Molecular Screening of Acute Myeloid Leukemia Patients of Pakistani Origin

By

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Dedicated to the ones I love.

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Minahil Mehdi

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LIST OF ACRONYMS

EMP	Erythroid-Myeloid Progenitor
HSC	Hematopoietic Stem Cell
AML	Acute Myeloid Leukemia
WHO	World Health Organization
FAB	French-American-British
CBC	Complete Blood Count
FISH	Fluorescence In Situ Hybridization
RT-PCR	Reverse Transcription Polymerase Chain Reaction
CN-AML	Cytogenetically Normal-Acute Myeloid Leukemia
DNA	Deoxyribonucleic acid
PcGs	Polycomb Group
HDAC	Histone Deacetylase
FTI	Farnesyl Transferase Inhibitors
FLT3	FMS-like receptor tyrosine kinase-3
NPM1	Nucleophosmin 1
CEBPa	CCAAT/Enhancer Binding Protein Alpha
MLL-PTD	Mixed Lineage Leukemia- Partial Tandem Duplication
ASXL1	Additional Sex Combs Like 1

RUNX1	Runt Related Transcription Factor 1
IDH1/2	Isocitrate Dehydrogenase 1
EZH2	Enhancer of zeste homolog 2
JAK2	Janus Kinase 2
KIT	Proto-Oncogene Receptor Tyrosine Kinase
RAS	RAS Oncogene
RTK	Receptor tyrosine kinase
PDGFR	Platelet-derived growth factor receptor
CD34	Hematopoetic progenitor cell surface antigen
STAT-5	Signal transducer and activator of transcription 5
PI3K	Phosphoinositide 3-kinase
AKT	Protein kinase B
mTOR	Mammalian target of rapamycin
FLT3/ITD	FMS-like receptor tyrosine kinase-3/Internal tandem duplication
FLT3/TKD	FMS-like receptor tyrosine kinase-3/Tyrosine kinase domain
Kb	Kilobases
RHD	Runt homology domain
TA	Transactivation domain
ID	Inhibitory domain
CBFβ	Core-binding factor subunit beta

CBF	Core-binding factor
PEBP2 β	Polyomavirus enhancer binding protein subunit beta
MYB	MYB Proto-oncogene
STAT3	Signal transducer and activator of transcription 3
TCRA	T-cell receptor Alpha
TCRB	T-cell receptor Beta
CSF	Colony stimulating factor
BLK	B-lymphocyte kinase
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A
BCL2	B-cell lymphoma
IL3	Interleukin-3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
MPO	Myeloperoxidase
DNMT3B	DNA (Cytosine-5-)-Methyltransferase 3 Beta
Asx	Additional sex comb protein
HARE-HTH	Hyaluronic Acid Receptor For Endocytosis-helix-turn-helix
ASXH	Asx homology domain
DUB	Deubiquitinating enzymes
PHD	Plant homeodomain

ALL	Acute lymphoblastic leukemia
MDS	Myelodysplastic syndrome
JM	Juxtamembrane
WBC	White blood cells
%	Percentage
CR	Complete remission
OS	Overall survival
ES	Event-free survival
T	Threonine
Inv	Inversion
D	Aspartic acid
G	Glycine
R	Arginine
Q	Glutamine
K83N/WT	Lysine83Asparagine/wild type
FPD	Familial platelet disorder
R177Q/WT	Arginine177Glutamine/wild type
RUNX1T1	RUNX1 Translocation partner 1
C	Coding
Dup	Duplication
NK	Normal Karyotype
RBC	Red Blood Cell
$MgCl_2$	Magnesium Chloride

NaOH	Sodium Hydroxide
SDS	Sodium dodecyl sulphate
Rpm	Revolutions per minute
µl	Microliter
Nm	Nanometer
A	Absorbance
Ng	Nanogram
KCl	Potassium Chloride
dNTPs	Deoxyribonucleotide Triphosphate
U	Unit
µg/ml	Microgram per milliliter
Gel Doc	Gel Documentation
Chemo	Chemotherapy
PCR	Polymerase Chain Reaction
PROVEAN	Protein Variation Effect Analyzer
mg/m^2	Milligram per meter square
CN	Controls
N	No template control
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant

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ABSTRACT

Acute Myeloid Leukemia is a heterogenous hematological malignancy resulting in rapid increase of immature myeloid cells or blasts in peripheral blood and bone marrow. Largely undocumented in Pakistan, AML has little treatment options and diagnostic measures. The aim of the study was to screen Pakistani sample of AML patients for FLT3/ITDs, ASXL1 and RUNX1, all genes relevant with worst prognosis of the disease and whether this triple gene signature can be used as a predictor of response to chemotherapy. Peripheral blood samples of 32 AML patients and 10 healthy controls were taken. To detect mutations PCR amplification and gel electrophoresis was used for FLT3/ITD and sanger sequencing technique was utilized for detecting mutations in RUNX1 (Exon 3 and 5) and ASXL1 (Exon 12). Six patients were FLT3/ITD mutant positive whereas 5 non-coding variants were identified in 3 patients respectively. It may be concluded from this study, that non-coding variants have a role to play in bringing about AML in Pakistani populatio

INTRODUCTION

1.1 Hematopoiesis

Hematopoiesis is the formation of blood cells. The understanding of hematopoiesis has greatly evolved over the years and can be classified broadly into two categories: primitive wave and definitive wave. In humans, the primitive wave of hematopoiesis occurs at the embryonic stage within the yolk sac and later in the fetal liver. At this stage erythroid-myeloid precursors (EMP) have been observed to originate in blood islands (McGrath *et al*., 2011). These precursors are not renewable or pluripotent. However, this phase transitions to a more definitive wave of hematopoiesis at adulthood occurring in the spongy bone and thymus.

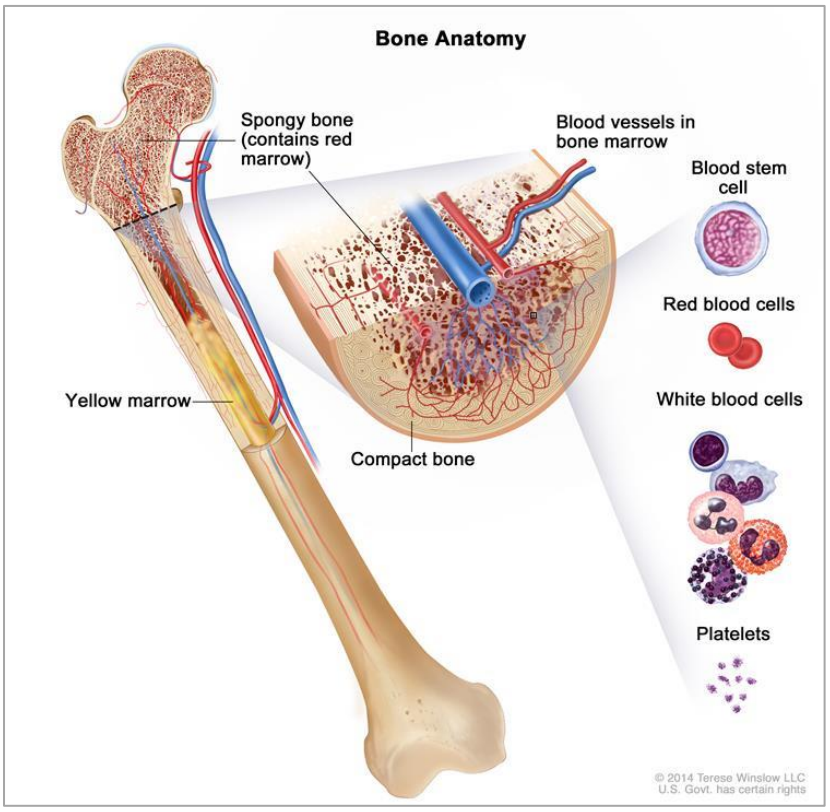

Figure 1.1: Anatomy of the bone. The internal spongy bone that contains red marrow is the site for hematopoiesis (anatomy-bodychart.us, 2016).

The bone (Figure 1.1) consists of an outer compact region and an inner spongy region consisting of red bone marrow. The spongy bone marrow gives rise to the earliest progenitor stem cells or hematopoietic stem cells (HSC's) which are multipotent in nature and express the capability of differentiating into various blood cell lineages. In normal hematopoietic process, initially HSCs differentiate into lymphoid and myeloid progenitors. These are further classified into T-cells, B-cells, natural killer cells, erythrocytes, macrophages, granulocytes and megakaryocytes (Tavian *et al*., 2010).

1.2 Leukemia

Leukemia or blood cancer is primarily a malignancy consisting of abnormal blood forming cells in blood-forming tissue i.e. bone marrow which overcrowd the marrow eventually spilling into the blood stream (Pubmed Health Glossary, 2016). Leukemia is further divided into various classes depending on the cell of origination (myeloid or lymphoid) and the rate at which the malignancy develops (acute or chronic). In leukemia (Figure 1.2) the maturation stages involved during conversion of myeloid-lymphoid progenitors into mature blast stage are disrupted. Major classification of leukemia is as follows:-

1. Acute myeloid leukemia
2. Acute lymphoblastic leukemia
3. Chronic myeloid leukemia
4. Chronic lymphoblastic leukemia
5. Hairy cell leukemia

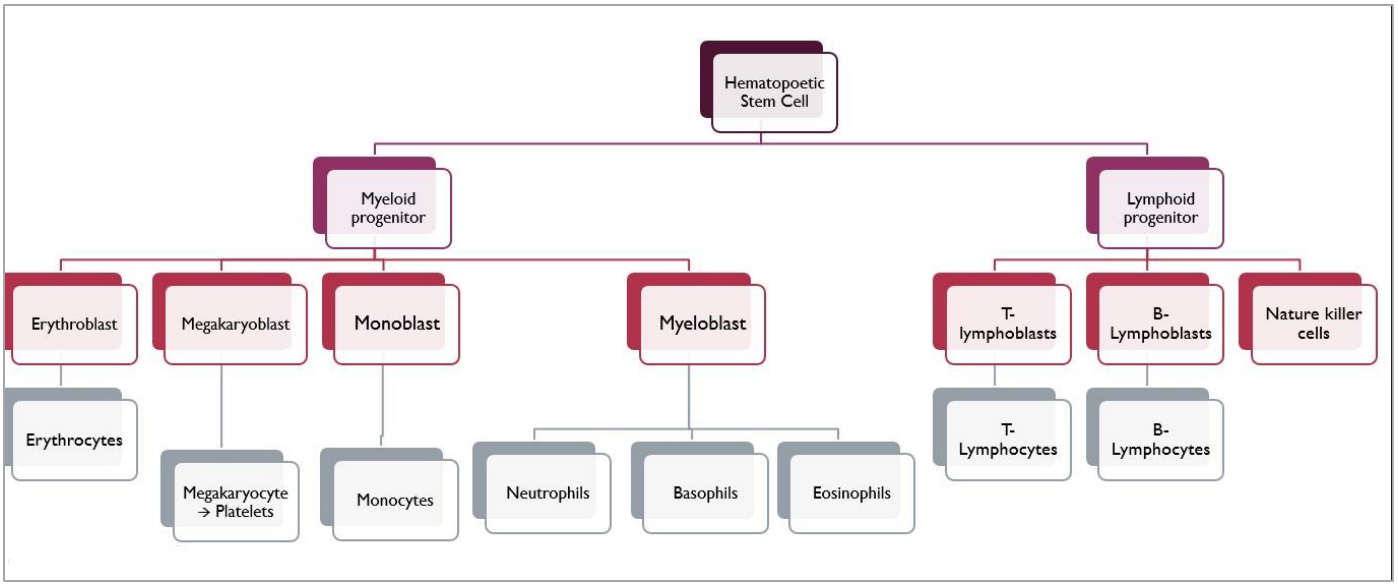

Figure 1.2: General overview of the hematopoietic process in the bone marrow.

1.3 Acute Myeloid Leukemia

One of the most commonly occurring subtype of leukemia is acute myeloid leukemia (AML). Acute myeloid leukemia is also referred to as acute myelogenous leukemia, a diseased condition characterized by abnormal increase in the amount of immature myeloid cells or blasts in the bone marrow and peripheral blood (Pubmed Health Glossary, 2016). Acute myeloid leukemia is found to have rapid progression that leads to greater number of fatalities thus it is a focal point for devising effective and quickly administrable treatments.

Acute Myeloid leukemia begins with the dysfunction of cells that make up the blood tissue. During AML, maturation and differentiation of particularly myeloid stem cells halts, resulting in immature myeloid cells or immature blasts (Figure 1.3) that accumulate in the bone marrow and blood.

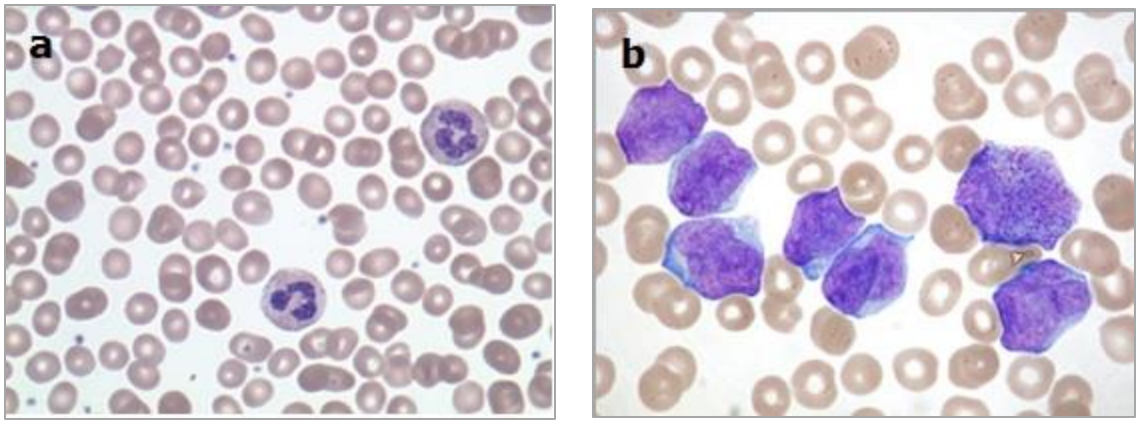

Figure 1.3: Blast cells**.** a) Normal peripheral blood picture b) Immature blast cells circulating in blood (Cancer.net, 2016).

Commonly, this abnormality causes production of immature white blood cells however, many times, immature red blood cells and platelets have been associated with AML as well. These immature blood cells lack the capacity to carry out their normal functions of immunity, carrying-oxygen and thrombosis. This irregularity in the blood is registered by the body and results in showcasing a variety of symptoms including fatigue, infections, pallor, anemia, dyspnea, unusual bleeding, and weight loss including various other signs and symptoms (Cancer.net, 2016).

This heterogeneous and aggressive hematological malignancy is characterized by accumulation of acquired genetic mutations HSC's resulting in modification and rapid increase of immature myeloid cells or blasts in peripheral blood and bone marrow. This hampers the normal functions of cell proliferation and differentiation. Since the molecular nature of AML is of great interest to study the dynamics of this complicated malignancy, genetic testing is currently being employed to check for mutations that collect in the genes (FLT3, NPM1, c-KIT, IDH1 etc.) that are frequently found in association with AML.

1.4 Prevalence

Acute myeloid leukemia is predominantly found in adults, irrespective of gender. The incidence of AML worldwide was estimated to have affected 351,965 individuals in 2012. In 2014, WHO reported an estimated 18,860 diagnosed cases in the US with a median age of 66 years (UICC EML Review, 2014). In Pakistan, AML prevalence is found both in children and adults however, the scope of the disease remains largely unknown.

Acute myeloid leukemia is a genetically heterogeneous clonal disorder that requires insight into its molecular aspects to devise simpler diagnostic methods and facilitate targeted treatment (Tayyab *et al*., 2014).

