reappearance of more than 5% leukemia cells in bone marrow. A minimum of two years and six months follow up was obtained on all patients. Complete remission, remission duration and survival were further categorized in various chromosome groups. Remission duration and survival were calculated and plotted by method of Kaplan and Meirer (Kaplan-Meirer; 1958).

3.8.1. Complete Remission (CR):

Complete remission was defined as less than 5% morphologically identified blasts in a bone marrow. The number and percentage of patients with CR was calculated and categorized into various chromosome groups.

3.8.2. Remission duration:

The remission duration of patients was measured from the date of complete remission to the end of remission or at the last date of follow up. Duration of initial remission of various chromosome groups was plotted from life tables, calculated by the method of Kaplan and Meier (Kaplan-Meirer; 1958). Differences in median remission duration were tested by long rank test (Cox; 1972).

3.8.3. Overall Survival:

The time was recorded for each patient from the start of treatment to the event of interest like relapse, death or to the last follow up. Duration of survival of various chromosome groups was plotted from life tables, calculated by the method of Kaplan and Meier (Kaplan-Meirer; 1958). Differences in median survival were tested by long rank test (Cox; 1972).

Appendix-I: Case History Form of Acute Myeloid Leukemia Patients
Appendix-II: Requirements
Appendix-III: Precautions
Appendix-IV: Statistical analysis