1.5 Classification of AML

Patients of AML cannot be categorized according to the stage of cancer; subtypes have been created for accurate characterization. Due to its heterogeneity and variation, AML has been classified by two systems presently; French-American-British (FAB) classification and World Health Organization (WHO) classification.

1.5.1 French-American-British (FAB) classification

Widely used, FAB distinguishes AML into eight subgroups on the basis of leukemic blasts morphology and differentiation ranging from M0 to M7. First introduced in 1976, the classification was proposed by seven French, American and British hematologists who studied 200 films of bone marrow and peripheral blood of AML patients (Bennett *et al.,* 1976). The revision report was also provided by the same group of hematologists updating the status of AML subtypes (Bennett *et al*., 1985).

Table 1.1: FAB classification for AML

	Name	FAB Subtype
1.	Undifferentiated acute myeloblastic leukemia	M0
2.	Acute myeloblastic leukemia with minimal maturation	M1
3.	Acute myeloblastic leukemia with maturation	M2
4.	Acute promyelocytic leukemia	M3
5.	Acute myelomonocytic leukemia	M4
6.	Acute myelomonocytic leukemia with eosinophilia	M4 eos
7.	Acute monocytic leukemia	M5
8.	Acute erythroid leukemia	M6
9.	Acute megakaryoblastic leukemia	M7

1.5.2 World Health Organization (WHO) classification.

WHO classification unlike FAB system depends on the prognostic factors associated with outlook of the patients (Vardiman *et al.*, 2009). This newer system includes multiple groups and is more efficient because it takes into account multiple factors that would help in determining the classification of the AML patient.

Table 1.2: WHO classification for AML

1.	**AML with certain genetic abnormalities**
	AML with a translocation between chromosomes 8 and 21
	AML with a translocation or inversion in chromosome 16
	AML with a translocation between chromosomes 9 and 11
	APL (M3) with a translocation between chromosomes 15 and 17
	AML with a translocation between chromosomes 6 and 9
	AML with a translocation or inversion in chromosome 3
	AML (megakaryoblastic) with a translocation between chromosomes 1 and 22
2.	**AML with myelodysplasia-related changes**

3.	**AML related to previous chemotherapy or radiation**
4.	**AML not otherwise specified***
	AML with minimal differentiation (M0)
	AML without maturation (M1)
	AML with maturation (M2)
	Acute myelomonocytic leukemia (M4)
	Acute monocytic leukemia (M5)
	Acute erythroid leukemia (M6)
	Acute erythroid leukemia (M6)
	Acute megakaryoblastic leukemia (M7)
	Acute basophilic leukemia
	Acute panmyelosis with fibrosis
5.	**Myeloid sarcoma****
6.	**Myeloid proliferations related to Down syndrome**
7.	**Undifferentiated and biphenotypic acute leukemias*****

*includes cases that do not share similarity with any other groups mentioned and are similar to FAB classification

** also known as granulocytic sarcoma or chloroma

***have both lymphoid and myeloid features. Also known as mixed phenotype acute leukemias

1.6 Diagnosis

Acute Myeloid Leukemia diagnosis is not a trivial task. Initially a patient is checked for leukemia and further tested to differentiate whether he is suffering from myeloid or lymphoblastic leukemia. Diagnosis checks AML patients for subtypes to check for prognostic factors and give clinicians a fair idea of the condition and stage of the disease to implement possible treatments. Currently AML is diagnosed using the patient's peripheral blood or bone marrow. Conventional methods include a complete blood count (CBC) to study anomalous numbers of erythrocytes, leukocytes, blast cells and platelets as indicators of the disease. Techniques to elucidate AML aberrations from

other leukemia's include fluorescence in-situ hybridization (FISH), flow cytometry and karyotyping.

Since the molecular nature of AML is of great interest to study the dynamics of this complicated malignancy, genetic testing is currently being employed to check for mutations that collect in the genes (FLT3, NPM1, c-KIT, IDH1 etc.) that are frequently found in association with AML. Cytogenetics and molecular techniques such as direct sequencing, reverse transcription-polymerase chain reaction (RT-PCR), and global expression profiling have been used to find genetic causes and implications of the disease (Mrózek *et al.*, 2007).

1.7 Causative Agents

Multiple factors are responsible for the occurrence of AML. The disease is normally caused by exposure to chemotherapeutic agents (alkylating agents, anthracyclines, benzene and epipodophylotoxins), high ionizing radiations, genetic factors, and epigenetics, frequently found in patients of other blood diseases that can worsen into AML.

1.7.1 Genetic factors

Acute myeloid leukemia is known to be caused by genetic factors like gene rearrangements, chromosomal aberrations (monosomies, trisomies) and various mutations such as point mutations, insertions, deletions, translocations etc. These somatic mutations accumulate within genes and result in discontinuation of signaling and regulatory pathways that drive hematopoiesis. In cytogenetically normal-acute myeloid leukemia (CN-AML) patients subtle mutations have been observed to cause disease and poor prognosis (Tayyab *et al*., 2014).

1.7.2 Epigenetic factors

In year 2010, Figueroa *et al*., studied DNA methylation in 344 AML patients. According to their findings some of the AML patients exhibited hyper-methylation in AML associated genes while others did not. The observed methylation signatures divided AML patients into 16 subclasses of which many were coherent with molecular and cytogenetic subgroups. The same group found aberrant methylation in 45 cases of AML as well which resulted in down regulation of gene expression.

Genes coding polycomb group complexes (PcGs) have been observed to have undergone histone modification. PcGs play a role in stem cell renewal thus any mutations in the related genes lead to loss of function and eventually myeloid cancers. PcGs are also associated with DNA methylation and histone modification (Boukarabila *et al*., 2009).

1.8 Treatment

Being the most common acute leukemia, treatment options for AML are fairly limited and still under investigation. Since the disease progresses at such a rapid rate, treatment and monitoring go hand in hand. Chemotherapy is the major route for handling the disease. It involves drugs that promote antitumor and cytotoxic effects. It further includes induction and post-remission therapy using high doses of cytarabine (Schlenk, 2014).

Many clinical trials are under way where drug tests are being carried out to cater to various prognostic factors and subtypes of AML patients. Numerous AML patients carrying mutations in single or multiple genes have previously responded to autologous and allogenic human stem cell transfer along with anthracycline and cytarabine induction therapy but still require testing on larger cohorts of patients. Drugs like sorafenib, histone deacetylase (HDAC) inhibitors, imatinib, farnesyl

transferase inhibitors (FTIs), ruxolitinib, panobinstat and many others are being studied extensively to aid targeted therapies of patients carrying subtle mutations in particular AML associated genes. Prior to chemo, patients are subjected to doses of ondansetron, decadron and avil to minimize nausea and discomfort caused by chemotherapy. These treatment options may be instrumental in controlling the presence and progression of the cancer however, they are not permanent or long-term solutions. As relapse and overall survival is a deep concern for clinicians treating the disease, its molecular basis is being questioned to help decipher the best way forward when it comes to providing the patient suitable treatment and care (Tayyab *et al*., 2014).

1.9 Genes Involved in AML

Acute Myeloid Leukemia's heterogeneity roots from the alterations that occur in multiple genes (Kumar, 2011). Concurrence of such mutated genes have been studied to comprehend the nature and complexity of AML however, the association and co-existence of many of these aberrations remain largely unreported. Various studies have been carried out to characterize the role of multiple genes that are involved in orchestrating the onset and progression of AML for example. FLT3, NPM1, CEBPA, MLL-PTD, ASXL1, KIT, RAS, RUNX1, IDH1/2, JAK 2 and EZH2 in association with AML. The aberrations related with AML are key prognostic factors which on further study can hail treatment strategies for overcoming relapse of disease, countering poor prognosis, pathogenesis of AML and to improve the overall survival of patients (Wander *et al*., 2014).

1.9.1 FLT3

Most frequent mutations related to the disease appear in the *FLT3* (FMS-like receptor tyrosine kinase-3) gene. It is one of many genes that undergoes dysfunction in AML. FMS-like receptor tyrosine kinase-3 is a cytokine which primarily plays a role in hematopoiesis and brings about normal cell differentiation and proliferation in HSCs (Bonnet and Dick, 1997). Located on chromosome 13q12, the *FLT3* gene encodes a protein belonging to the class III tyrosine kinase receptor family (RTK). It further shares a structural homology with certain other receptors namely PDGFR, KIT and FMS (van der Geer *et al.*, 1994); Carow *et al.* 1996). This protein contributes to the differentiation and growth of hematopoetic precursor cells and is expressed on CD34+ cells with a virtually ubiquitous ligand (Lyman, 1995). At the plasma membrane FLT3 receptor undergoes dimerization, like other RTKs, once the ligand binds to it. This as a result leads to auto-phosphorylation generating a cascade of signals downstream. These downstream signaling pathways inhibit apoptosis, promote progression of cell cycle and activate differentiation are namely the RAS/MEK, STAT-5 and PI3K/AKT/mTOR pathways (Hay and Sonenberg, 2004).

Accumulation of varying types of mutations in *FLT3* gene hampers its role and cause uncontrolled and abnormal hematopoiesis. Thus *FLT3* is a major player in causing AML. The structural formation of *FLT3* includes an extracellular region consisting of five loops similar to that of immunoglobulins, an intracellular region termed as the juxtamembrane region and two domains of tyrosine kinase (Figure 1.4).

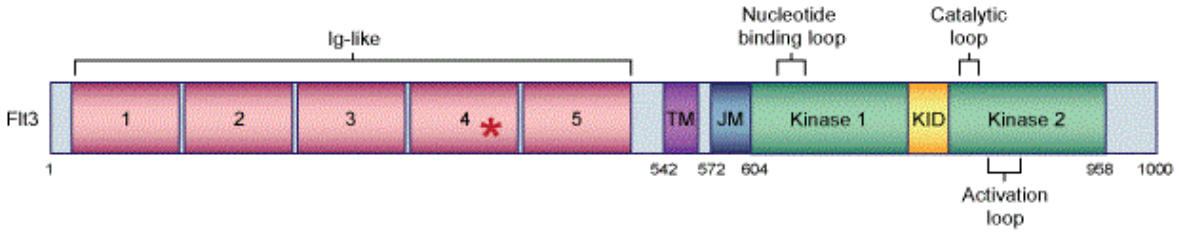

Figure 1.4: FLT3 domain structure (mutagenetix.utsouthwestern.edu).

Mutations that are known to occur in the gene are: *FLT3/ITD* (Internal Tandem Duplications) (Nakao *et al*., 1996) and *FLT3/TKD* (Tyrosine Kinase Domain) (Frohling *et al*., 2002). Most frequently occurring mutations are the ITDs in the juxta membrane region between exon 14 and 15 (Figure 1.5) with an occurrence rate of 20-30%. These mutations are in the form of duplications where a portion of the juxta-membrane repeats and these are found to be of varying lengths. Whereas, *TKD*'s are relatively rare point mutations found to have 10% occurrence rate (Rosnet *et al*., 1993).

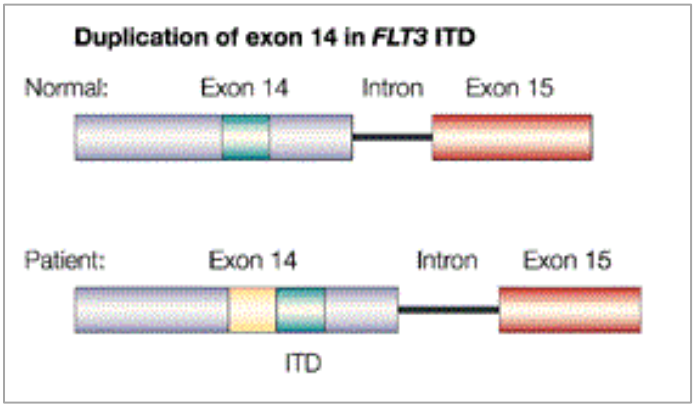

Figure 1.5: Normal structure of FLT3 exon 14-15 in comparison with patient exhibiting FLT3/ITD mutation (Stirewalt and Radich, 2003).

FMS-like receptor tyrosine kinase-3 is ligand dependent and its dimerization facilitates the cascade of signaling pathways however when it undergoes mutations, the receptor becomes ligand-

independent and constitutively autophosphorylates causing unchecked downstream signaling (Rosnet *et al*., 1993). FMS-like receptor tyrosine kinase-3 in association with several genes cause AML. Some genes do not show association with *FLT3* and alone cause the presentation of AML. These are involved in activating downstream signaling pathways which regulate the process of hematopoietic cell differentiation and maturation.

1.9.2 RUNX1

The *RUNX1* (Runt related transcription factor 1) gene also known as *AML1* is present on chromosome 21q22.3. The *RUNX1* gene has a length of 260 kb's. Various isoforms of *RUNX1* have been observed as transcription takes place via alternative promoters which include a distal promoter i.e. promoter 1 and a proximal promoter i.e. promoter 2. Thus alternative splicing gives rise to a number of isoforms (Fukunaga *et al*., 2013). Eight exons makeup the *RUNX1* gene (Figure 1.6) and the structure consists of 2 domains. First domain is coded by exon 3, 4 and 5 known as the runt domain or runt homology domain (RHD) whereas the second domain is coded by exon 7 and 8 know as the transcriptional regulatory domain. This domain includes the transactivation domain (TA) and inhibitory domains (ID).

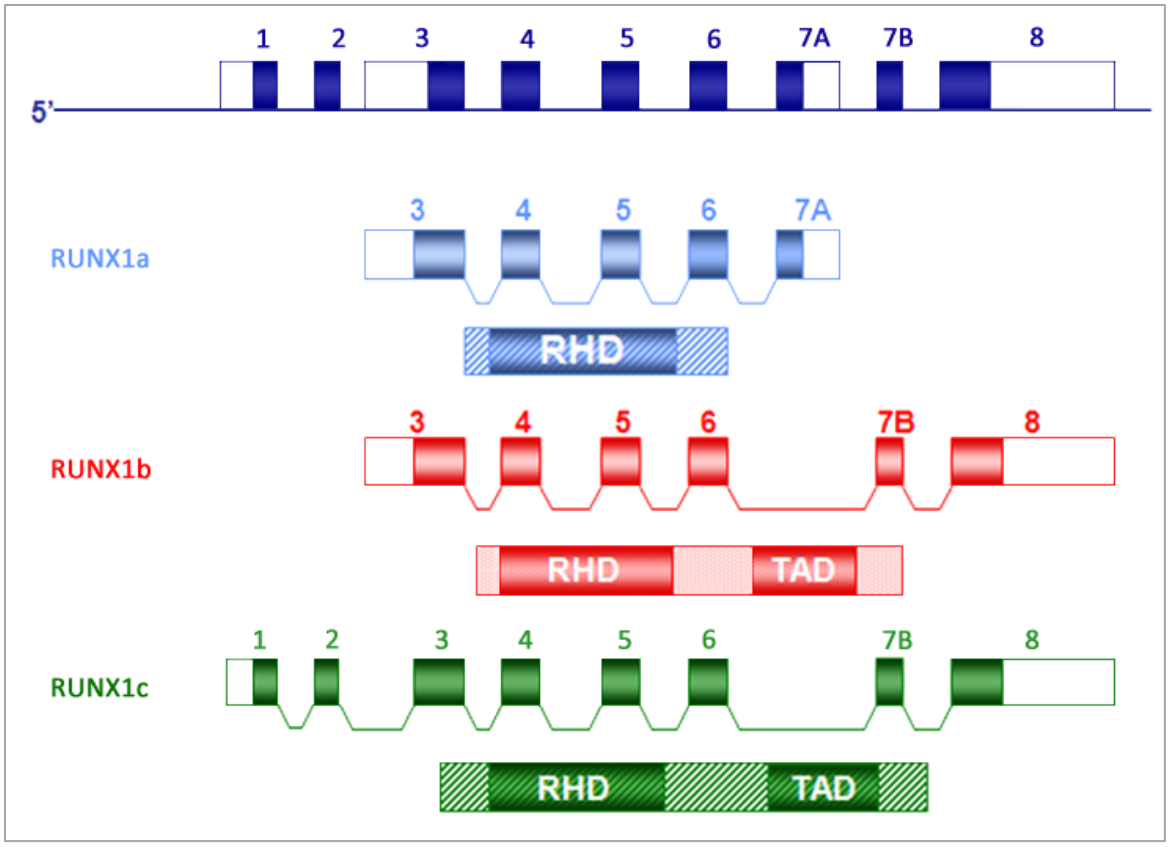

Figure 1.6: RUNX1 gene structure and possible isoforms.

In the hematopoietic system, *RUNX1* plays an active role by facilitating the interaction between proteins and mediating DNA binding. Regulatory elements 1 and 2 are the enhancers responsible for transcription of the gene. Binding of regulatory proteins that may be erythroid or lymphoid in nature is also enabled by these tissue specific enhancers making *RUNX1* gene, a key player in hematopoiesis (Asou, 2003).

The *RUNX1* gene codes for the protein known as runt-related transcription factor 1, RUNX1, which is primarily found in the nucleus. It expresses, except for the heart and brain, in all the tissues of the human body but majorly in bone marrow, peripheral blood and thymus at high levels. It plays a crucial role in hematopoiesis as it belongs to the runt family of transcription factors (Zhang *et al*., 2010). The RUNX1 protein binds directly to the DNA at specific regions, its function

being synonymous to that of other transcription factors thus orchestrating the activity of other genes in bringing about hematopoiesis. To maintain the integrity of the DNA, the interaction between RUNX1 and another protein known as the core binding factor beta (CBFβ) comes into play. The *CBFB* gene produces CBFβ protein which assists RUNX1's binding with DNA. A complex is formed by the interaction of these proteins termed as the core binding factor (CBF). It is safe to say that RUNX1 plays an instrumental role in bringing about hematopoiesis as it activates genes that control the development of the process. Specifically, it helps in the development and maturation of HSC's that are the potential precursors of producing mature blood cells for example red blood cells, white blood cells and platelets (Fukunaga *et al*., 2013).

For DNA binding and to form a transcription factor, it is necessary for RUNX1 to heterodimerize through RH domain with PEBP2 β also known as core-binding factor β (Ito, 2004). Michaud et al. described the transactivation domain of RUNX1 as the second essential part of this protein. A 100% conserved 5- amino acid sequence (VWRPY) follows the transactivation domain at the C-terminal end. Depending on the interacting partners of *RUNX1* that may be corepressors, transcription factors and coactivators, it performs its function as a repressor or activator of target gene expression, accordingly. Various hematopoietic genes are regulated by *RUNX1* that include transcription activators like MYB, STAT3; surface receptors like FLT3, TCRA, TCRB, CSF receptor; signaling molecules like BLK, CDKN1A, BCL2 and growth factors like IL3, GM-CSF, MPO. Thus all cell lineages definite hematopoiesis is essentially dependent on *RUNX1* regulated target genes (Michaud *et al*., 2003) (Ito, 2004).

1.9.3 ASXL1

The ASXL1 (Additional Sex Comb Like 1, Transcriptional Regulator) gene is present on chromosome 20q11.21 and consists of 12 exons (Fischer *et al*., 2003). First reported in 2009, the mutations in the ASXL1 gene were confirmed in myelodysplastic syndromes. The ASXL1 gene resides near to the DNMT3B gene on chromosome 20 which is also a key player in causing AML and other forms of leukemia. Expressed in majority of the hematopoietic cell types, ASXL1 is one of the three paralogs (ASXL2 and ASXL3) that has been studied in relation with cancer genomics (Gelsi-Boyer *et al*., 2009).

The ASXL1 is the human homologue for drosophila Asx gene and codes for the ASXL1 protein that consists 1084 residues. ASXL protein 1 also termed as putative polycomb group protein ASXL1 is found primarily in the nucleus. It is widely expressed in peripheral blood, bone marrow, placenta, spleen, leucocytes, brain, small intestines, heart, pancreas, skeletal muscles, prostate, fetal liver and colon but at low levels. Whereas in testes it is expressed at high levels.

This nuclear protein consists of a few major domains (Figure 1.7) that help in protein-protein and protein-DNA interaction. The helix-turn-helix domain also known as the HARE-HTH domain present on the N-terminal spanning exon 1 of the gene. A plant homeodomain (PHD) helps in the probable binding of methylated lysines and is present on the C-terminal spanning exon 12. The third important domain is the ASXH which is globular in nature and is present centrally in the ASXL1 gene spanning exon 7-10 and is responsible for a probable interaction with polycomb-associated deubiquitinase (DUB). The functions of the ASXL proteins are not well known however it has been observed that the regulation of transcription and epigenetics is brought about by ASXL1 through its interaction with numerous transcription activators and repressors and polycomb complex proteins (Gelsi-Boyer *et al*., 2012).

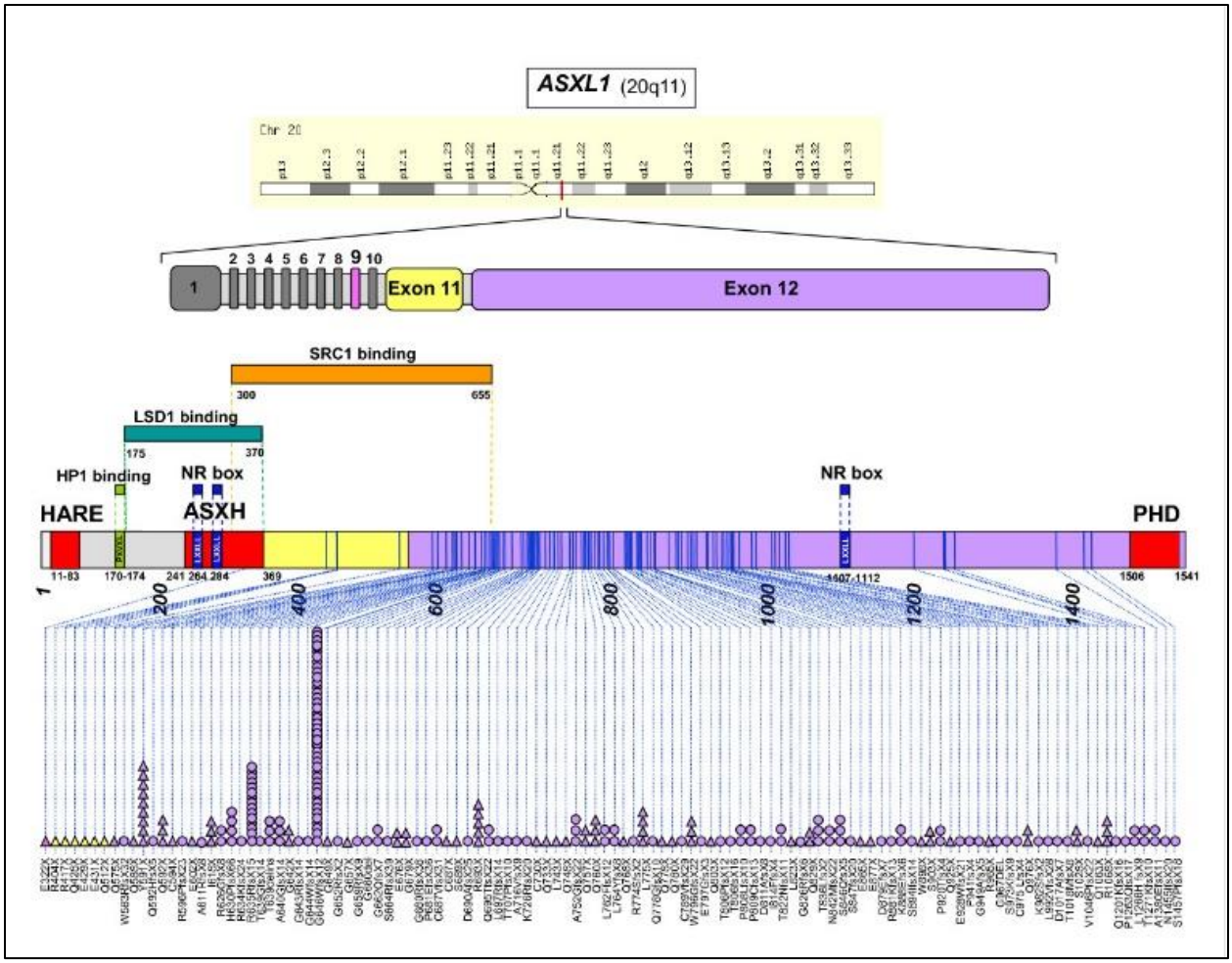

Figure 1.7: The ASXL1 gene structure.

Various transcript variants due to the alternative splicing process have been reported. The mutations frequently found in ASXL1 gene consist of nonsense as well as frameshift mutations. The dysfunctional gene is accounted to produce a C-terminal truncated protein which is described to occur upstream of the PHD domain. The occurrence of myelocytic and myelodysplastic syndromes have been associated with the mutations of ASXL1 gene (Abdul-Wahab *et al*., 2011).

1.10 Objectives and Impact of Study

Acute Myeloid Leukemia is a rare hematological malignancy however it is the most common acute leukemia. The current study will investigate the commonly mutated FLT3/ITD, relatively less known ASXL1 and RUNX1 genes in AML patients to check whether an association can be mapped for these three genes in causing AML in Pakistani patients. And whether the variations in these genes can predict a course of therapeutic action that can be taken relevant to the AML patients as per their classification of the disease. Also it will explore whether this triple gene signature can be used as a predictor of response to chemotherapy in newly diagnosed and relapsed cases of AML. Furthermore, the aim of the study is to assess the status of RUNX1 and ASXL1 mutation in FLT3/ITD negative patients.

Review of Literature

High expression of *FLT3* has been observed in early progenitors like CD34+ cells as well as in many hematologic disorders for example acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML) and most subtypes of AML (Adolfsson *et al*., 2005, Stirewalt and Radich, 2003, Turner *et al*., 1996). In AML patients, two major types of mutations in the *FLT3* gene have been observed. The internal tandem duplication (*FLT3*-ITD) mutations occur in a portion of the juxtamembrane region (JM) which is duplicated and tandemly inserted specifically between exons 14 and 15 (Nakao *et al*., 1996). These mutations form a functional protein and are always in frame. Reported in 13 to 32% adult patients, ITDs are the most commonly observed in AML (Gale, 2003). Similar mutations have been observed in myelodysplastic syndrome (MDS) and ALL (Horiike *et al.*, 1997; Yokota *et al*., 1997; Nakao *et al*., 2000).

2.1 FLT3 and Downstream Signaling

Fragment duplication in *FLT3/ITDs* is observed in the JM domain which is responsible for dimerization and phosphorylation of the tyrosine kinase receptor. Once *FLT3* is mutated its expression increases and its association with positivity of CD34 is lost. The receptor then becomes ligand independent and autophosphorylates constitutively thus continuously activating downstream signaling (Rosnet *et al.* 1996). Conformational changes induce blockade of the auto-inhibitory effect of the receptor when the ligand binds to it. Possibly the helix formation of the receptor is inhibited by the *FLT3*/ITD mutations thus restricting JMs collective auto-inhibitory effect and the kinase insert. The kinase is now constitutively activated. These mutations perhaps can be involved in blocking the differentiation as reported by two studies (Zheng *et al*., 2002; Mizuki *et al.*, 2003).

2.1.1 FLT3/ITD Size and Location

The independent and constitutive activation of the receptor happens regardless of the mutated fragment length and insertion site. The variability in the size and location of *FLT3*/ITD mutations is a prominent feature. The duplication length can vary from 3 to more than 400 bp (Schnittger *et al.*, 2002). Some other variations that have been reported range between 9 to 36 bp (Kiyoi *et al*., 1998; Frohling *et al*., 2002). In the reported duplicated regions, as such no similarity exists in the sequence however a tyrosine rich domain is common to all. Tyrosines 589, 591, 597 or 599 are present in at least one of the FLT3/ITD mutations which make up a part of the YEYDLK and YFYV motifs.

Other tyrosine kinases auto-phosphorylation sites present in their JM domains are also homologous to these motifs. Gilliland and Griffin suggested that the *FLT3*/ITD mutation is disrupts the negative regulatory role of JM domain. The helical formation of the wild type JM domain blocks the activation of the tyrosine kinase receptor by blocking its self-dimerization. The variability of the sizes and insertion sites of the ITDs have been observed in 28-34% cytogenetically normal-AML (CN-AML) (Mrózek *et al*., 2007, Schlenk *et al*., 2008). However this ligand dependent kinase becomes constitutively active i.e. it dimerizes and phosphorylates on its own accord regardless of the presence of the ligand continuously activating the AKT pathway (Griffith *et al*., 2004).

2.1.2 FLT3 Mutations Frequency

In 24% of the adult AML patients, the *FLT3*/ITD mutations can be found between ranges of 13 to 34%. High number of white blood cells and greater percentages of blast cells have been observed in patients positive for *FLT3*/ITD mutants. All FAB types have been reported to have the presence of ITD mutants however M3 and M5 subtypes have higher frequencies of FLT3/ITD mutations.

Conversely, lower frequencies have been reported for these mutations in M6 and M7 subtypes (Kottaridis *et al*., 2001; Noguera *et al*., 2002).

Paediatric patients exhibit a lower frequency of *FLT3*/ITDs ranging between 4.3 to 22.2% with a mean of 12%. Whereas, the incidence of mutations in adult patients is much higher especially associated with leukocytosis (elevated WBC count) and subtype M3, specifically M3v (Arrigoni et al., 2003). Of all patients positive for AML, 9 to 23% have been observed to have more than a single *FLT3/*ITD mutation. In various cases, up to five mutations have been recorded but mostly the occurrence of two mutations is observed (Kottaridis *et al.,* 2001; Noguera *et al*., 2002; Schnittger *et al*., 2002). The duplicated sequence varies in these mutations. Numerous studies have reported that wild type allele is entirely lost in AML patients (Kottaridis *et al*., 2001; Whitman *et al*., 2001; Schnittger *et al*., 2002; Thiede *et al*., 2002).

Different studies have reported the range 6.4 to 7.7% of the overall frequency in AML patients of *FLT3/TKD* mutations with a mean of 7.0%. Only a few studies have reported regarding the blast percentage and white blood cell count significant increase (Thiede *et al*., 2002; Frohling *et al*., 2002). TKD mutations have been reported to have higher frequencies in all FAB types however M4 and M5 subtypes show greater occurrence of these mutations. M2 subtype conversely has lower frequency of TKD mutations however in CN-AML patients 12 to 14% were found to have greater frequency of these mutations (Frohling *et al*., 2002; Thiede *et al.,* 2002).

2.1.3 Prognostic Relevance

The ITD mutation is considered as an important prognostic factor for relapse rate in patients. Since *FLT3* confers worse prognosis in patients, those harboring ITD mutations have 20-30% survival as compared to 50% of the cases that have no ITD mutations. (Meshinchi *et al*., 2006). *FLT3*/ITD

co-occurrence with other mutations leads towards favorable prognosis as observed in 40% of the cases with NPM1 mutations in co-existence with *FLT3*/ITD (Gale *et al*., 2008) as compared to *FLT3/*ITD aberration alone confers intermediate prognosis, complete remission (CR) and overall survival (OS) (Liu *et al*., 2014).

2.2 RUNX1 as a key regulator of definitive hematopoiesis

Its role in hematopoiesis makes it a key transcription factor belonging to the runt family (Wang *et al.*, 1996). As reported in a study by Bogdan and Zon, RUNX1 is crucial for regulating definitive hematopoiesis as observed in *RUNX1* knockout mice who had lost the definitive lymphoid, erythroid and myeloid cells. (Bogdan and Zon, 2013).

Okuda *et al*. reported a similar finding in their study of investigating *RUNX1* and its normal function in humans. In human leukemia, chromosomal rearrangements are most frequently found in *RUNX1*. Thus a mouse model that lacked *RUNX1* was generated. Normal morphogenesis was observed in mice embryos having a homozygous mutation in *RUNX1*. The results also showed that embryonic stem (ES) cells having targeted $RUNX1^{-/-}$ were able to retain the ability of differentiating into primitive erythroid cells where as in the mutant embryos yolk sac or fetal liver, no detection of the definitive hematopoiesis erythroid or myeloid progenitors was made. Similar effects were seen in chimeric animals in that hematopoiesis failed in $RUNX1^{-/-}$ ES cells. These results concluded that RUNX1 contribution to regulate target genes involved in definitive hematopoiesis is essential (Okuda *et al*., 1996).

Grief *et al.* described in his study that 8-13% de novo AML patients harbor a recurring translocation t(8;21)(q22;q22) in which *RUNX1* transcription factor and *RUNX1T1* (ETO) are fusion partners. Around 6-33% cytogenetically heterogeneous patients of AML were found to have

a spectrum of *RUNX1* mutations including C-terminal truncating mutations and N-terminal missense mutations that lead to deletion of the transactivation domain and affecting the RUNT domain, respectively. AML cohorts consisting of both these mutations were observed to have a loss of function of normal *RUNX1* and impacted the wild-type *RUNX1* transactivation capacity in a dominant negative manner. Deregulation of lymphoid and *RUNX1* mutations were also associated leading to linage infidelity as indicated in AML M0 subtype (Grief *et al*., 2012).

2.2.1 RUNX1 mutations

In AML, *RUNX1* has been discovered to undergo three types of acquired alterations that are translocations, intragenic mutations and amplification. Being the most frequently targeted gene for chromosomal translocation in leukemia, *RUNX1* has been linked with aberrant karyotypes and fusion genes. Chromosomal translocations in *RUNX1* have been associated with different types of leukemia but mutation in RUNX1 alone do not cause AML. Mostly observed translocation in *RUNX1* is t(8;21)(q22;q22) associated with de-novo AML. This particular translocation yields AML1-MTG8 (ETO) which is a chimeric protein (Miyoshi *et al.*, 1993). Around a quarter of all de novo acute leukemias include the disruption of *RUNX1* together with *PEBP2β* in inv(16) mutation (Erickson *et al.*, 1992). In AML patients, Trisomy 13 and trisomy 21 (Preudhomme *et al*., 2000) have also been associated with mutations in *RUNX1*. Few studies have reported the rare incidence of *RUNX1* mutations in the M0 subtype of de novo AML (Preudhomme *et al.*, 2000) (Langabeer *et al.*, 2002). An association between monosomy 7 and therapy related MDS progressing to AML has also been reported (Christiansen *et al*., 2004).

Another group of mutations observed in *RUNX1* are the intragenic mutations. These have been observed in AML (therapy-related) as well as patients belonging to M0 subtype. Preudhomme *et al*. have reported a mutation between intron 3 and exon 4 in one patient at the splicing site,

missense mutations in three patients i.e. D171G, G138D and R135G and a stop codon mutation in two patients i.e. R139 ter in M0 subtype AML (M0 AML). One of the patients was also reported to have a 23bp insertion at AA 135 and a missense mutation (R174Q) which corresponded to an intron 4 region (Preudhomme *et al*., 2000).

Since *RUNX1* is not responsible for single-handedly causing a full blown version of AML, it is studied in relevance with various other genes that have been regarded significant in causing AML for example, *FLT3-ITD, FLT3-TKD, MLL, CEBPA, NRAS* mutation etc (Schnittger *et al*, 2011).

Point mutations of the *RUNX1* gene have been observed in AML with a frequency of 8 to 10% in patients exposed to chemotherapy or radiation and in patients with MDS/AML (Greif *et al*., 2012). Osato *et al*. reported S114X/C72fsX111, H58N/WT, R177X/R177X mutations in M0 AML, K83N/WT in relapse patient of M3 subtype and R177Q/WT in M5a subtype (Osato *et al*., 1999). While studying development of leukemia, Osato described *RUNX1* point mutations as another factor in its progression. Patients predisposed to AML have higher chances of resulting in familial platelet disorder (FPD/AML) if monoallelic mutations in *RUNX1* are detected in their germlines. About half of point mutations occurring in AML M0 vary according to ethnicity and are biallelic. Three leukemic subtypes have been linked with sporadic point mutations namely M0, MDS-AML and secondary MDS/AML. A comparable incidence rate has been observed for *RUNX1* point mutations and incidence of AML subtypes: M2 (t(8:21)) and M4Eo (inv(16)) in patients that undergo anti-cancer treatments resulting in secondary (therapy-related) leukemias which is quite common. According to findings, Runt domain is found to be clustered with mostly *RUNX1* mutations that explains their dominant inhibitory effect because due to the change in three dimensional structure of the domain, it results in active binding of *B*-subunit however defective binding with DNA has been reported. *RUNX1* haplo-insufficiency leads to manifestation of

leukemias as compared to popular belief that biallelic inactivation is required by classical tumor suppressor genes in order to be leukemogenic (Osato, 2004).

2.2.2 Prognostic relevance

RUNX1 is an important candidate to study prognostic effects in AML and as detected in a study, its mutations have a strong adverse impact, independent of other factors involved in the manifestation of the disease (Schnittger *et al*., 2011).

Grief *et al.*, studied AML patients that were cytogenetically normal and under treatment to check the implications of *RUNX1* mutations. Three of ten patients that underwent intensive induction treatment (cytarabine) were able to achieve complete remission whereas 46 of 63 patients from the control group had complete remission. Primary refractory response was seen right after induction treatment in 3 /10 *RUNX1* positive mutations whereas this rate in the control group was 11.5%. The findings were in line with previous studies regarding poor prognosis in *RUNX1* mutation-positive AML patients however *RUNX1-RUNX1T1* mutations conferred positive prognosis (Greif *et al*., 2012).

Gaidzik *et al*. observed while studying expression patterns including *RUNX1* mutation-derived signatures as well as high risk AML cases like t(3;3), -7, inv(3) and 7q-, that patients exhibited an inferior outcome and event free survival, greater resistance to chemotherapy and showcased distinct genetic markings. Patients' positive for *RUNX1* mutations and under observation for relapse-free survival showed favorable impact of transplantation made from allogenic hematopoietic stem cells (Gaidzik *et al.*, 2011).

2.2.3 Mutational frequency and concurrence

Gaidzik *et al*. in their study found 59 *RUNX1* mutations and were able to establish a frequency rate of 5.6% in their sample set and these mutations were predominantly identified in exons 3, 4 and 8

of intermediate-risk cytogenetic group. In acute promyelocytic leukemia, core-factor binding AML and adverse-risk cytogenetics the frequency of *RUNX1* mutations was lower. *RUNX1* mutations were checked for concurrence with other mutated genes for causing AML in patients and an inverse relation was found between *RUNX1* with *NPM1* and *CEBPA1*. The association trend was mapped between *RUNX1*, *IDH1*/*IDH2* and *MLL*-partial tandem duplications (Gaidzik *et al*., 2011).

Schnittger *et al.* in their study of *RUNX1* mutations established occurrence of 164 mutations in FAB classification of AML with the following frequency: M0 (65.2%) > M2 (32.4%) > M1 (30.2%) in patients with non-complex chromosomal imbalances or normal karyotype. Patients with cytogenetic abnormality of +13 showed 90% of mutations whereas amongst other types of cytogenetic groups the frequency of *RUNX1* mutations was similar (26%-36). *NRAS* (9.5%), *FLT3*-ITD (16.3%) and *MLL*-tandem duplications (19.7%) were the frequent genetic markers associated with *RUNX1* mutations which lead to clinico-pathologic characteristics in AML patients including shorter event-free survival and overall survival in comparison with cases having *RUNX1* wild-type gene. It was also established that mutated *RUNX1* independently also caused adverse effect in AML patients similar to *FLT3*-ITD. It was observed that individual *RUNX1* mutations posed no effect if classified according to type or localization (Schnittger *et al*., 2011).

2.3 ASXL1 and Hematopoiesis

The *ASXL1* gene's twelfth exon consists maximum mutations that have been related with occurrence of myeloid malignancies. The twelfth exon alone spans over more than half of the gene. Other exons have been reported to have rare mutations (Abdel-Wahab *et al.*, 2011b).

2.3.1 ASXL1 and concomitant mutations

The nature of mutations that occur in *ASXL1* are nonsense and frameshift mutations. This results in protein truncation at the C-terminal which is upstream of the PHD finger. Certain missense mutations have been reported but their functional relevance has yet to be determined. One bonafide mutation of the *ASXL1* that has been reported to frequently occur, more than 50% of all mutations. This mutation is a duplication of guanine nucleotide leading to a frameshift (c.1934dupG → p.Gly646TrpfsX12). This mutation was previously termed as a PCR artefact by one study however it was neither found in control DNAs, other cancer types e.g. breast cancer nor in germ line DNAs hence its status as a bonafide mutation of *ASXL1* was maintained.

Mutations of this gene are mostly heterozygous that indicates the key pathological factor here is the haplo-insufficiency. Interacting proteins may be rendered inactive due to the dominant negative role of the truncated ASXL1 protein. *ASXL1* mutation has been termed as a loss-of-function mutation in a recent study which observed loss of the ASXL1 protein following the presence of the *ASXL1* mutation gene (Abdel-Wahab *et al*., 2011b). In AML, the occurrence of *ASXL1* mutations have been often associated with *RUNX1* and *CEBPA* (Chou et al., 2011) (Pratcorona *et al.*, 2012) (Metzeler *et al*., 2011). Another study by Gelsi-Boyer in 2012 reported greater possibility of the co-occurrence of *ASXL1* and *RUNX1* mutations as compared to other genes i.e. *FLT3*-ITD, *FLT3*-TKD, *NPM1* and *DNMT3A*.

2.3.2 ASXL1 mutational frequency, associated biologic features and prognostic relevance

In 2013 Schnittger *et al.* observed in their study which consisted of 740 AML patients that frequency of the *ASXL1* mutation has been found to be higher in males (23.5%) as compared to females (9.9%). Detection of this mutated gene was made in 17.2% of the patients i.e. 127/740.

According to AML risk stratification standards, the patients karyotype was divided into two: normal karyotype (NK) and intermediate risk aberrant cytogenetics (includes unfavorable cytogenetic aberrations). Mutations of the *ASXL1* gene were frequently found specifically in trisomy 8 cases (52.7%) and generally in patients with aberrant karyotypes (31%) as compared to NK patients (12.5%). The event free survival and overall survival (OS) was found to be shorter in patients harboring *ASXL1* mutations. The group concluded that *ASXL1* mutations independently are strong determinants of poor prognostic impact which is why including its work-up during AML diagnosis is important.

Pratcorona *et al*. studied *ASXL1* exon 12 mutations to analyze the significance of its role in AML patients and how indispensable is the characterization of the mutations for diagnostic and clinical purposes. The prognostic impact and prevalence of these mutations was made in 882 patients. As a conclusion, 46 cases harbored truncating *ASXL1* mutations all of which suggested inferior overall survival and poor complete remission rate leading to the idea that therapy outcome for such patients is poor and unfavorable in the long run (Pratcorona *et al*., 2012).

Paschka *et al.* in 2015 studied AML patients ranging between the ages of 18 to 61. The occurrence of *ASXL1* mutations was 6.1% and was associated particularly with males, secondary AML and old age. It was also noticed that these patients had lower circulating blasts and bone marrow values. All cytogenetic groups of AML were observed to have *ASXL1* mutations including low—risk, and high risk as well as intermediate-risk and normal karyotype cytogenetics. Associations were established between *ASXL1*, *RUNX1* and *IDH1* mutations whereas an inverse relation was observed between *ASXL1* and *NPM1*, *FLT3*-ITD, *DNMT3A* mutations. Furthermore, inferior survival rates and decreased complete remission rates as compared to *ASXL1* wild type confirmed the coherence of these notions as previously reported by studies on *ASXL1*.

2.4 Association between FLT3/ITD, RUNX1 and ASXL1 genes

FlT3-ITD mutations have been studied in juxtaposition with numerous other mutations to establish a direct or inverse relation to determine their impact on the prognosis and treatment options for AML. Paschka *et al.* in their study inversely correlated *ASXL1* mutations with *FLT3*-ITD but directly with *RUNX1*. Independently the presence of *ASXL1* and *RUNX1* mutations did not impact the prognosis significantly however adverse prognostic impact was determined in context of co-existence of *ASXL1* and *RUNX1* mutations. Risk of death was higher in patients with the genotype $ASXL1^{mutated}/RUNX1^{mutated}$ in comparison to those without it (Paschka *et al*., 2015). Schnittger *et al.* reported that in *RUNX1*-mutated AML cases the second most frequent aberration after *MLL*-PTD is *FLT3*-ITD mutations (Schnittger *et al*., 2011).

METHODOLOGY

3.1 Study Subjects

This case-control study was carried out to compare the mutational profile of genes between Acute Myeloid Leukemia (AML) patients and wild type genes in healthy non- AML controls. The study was approved by Ethical Review Board of National University of Science and Technology (NUST) and Advanced Studies & Research Board and Ethical Review Committee of Forman Christian College (FCCU) (A Chartered University). Prior approval was also obtained from the ethical review boards of the participating hospitals INMOL, MAYO and Jinnah hospital. Whole blood samples of 35 clinically diagnosed AML patients and 10 non-AML controls were obtained. AML patients were further classified as newly diagnosed (Non-chemotherapy) and treated patients (Induction phase/chemotherapy). An informed consent was produced per sample collected and signatures were taken. Data collection form was used to record relevant details of patient's blood profile.

3.2 Patient Data Collection Form

To record patient data, a form was developed which consisted of the following:-

1. Patient data sheet including case history and blood profile (WBC count, blast cell%, RBC count, platelets etc.).
2. Consent form.
3. Treatment details (if the patient was on chemotherapy).
4. Contact details.

3.3 Sample Collection and Storage

Whole blood samples of approximately 3 to 5ml were collected in vacutainers/EDTA tubes with the help of hospital staff. On arrival in FCCU, aliquots were made of 500µl in 1.5ml microfuge tubes and stored at -20°C for DNA extraction later on.

3.4 DNA Extraction

Cellular DNA was extracted from whole blood using manual method. The method consisted of the following steps of sample homogenization, lysis, phase separation, DNA precipitation, washing and solubilization of extracted DNA in TE buffer for stability and long term storage.

3.4.1 Reagent preparation

- **Red blood cell lysis buffer (RBC lysis buffer)**

  In 1000ml reagent bottle, 0.01M Tris-HCl pH 7.6 (10ml 1M Tris), 320mM sucrose (109.54g sucrose) and 5mM $MgCl_2$ (1.01g $MgCl_2$) was added and pH was adjusted to 8.0 using NaOH pellets. After which 1% Triton X 100 (10ml Triton X 100) was added to 800ml of distilled water. The mixture was gently shaken and distilled water was further added to make up to 1 liter mark. Buffer was given for autoclaving at 15 p.s.i for 10min to avoid caramelization (browning) of sugars.

- **Nucleic lysis buffer**

  In 1000ml reagent bottle, 0.01M Tris-HCl pH 7.6 (10ml 1M Tris-HCl), 11.4 mM sodium citrate (2.94g sodium citrate) and 1mM EDTA pH 8.0 (3.75g anhydrous EDTA) and 1% sodium dodecyl sulphate (SDS) (10g SDS) was added to 800ml of distilled water and pH

was adjusted to 8.0 using concentrated HCl. The mixture was gently shaken and distilled water was further added to make up to 1 liter mark. Buffer was given for autoclaving at 15 p.s.i. for 15min.

- **TE buffer pH 8.0**

  In 1000ml reagent bottle, 5ml 1M Tric-HCl (pH 7.6), 2ml 0.5M EDTA (pH 8.0) and distilled water was added to make up 1 liter mark. pH was adjusted to 8.0 and the buffer was autoclaved at 15 p.s.i. for 15min.

The extraction method proceeded by first shaking the whole blood vials/vacutainer tubes gently using a vortex. Blood samples (500µl) were then transferred to 1.5ml microfuge tubes. To it 1000µl of RBC lysis buffer was added. The tube was gently shaken to homogenize blood with the buffer and then subjected to centrifugation for 2 minutes at 7000 rpm. The supernatant was discarded and the pellet was rinsed with 1000µl RBC lysis buffer. The pellet was broken down in RBC lysis buffer by gentle vortexing and was then subjected to centrifugation (2min; 7000rpm). The step was repeated thrice to wash out the hemoglobin and a clean white blood cell (WBC) pellet was obtained. The tubes were then placed inverted onto a clean tissue paper for a few seconds. To avoid cross-contamination it was made sure that samples were placed apart. To each tube 400µl of nucleic lysis buffer was added and the pellet was pipetted until dissolved. Next, 100µl of saturated NaCl (5M) and 600µl of chloroform was added to the tubes which were then gently mixed (vortex) at room temperature and centrifuged at 7000rpm for 2 minutes. At this stage phases were visible. From each tube, all the while carefully staying clear off of the lower phase, 400µl supernatant was transferred into new 1.5ml tubes. To each tube containing the supernatant, 800µl of cold (-20°C) absolute Ethanol was added. After gently shaking, the tubes were vortexed. At this stage, mucus like strands of DNA were visible in the solution phase. Final step was to spin the microfuge tubes

for 1 minute at 12000 rpm to precipitate the DNA. The supernatant was discarded and the tubes were left to dry completely at room temperature. The tubes were placed downward on a clean tissue paper for drying. Once the tubes were completely dry, 50µl of TE buffer was added to each tube and then vortexed. DNA was left over night at 4°C and then stored at -20°C for later use.

3.4.2 DNA Quantification

Extracted genomic DNA was quantified at FCCU using Thermo Scientific Nano Drop ND-2000/2000c Spectrophotometer. The absorbance wavelength was set at 260nm and TE buffer was used as a blank. The absorbance ratios; $A_{260/280}$ and $A_{260/230}$ were also determined to check the purity level of DNA.

3.5 Primers Used

Primers for FLT3, RUNX1 (Exon 3, Exon 5) and ASXL1 were picked from literature and were checked for any discrepancies using primer3 tool available online. Following were the primer pairs used:-

Table 3.1: Primers used for exon amplification

Gene/Exon	**Primer Sequence**	**Product size**
FLT3/14-15	F: 5'- GCAATTTAGGTATGAAAGCCAGC -3' R: 5'- CTTTCAGCATTTTGACGGCAACC- 3'	329 bp
RUNX1/3	F: 5'- AACCACGTGCATAAGGAACAG -3' R: 5'- GCAGAAACAGCCTTAATTATTTGG -3'	363bp
RUNX1/5	F: 5'- TCACTACACAAATGCCCTAAAAG -3' R: 5'- TTGAAATGTGGGTTTGTTGC -3'	292bp

ASXL1	F: 5'- CCCAGTCAGTTAAAACTATTTTCT- 3' R: 5'- TTTCTCAGAAGGCAGGTCCTCT -3'	496bp

3.6 Mutation Screening Methodology

Multiple PCR reactions were done to achieve optimization. Each exon had a different reaction profile except RUNX1 gene whose exons 3 and 5 were first optimized using gradient PCR after which a single annealing temperature was determined for amplifying both exons.

3.6.1 Conventional PCR for FLT3 Amplification

Cycling conditions for FLT3 were taken from literature (Ali, 2011). Amplification profile optimized for FLT3/exon 14-15 using conventional PCR (ICCC- Programmable Thermal Cycler-PTC 06) consisted of initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds and a final extension at 72°C for 5 minutes.

Concentration of template DNA was kept constant for each reaction at 100ng. Primers having 10µM working concentration were added to the reaction to make a final concentration of 0.5µM. 2µl of 10x Taq buffer containing KCl, 25mM of $MgCl_2$, 2mM dNTPs mix, 0.5µM of each primer, 100ng of template DNA, 1U Taq polymerase. In terms of reaction volumes the reaction prepared had 1µl of DNA, 2µl of the provided Taq buffer, 2.5µl $MgCl_2$, 2.5µl of dNTPs mix, 2µl of each primer and 2µl of Thermo Scientific Taq DNA polymerase and Nuclease free water was used to raise the volume up to 25µl of the total reaction. Double round of amplifications were made for

each template DNA extracted from AML patients that particularly underwent chemotherapy carrying low percentage of blast cells in blood.

3.6.2 Gradient PCR for Optimization and Amplification of RUNX1 Primers

Veriti 96-well thermal cycler (Applied Biosystems) was used to optimize primers for RUNX1 exon 3 and 5. On successful optimization, one annealing temperature was determined for both exons. Amplification profile for RUNX1 exon 3 and exon 5 consisted of initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, amplification at 52°C for 30 seconds, extension at 72°C for 30 seconds and a final extension of 72°C for 5 minutes.

Concentration of template DNA was kept constant for each reaction at 100ng. Primers having 10µM working concentration were added to the reaction to make a final concentration of 0.5µM. 2µl of 10x Taq buffer containing KCl, 25mM of $MgCl_2$, 2mM dNTPs mix, 0.5µM of each primer, 100ng of template DNA, 1U Taq polymerase. In terms of reaction volumes the reaction prepared had 1µl of DNA, 2µl of the provided Taq buffer, 2.5µl $MgCl_2$, 2.5µl of dNTPs mix, 2µl of each primer and 2µl of Thermo Scientific Taq DNA polymerase and Nuclease free water was used to raise the volume up to 25µl of the total reaction. Double round of amplifications were made for each template DNA extracted from AML patients that particularly underwent chemotherapy carrying low percentage of blast cells in blood.

3.6.3 Touchdown PCR for Amplification of ASXL1

The method was taken from literature (Li et al., 2014). Reaction conditions for amplification consisted of initial denaturation at 95°C for 10 min; 16 cycles of denaturation at 94°C for 30 seconds, annealing at 64°C for 90 seconds, extension at 72°C for 30 seconds, 24 cycles of

denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, elongation at 72°C for 30 second. Final extension at 72°C for 10 minutes.

Concentration of template DNA was kept constant for each reaction at 100ng. Primers having 10µM working concentration were added to the reaction to make a final concentration of 0.5µM. 2µl of 10x Taq buffer containing KCl, 25mM of $MgCl_2$, 2mM dNTPs mix, 0.5µM of each primer, 100ng of template DNA, 1U Taq polymerase. In terms of reaction volumes the reaction prepared had 1µl of DNA, 2µl of the provided Taq buffer, 2.5µl $MgCl_2$, 2.5µl of dNTPs mix, 2µl of each primer and 2µl of Thermo Scientific Taq DNA polymerase and Nuclease free water was used to raise the volume up to 25µl of the total reaction. Double round of amplifications were made for each template DNA extracted from AML patients that particularly underwent chemotherapy carrying low percentage of blast cells in blood.

3.7 Gel electrophoresis

The PCR product was confirmed using 2% agarose gel made by 2g of agarose in 100ml of 1X TBE buffer. Before the gel was poured into the caster for solidification, 5µl of ethidium bromide (0.5µg/ml) was dissolved into it. 3µl of each PCR product was stained with 2µl of loading dye (0.25% Bromophenol blue in 40% sucrose solution) and loaded into wells. For each gel a Thermo Scientific Gene Ruler 50bp DNA ladder was loaded alongside the pcr products. The gel was then run at 80V for 60 minutes by Elite 300 plus (Weal Tec) power supply unit. Later for analyzing the gel Gel Doc-It gel documentation system (UVP) was utilized and the length of the product was determined by comparison to the ladder.

3.8 DNA Sequencing

3.8.1. Sample Preparation and Purification

Ten samples of non-chemo patients were amplified for RUNX1/Exon 3, RUNX1/Exon 5 and ASXL-1. The final volume for each PCR product was 20µl which were purified using Thermo scientific PCR purification kit. The kit consisted of silica-based membrane spin columns, binding buffer, wash buffer and elution buffer. For every pcr product 1:1 volume of binding buffer was added to the PCR tube, i.e. to 20µl PCR product, 20µl of binding buffer was added. Yellow colour of the binding buffer determined optimal pH of PCR product. The tube was gently tapped to ensure mixing. The mixture was then transferred to the purification column and centrifuged for 1 minute at 13000 rpm. The flow-through was discarded by taking off the removable collection tube of the spin column. The column was placed back into the collection tube and 250µl of wash buffer was added to the column. The purification column was centrifuged at 13000 rpm for 1 minute and the flow-through was discarded as before. The empty purification column was centrifuged an additional 1 minute at 13000 rpm to remove any residual wash buffer. The purification column was placed into a new 1.5ml microfuge tube. To it 15µl elution buffer was added and the column was incubated at room temperature for 1 minute. The purification column along with the microfuge tube was centrifuged for 2 minutes at 13000 rpm. The column was discarded and the purified sample collected in the microfuge tube was stored at -20°C for later use.

Sequencing tubes (Eurofins) were loaded with 10µl of the purified sample and 100-200µl of each primer was freshly constituted from the stock and placed in 1.5ml microfuge tubes. All the primer containing tubes were properly labeled and wrapped with parafilm to avoid evaporation. Codes

were noted off of the sequencing tubes and recorded. Tubes were packaged and sent for DNA sequencing.

Table 3.2: Primers used for DNA sequencing

Gene/Exon	Strand	Sequencing primer
RUNX-1/3	Forward	5'- AACCACGTGCATAAGGAACAG -3'
	Reverse	5' - GCAGAAACAGCCTTAATTATTTGG – 3'
RUNX-1/5	Forward	5'- TCACTACACAAATGCCCTAAAAG -3'
	Reverse	5' – TTGAAATGTGGGTTTGTTGC – 3'
ASXL-1	Forward	5'- CCCAGTCAGTTAAAACTATTTTCT- 3'
	Reverse	5' – TTTCTCAGAAGGCAGGTCCTCT - 3'

3.8.2 DNA Sequencing Analysis

3.8.2.1. Multiple Sequence Alignment and Visualization

DNA sequencing results for each gene were received in the form of a zip folder containing 5 files for each sample that was sequenced (.ab1, .pdf, .scf, .seq and .1). Sequencing results were analyzed using DNASTAR SeqMan software. For each gene, multiple alignment was carried out in separate windows. For each gene, all 10 sequence files in AB1 format were uploaded simultaneously in one window along with reference sequence file taken from GenBank (FASTA format). After aligning the sequences, the consensus sequence was observed to find any mismatched bases. The variants were then observed by viewing chromatograms in contig form using DNASTAR SeqMan software. For better visualization of the peaks, the trace data (chromatograms) was also viewed using BioEdit software available for free download online.

3.8.2.2 Variant Analysis

Variant analysis was performed using PROVEAN Genome Variants (PROVEAN v1.1.3.), a web server that allows variants to be classified as coding or non-coding variants that can be further linked with amino acid frameshifts, nonsense mutations, deletions, insertions, substitutions and analyzing how and whether the variants impacted the functional protein. PROVEAN web server was provided chromosome number, base number, variant and reference allele for processing.

RESULTS

4.1 Patient cohort

Whole blood samples of 32 patients (9-54 years) of AML were collected from different hospitals of Lahore (INMOL Hospital, JINNAH Hospital and MAYO Hospital).

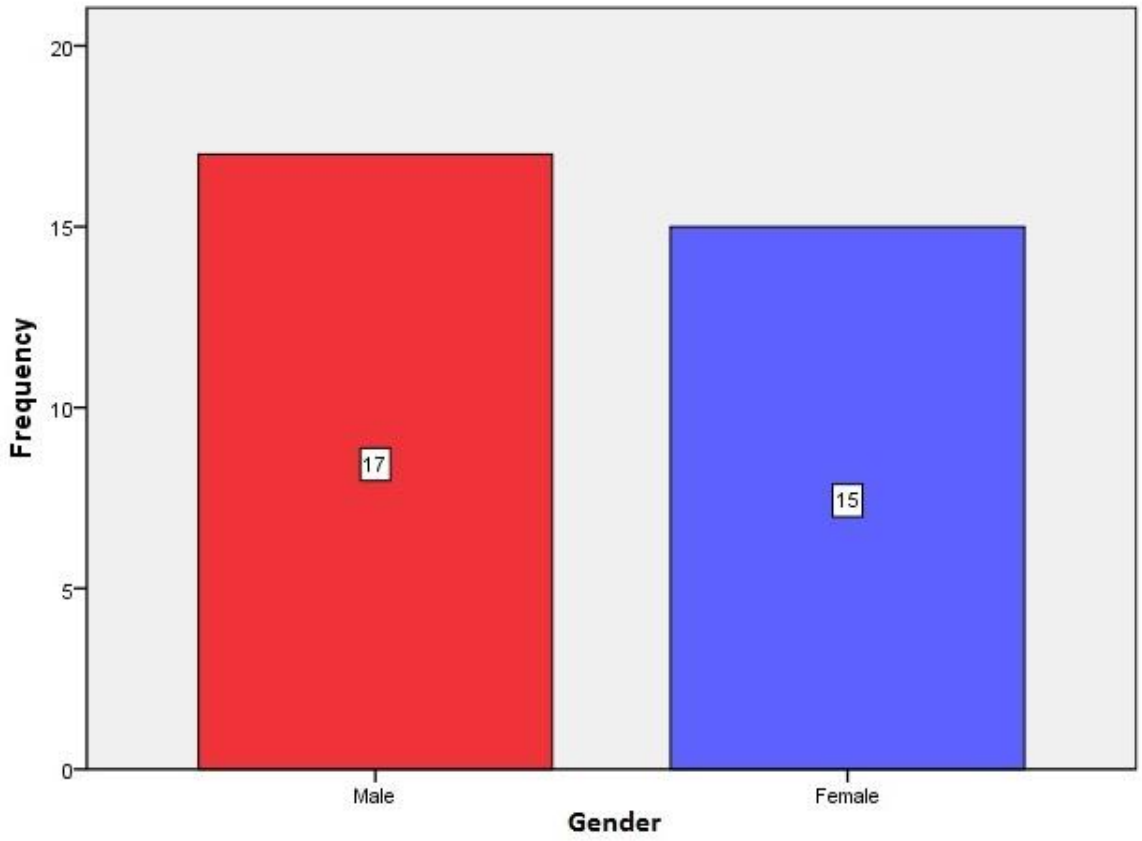

Figure 4.1: Frequency of males and females in AML patient cohort.

Out of these 32 patients, 17 were males whereas 15 were females. Ten control samples (non-AML) of which 5 were male and 5 were female were also included in the study.

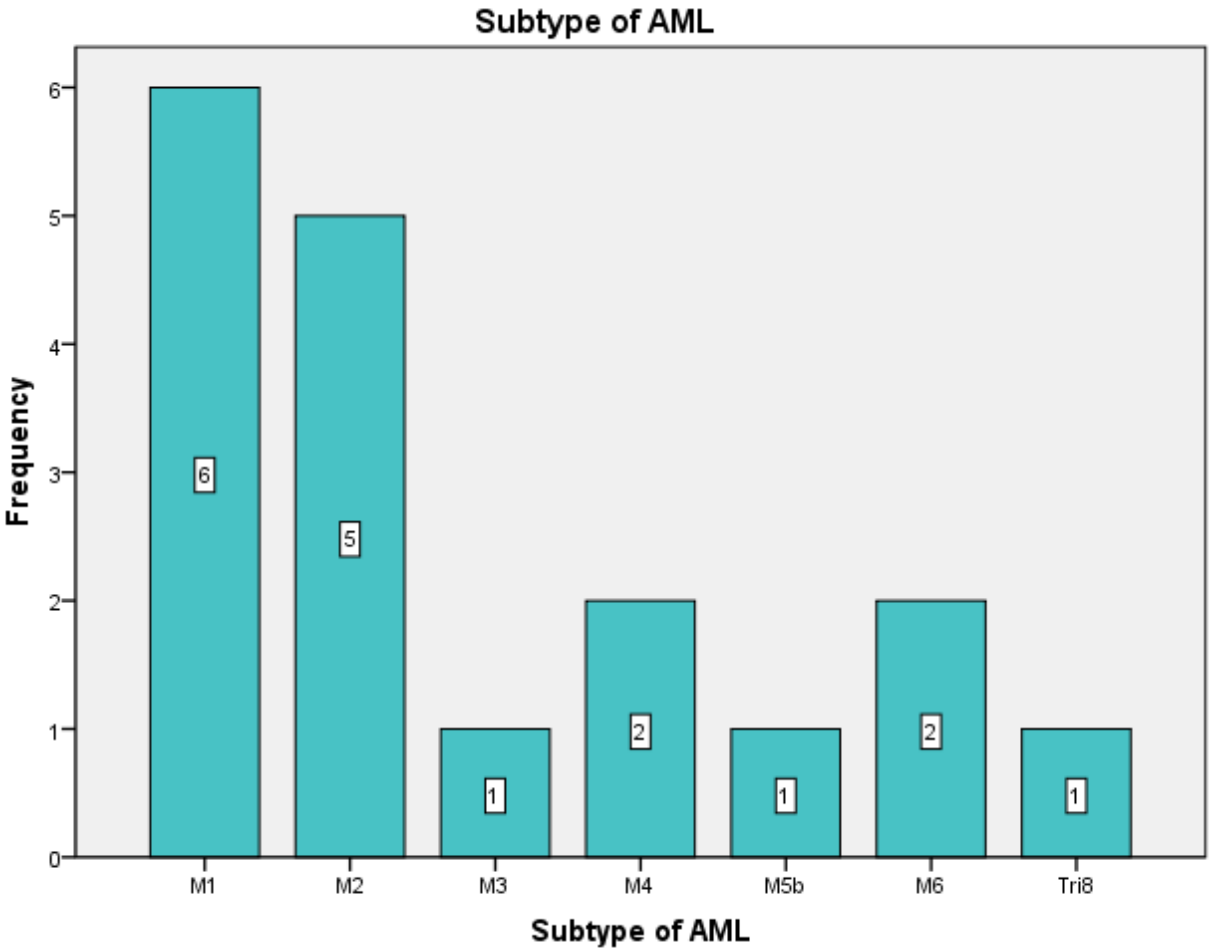

Figure 4.2: Patient frequency with respect to AML subtype. Subtypes of 18 patients were known of which 6 were M1 subtype (4 males; 2 females), 5 were M2 (1 male; 4 females), 1 male was M3, 2 were M4 (both males), 1 female was M5b, 2 were M6 (both males) and 1 male had trisomy 8 (cytogenetic abnormality). Subtypes of 14 patients were unknown.

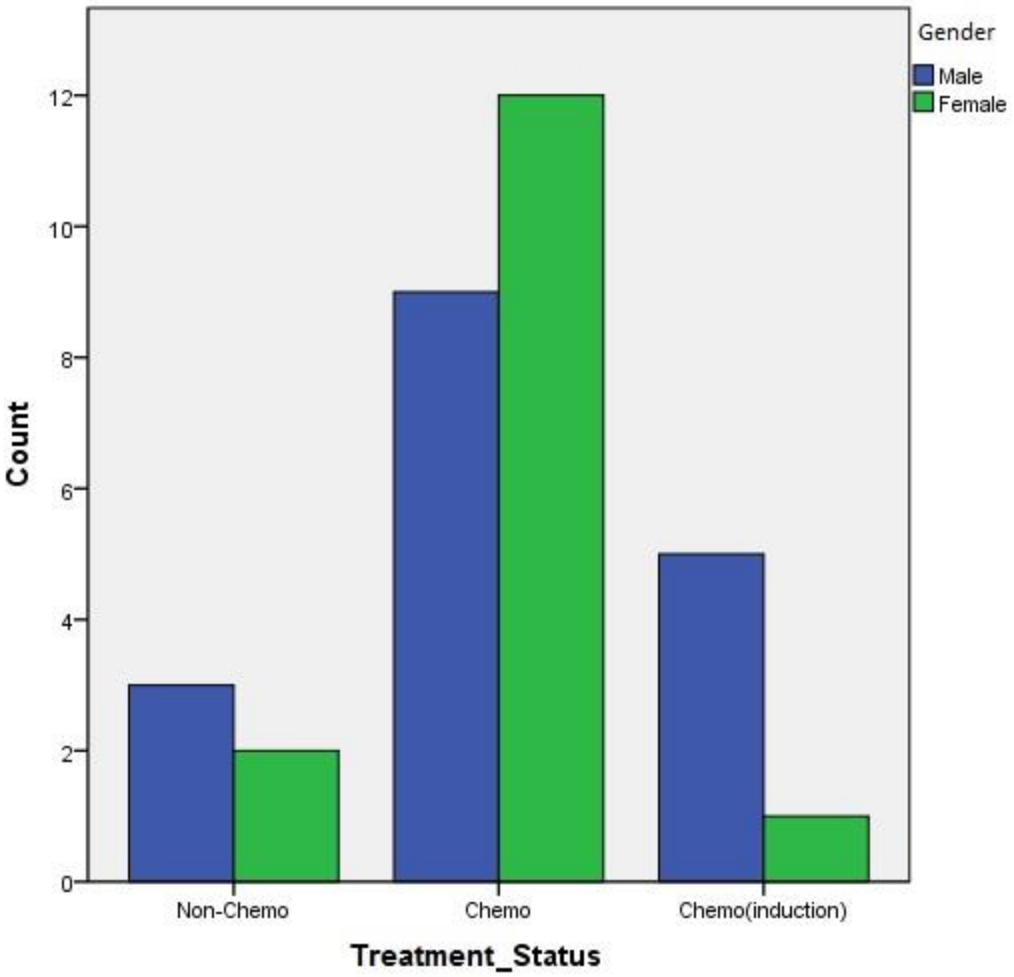

Figure 4.3: Patient count with respect to treatment status (Non-chemotherapy patients, Chemotherapy patients (long term), Chemotherapy patients (Induction phase)) in males (blue) and females (green).

Patients were stratified according to treated and non-treated groups at the time of sample collection. Three males and 2 females patients were newly diagnosed for AML and had not started chemotherapy as yet. Nine males and 12 females were receiving chemotherapy with varying doses of cytosar (300mg/m^2 – 380mg/m^2) and daunorubicin (70mg/m^2) over a period of 5 to 12 months approximately. Five males and 1 female were in induction phase of chemotherapy receiving controlled dosage of cytosar (100mg/m^2 - 200 mg/m^2) and daunorubicin (70mg/m^2). All

chemotherapy patients also received intravenous injections of ondansetron HCl (8mg), decadron (4mg) and avil (9mg) as premedication.

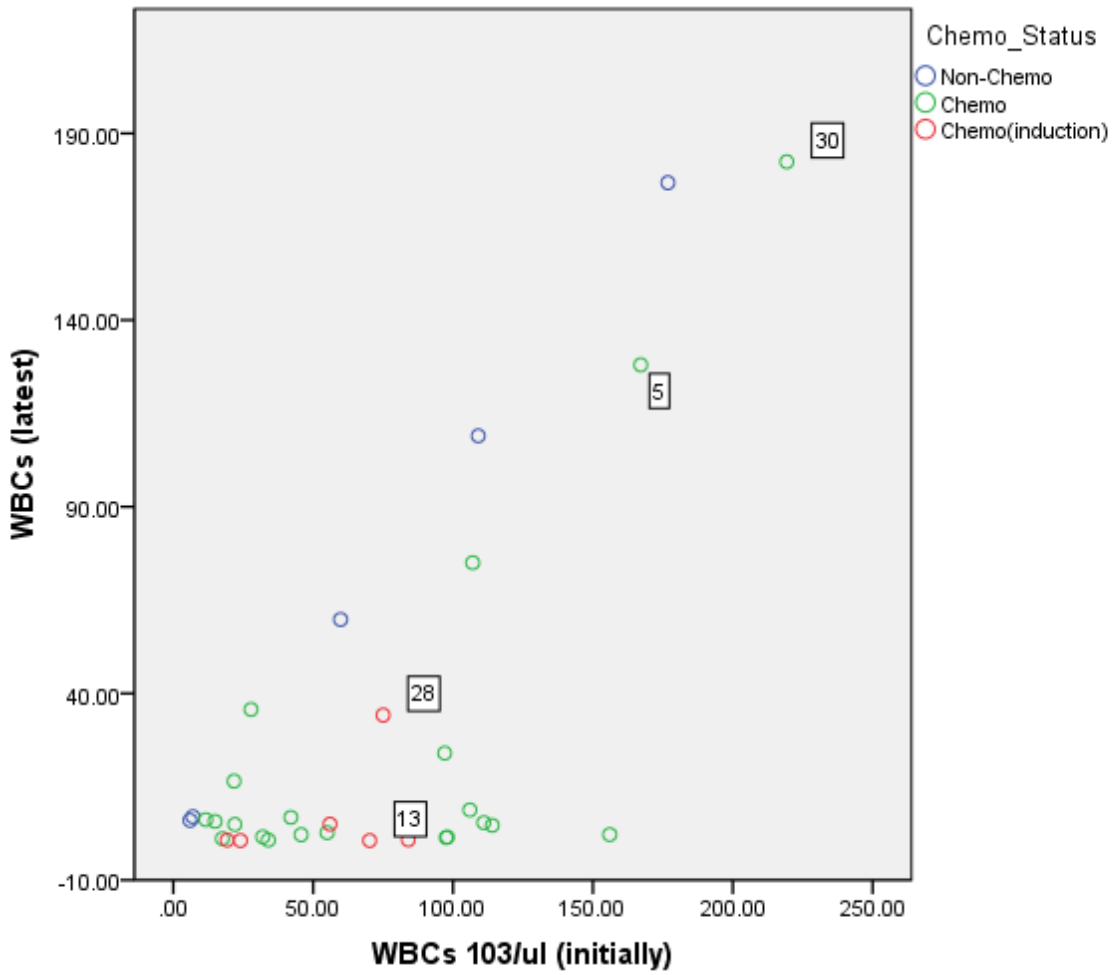

Figure 4.4: Dot plot graph showing WBC counts initial (x-axis) and latest (y-axis) in patients grouped according to treatment status: Non-Chemotherapy (blue), Chemotherapy-Long-term (green) and Chemotherapy-Induction phase (red). The graph shows a sharp decrease in latest WBC count as compared to initial counts in patients that were in induction phase whereas long term chemotherapy patients show gradual decrease of WBCs. For newly diagnosed or non-chemotherapy AML patients only the preliminary CBC report was treated as recent. Patient 13 initial WBCs were $97x10^3/\mu l$ and after induction therapy they decreased to $24x10^3/\mu l$. However

patient 30 receiving long term chemotherapy had a gradual decrease in WBC's from $24x10^3/\mu l$ to $0.6x10^3/\mu l$.

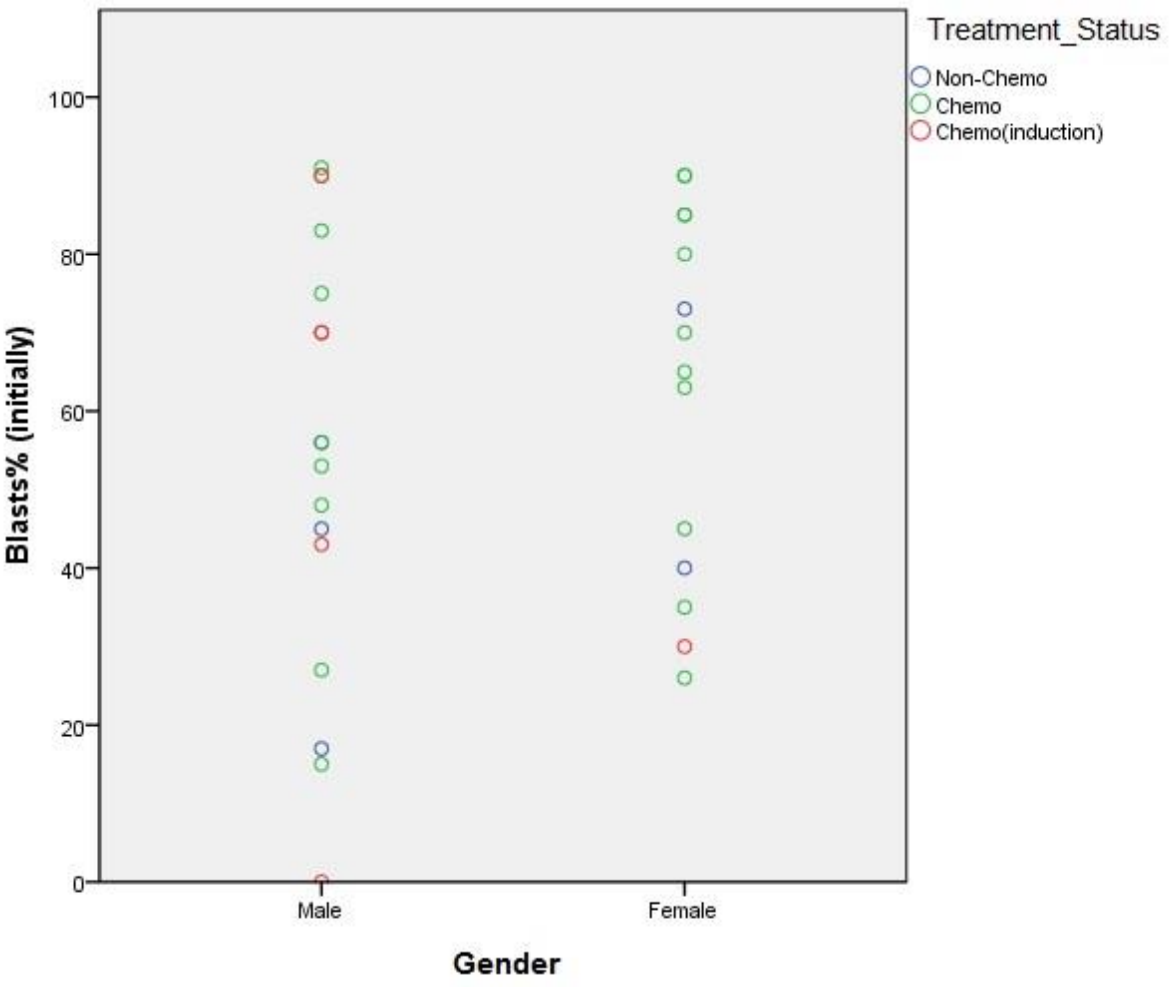

Figure 4.5: Dot-plot graph representing initial blast percentage with respect to treatment status.

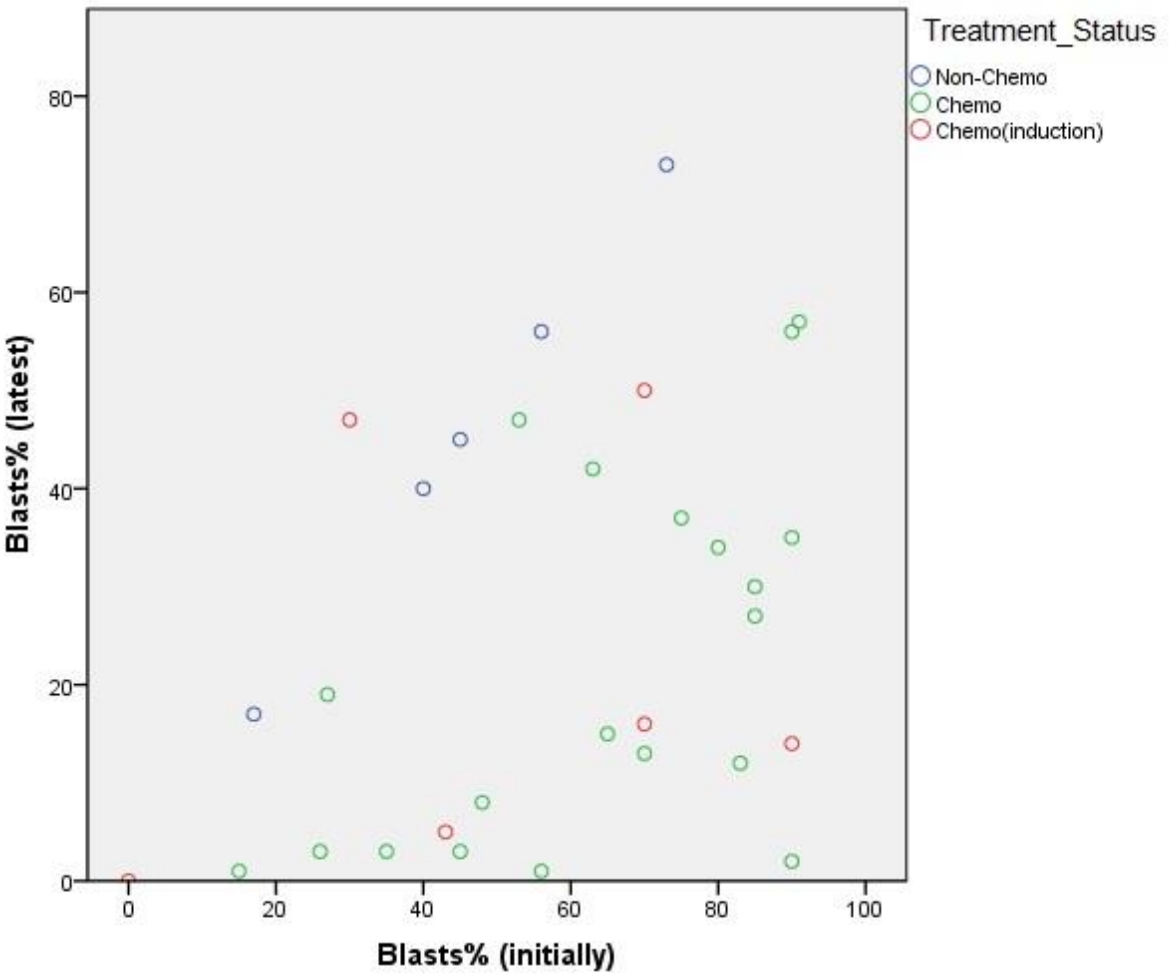

Figure 4.6: Dot plot graph showing relationship between initial blast percentage and latest blast percentage with respect to treatment status. Blast percentages were recorded from patient reports before and during chemotherapy. Patients subjected to long term chemotherapy (green dots) and induction phase (red dots) show marked decrease in blast cells percentage during treatment.

4.2 Clinicopathological characteristics of AML patients

Table 4.1: All patient's profiles evaluated statistically with respect to treatment status.

	Chemo N = 21 (65.6%) Median (Q_3-Q_1)	**Induction N = 6 (18.7%) Median (Q_3-Q_1)**	**Non-Chemo N = 5 (15.6%) Median (Q_3-Q_1)**	**Total N = 32 Median (Q_3-Q_1)**	**P-Value***
Gender					
Male [N(%)]	9	5	3	17	0.204**
Female [N(%)]	12	1	2	15	
Age	35 (41 – 20)	20 (26.25 –14.75)	40.00 (48.00 – 36.50)	31 (40 – 20)	0.009
Weight (Kg)	55.00 (73.00 – 42.00)	51.00 (63.50 – 45.25)	61.00 (70.00 – 51.00)	55.00 (70.75 – 43.25)	0.705
Height (cm)	162 (167 – 154)	165.50 (167.75 – 164.00)	160.00 (169.25 – 156.75)	163.00 (166.75 – 158.50)	0.236
BSA	1.56 (1.80 – 1.36)	1.53 (1.70 – 1.45)	1.69 (1.78 – 1.50)	1.57 (1.79 – 1.41)	0.683
Blast (Initial)	67.50 (85.00 – 45.47)	56.5 (75.0 – 22.5)	45.00 (64.50 – 28.50)	63.00 (83.00 – 40.00)	0.292
Blast (Latest)	17.0 (36.5 – 3.0)	15.00 (47.75 – 3.75)	45.00 (64.50 – 28.50)	19.00 (45.00 – 5.00)	0.093
RBC	3.150 (3.795 – 2.565)	2.85 (3.00 – 2.07)	2.67 (4.38 – 1.76)	2.99 (3.56 – 2.51)	0.376
WBC (Initial)	55.55 (109.00 – 24.90)	63.10 (77.25 – 22.81)	59.80 (142.90 – 6.49)	57.90 (106.75 – 22.50)	0.800
WBC (Latest)	5.40 (20.25 – 1.87)	0.75 (12.30 – 0.75)	59.80 (142.90 – 6.49)	5.55 (31.65 – 1.53)	0.014
Hb	9.10 (10.70 – 7.85)	8.00 (9.90 – 6.13)	7.90 (12.80 – 5.70)	9.00 (10.40 – 7.40)	0.438
Plt	32.00 (45.50 – 16.50)	54.50 (61.25 – 38.00)	65.00 (193.00 – 25.00)	38.00 (58.00 – 21.00)	0.062

* P-values obtained from Kruskal Wallis test to make between group comparisons (Chemo, Induction and Non-chemo).

** P-value obtained from Chi-Square test to observe the independence between gender and treatment groups.

In Table 4.1, there is no significant association between gender and treatment groups. Age is significantly different among treatment group, non-chemo patient with highest median age i.e 40 years. WBC percentages (latest) are also significantly different among the groups with significance value 0.014, which shows that the median WBC counts in induction group are the smallest i.e 0.75. Moreover, multiple comparisons test for WBC counts between chemo (long-term) and induction group shows that there is a significant reduction in the WBC counts after chemo therapy for induction group in comparison with chemo group with significance value 0.042.

Blast percentages after the chemotherapy are clinically significant among three treatment groups with significance value 0.093, this shows that median blast percentage for non-chemo is significantly higher as compared with chemo and induction groups. Multiple comparison tests show that median blast percentage for chemotherapy and induction groups are not statistically significant with p-value 0.976.

Platelet counts are also clinically significant among three treatment groups with significance value 0.062, this also shows that the platelet counts in the chemo group are significantly smaller than non-chemo group.

4.3 FLT3/ITD Mutations Detected in non-treatment and treated groups

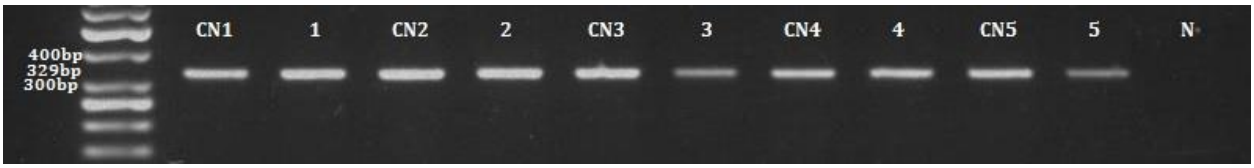

Figure 4.7: Electropherogram of wild type product of FLT3 gene (exon 14-15). Lanes labelled CN1, CN2, CN3, CN4 and CN5 were loaded with control samples whereas lanes labelled 1, 2, 3, 4 and 5 were loaded with patient's samples. Wild type gene product was obtained at 329bp for each control and AML patient. There was no amplification in non-template negative control (N).

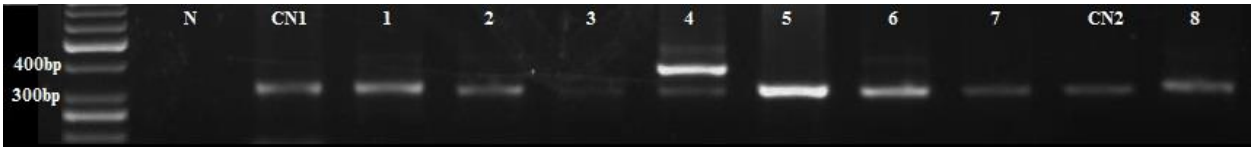

Figure 4.8: Electropherogram of wild type and mutated product of FLT3 gene (exon 14-15). Lanes labelled CN1 and CN2 were loaded with control samples whereas lanes labelled 1, 2, 3, 4, 5, 6, 7 and 8 were loaded with patient samples. Band size of 329bp depicts the wild type gene product as shown in CN1, 1, 2, 3, 5, 7, 8 and CN2. A mutant band greater than 329bp is visible in lane 6. Two mutant bands of size greater than 329bp is visible in lane 4 which shows possibility of 2 duplication mutations. There was no amplification in no template control- negative control (N).

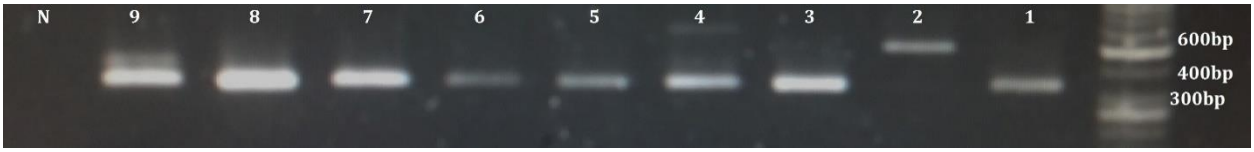

Figure 4.9: Electropherogram of wild type and mutated product of FLT3 gene (exon 14-15) of non-chemo patients. Lane labelled 1 was loaded with control sample whereas lanes labelled 2-9 were loaded with AML non-chemotherapy samples. Mutated products of size greater than 329bp

are visible in lanes 2, 4 and 9. Band size of 329bp depicts the wild type gene product as shown in 1, 3, 5-8. There was no amplification in no template control- negative control (N).

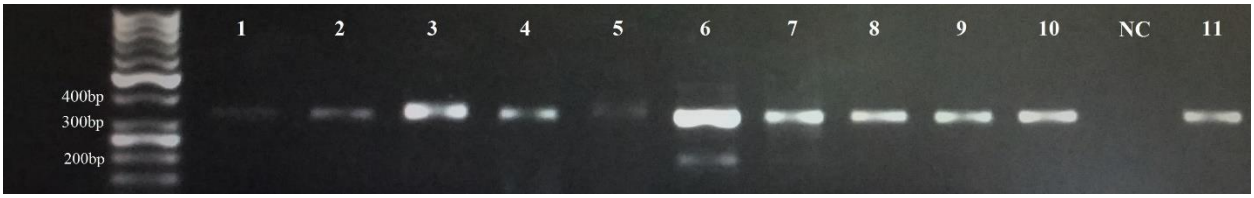

Figure 4.10: Electropherogram of wild type and mutated product of FLT3 gene (exon 14-15) of chemo patients. Lanes labelled 1-2 were loaded with control samples whereas lanes labelled 3-10 and 11 were loaded with PCR product of DNA samples of AML patient that underwent chemotherapy. Mutated product of size lesser than 329bp was visible in lane 6 (approximately 200bp). Band size of 329bp depicts the wild type gene product as shown in 1-5, 7-10 and 11. There was no amplification in non-template negative control (N).

4.4 RUNX1 (Exon 3) Amplification

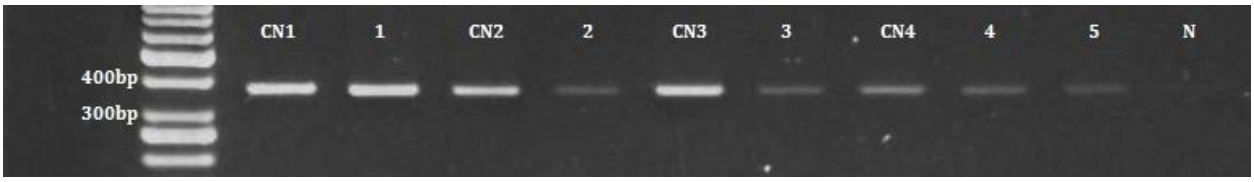

Figure 4.11: A representative agarose gel showing wild-type bands for Exon 3 of RUNX1 gene. Lanes 1-9 show PCR products from 4 controls (CN) and 5 newly diagnosed AML patients, simultaneously that were not subjected to chemotherapy as yet. Wells labelled CN1-CN4 contain control sample whereas wells labelled 1-5 contain patient samples. Band size of 363bp was observed for each sample. PCR products of patient samples (only) were purified and sent further for DNA sequencing. No amplification was observed in non-template negative control (NC).

4.4.1 Trace Data Visualization (Exon 3)

All 10 samples that were sequenced for RUNX1 gene, their chromatograms (trace data) were visualized on DNASTAR SeqMan software. Two patient sequences (AML 16 and AML 20) were observed to have single base substitutions.

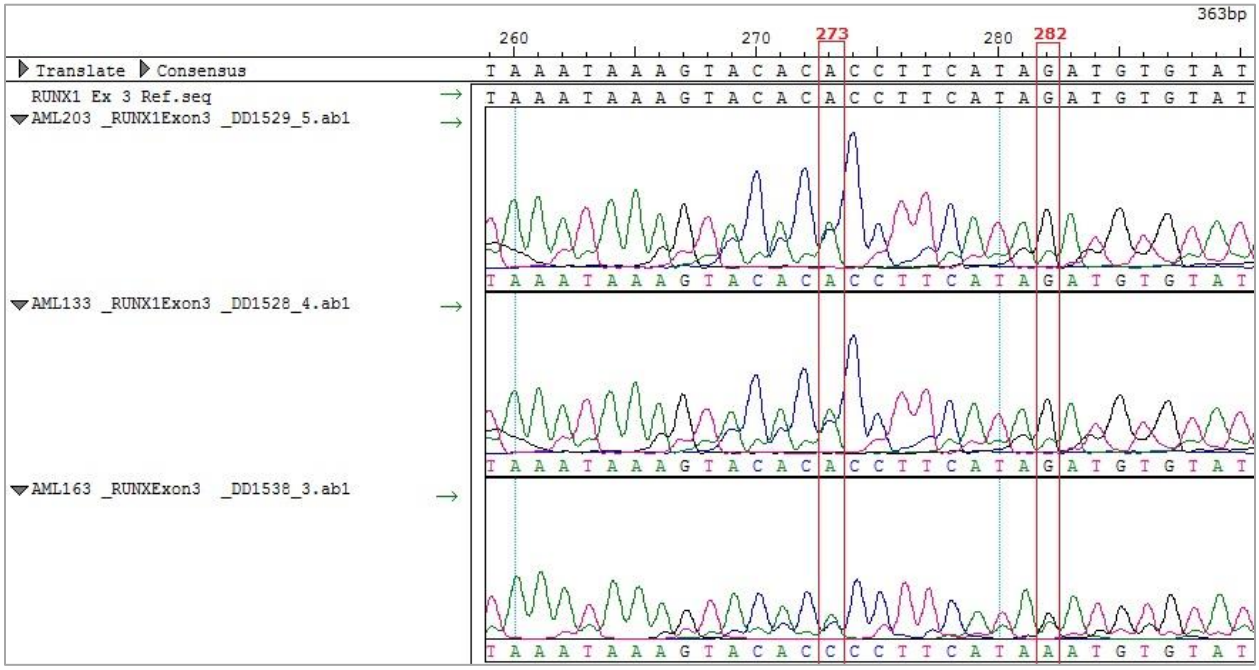

Figure 4.12: Chromatogram for AML 16 showing single base substitutions at position 273 and 282. Top to bottom: consensus sequence, RUNX1 gene reference sequence, AML 20 and AML 13 sequences (respectively) without any variations, AML 16 sequence shows substitutions at position 273 (C instead of A) and 282 (A instead of G).

4.5 RUNX1 (Exon 5) Amplification

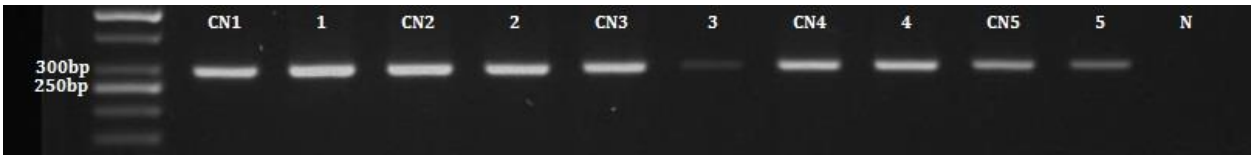

Figure 4.13: A representative agarose gel showing wild-type bands for Exon 5 of RUNX1 gene. Lanes 1-10 show PCR products from 5 controls (CN) and 5 newly diagnosed AML patients (not

subjected to chemotherapy as yet). Band size of 292bp was observed for each sample of RUNX1 Exon 5. PCR products of patient samples (only) were purified and sent further for DNA sequencing. No amplification was observed in non-template negative control (NC).

4.5.1 Trace Data Visualization (Exon 5)

All 10 samples that were sequenced for exon 5, their chromatograms (trace data) were visualized on DNASTAR SeqMan software. No variation was observed.

4.6 ASXL1 Amplification

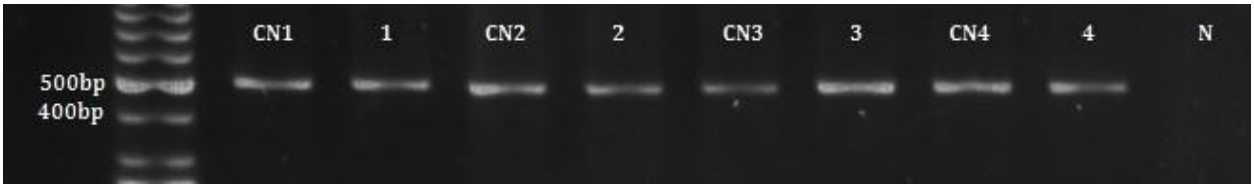

Figure 4.14: An electropherogram showing wild-type bands for ASXL1 gene. Lanes labelled CN1, CN2, CN3 and CN4 consist of control samples (CN) whereas lanes labelled 1, 2, 3 and 4 show PCR products from 4 different newly diagnosed AML patients (not subjected to chemotherapy as yet). Band size of 498bp was observed for each sample. PCR products of patient samples were purified and sent further for DNA sequencing. No amplification was observed in non-template negative control (N).

4.6.1 Trace Data Visualization

All 10 samples that were sequenced for ASXL1 gene, their chromatograms (trace data) were visualized on DNASTAR SeqMan software. Two patient sequences (AML 14 and AML 20) were observed to have single base substitutions.

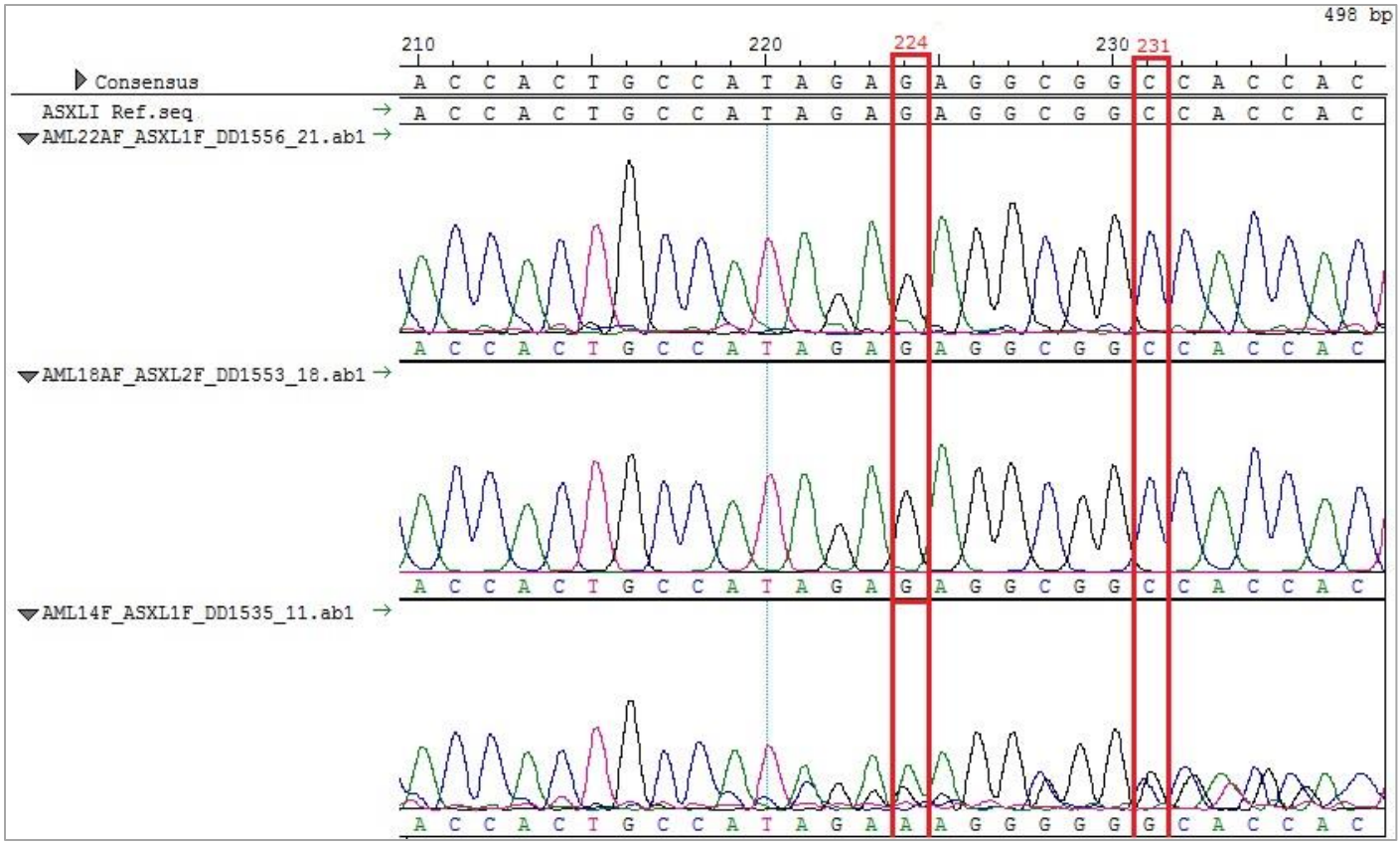

Figure 4.15: Chromatogram for AML 14 showing single base substitutions at position 224 and 231. Top to bottom: consensus sequence, ASXL1 gene reference sequence, AML 22 and AML 16 sequences (respectively) without any variations, AML 14 sequence shows substitutions at position 224 (A instead of G) and 231 (G instead of C).

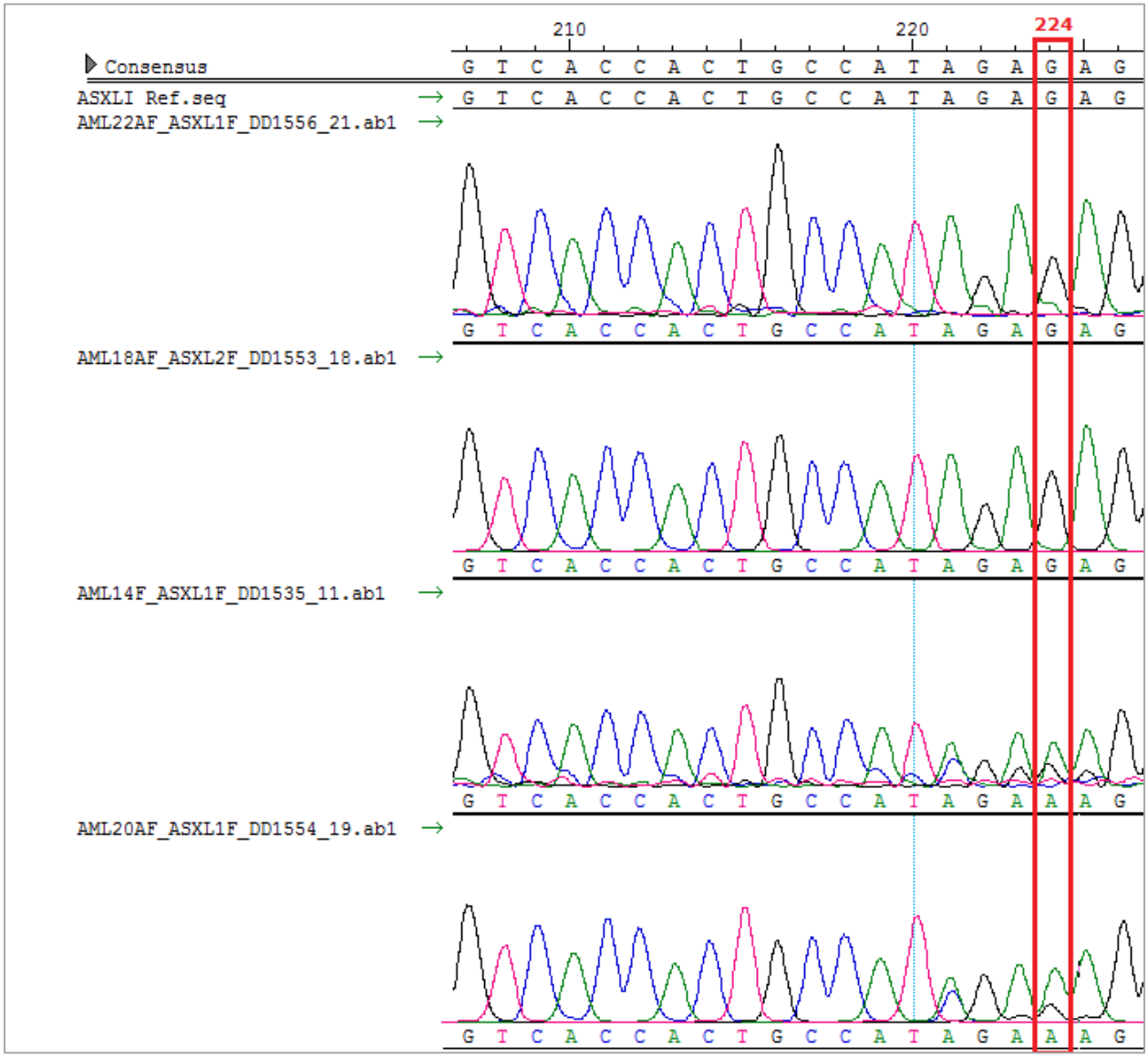

Figure 4.16: Chromatogram for AML 20 showing single base substitution. Top to bottom: consensus sequence, ASXL1 gene reference sequence, AML 22 and AML 16 sequences (respectively) without any variations, AML 14 sequence shows substitution at position 224 (A instead of G), AML 20 shows substitution at position 224 (A instead of G).

4.7 Detected Variants

Table 4.2: FLT3/ITD, RUNX1 and ASXL1 mutations observed in AML patients.

FLT3/ITD		**Males**	**Females**
Wild type	26	15	11
Mutant	6 (18%)	2 (6%)	4 (12%)
RUNX1 (Exon 3)			
Wild type	9	4	5
Mutant	2 (20%) Single nucleotide variant	2 (20%)	0 (0%)
RUNX1 (Exon 5)			
Wild type	10	5	5
Mutant	0	0	0
ASXL1			
Wild type	8	4	4
Mutant	3 (30%) Single nucleotide variant	2 (20%)	1 (10%)

Table 4.3: Clinical and demographic characteristics of mutant-positive patients.

S. N	Patient ID	Gender	Age	WBC Count (10^3/ul)	Platelet Count	Blasts %	FAB	Insert size bp
FLT3								
1	AML 9	M	27	27.8	49	91%	-	400bp
2	AML 10	F	40	55	8	70%	-	390bp
3	AML 12	F	42	59.8	25	73%	-	350, 410
4	AML 14*	F	20	42	27	90%	M2	400bp
5	AML 22	F	15	75	51	30%	M2	400bp
6	AML 30	M	26	24	45	43%	M6	200bp
RUNX1								
7	AML 16	M	36	22	38	75%	M2	Single base substitution
ASXL1								
8	AML 20	M	34	176.8	113	17%	-	Single base substitution

*Patient exhibited a single base substitution for ASXL1 also.

4.8 Bioinformatics Tool for Variant Analysis

PROVEAN genome variants web server was used to analyze whether the variants were present in coding or non-coding region of DNA and how would they impact the functional protein.

Table 4.4: Input format (<chromosome>, <base location>, <mutant allele>, <reference allele>)

Gene	Input Format
RUNX1 (Exon3)	21,1096828,C,A
	21,1096837,A,G
ASXL1	20,81272,A,G*
	20,81279,G,C

*Two patients (AML 20 and AML 14) exhibited the same variant for ASXL1

PROVEAN Result - Summary

Total number of input variants: 4

		Found in dbSNP?		PROVEAN Prediction			SIFT Prediction*		
	Total	Yes	No (novel)	Neutral	Deleterious	Not available	Tolerated	Damaging	Not available
1. Protein-coding	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
(1) Single AA Change	0	0	0	0	0	0	0	0	0
(2) Synonymous	0	0	0	0	0	0	0	0	0
(3) Deletion	0	0	0	0	0	0	0	0	0
(4) Insertion	0	0	0	0	0	0	0	0	0
(5) Multiple AA Change	0	0	0	0	0	0	0	0	0
(6) Frameshift	0	0	0	0	0	0	0	0	0
(7) Nonsense	0	0	0	0	0	0	0	0	0
(8) Unknown	0	0	0	0	0	0	0	0	0
(9) Input error	0	0	0	0	0	0	0	0	0
2. Non protein-coding	**4**	**0**	**4**	**0**	**0**	**4**	**0**	**0**	**4**
3. Input format error	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

Figure 4.17: PROVEAN result summary showing all 5 variants are non-protein coding. None of the variants have been reported thus they were not found in the SNP database indicating them to be novel in nature.

DISCUSSION

Acute Myeloid Leukemia is a heterogeneous blood disorder involving myeloid cells or immature blast cells that are rampant in the bone marrow and as result spill into the blood stream occupying healthy blood cell space. In AML hematopoietic stem cells in the bone marrow that give rise to myeloid progenitors are halted causing inhibitory effect on the differentiation and maturity of early myeloid progenitors. These immature blasts are produced in great numbers and lack normal function causing death of nearby healthy blood cells by competing for nutrition. This can explain the great rise in blast cells in AML patients before receiving chemotherapy. The causes for AML are genetic mostly, involving over 45 or more genes. However epigenetics, exposure to chemotherapeutic agents and radiations are also causative agents of AML.

In Pakistan, AML remains largely undocumented which is why this study involves mutation detection in FLT3, ASXL1 and RUNX1. Patient characteristics and variant analysis was also made in an effort to establish an association between FLT3, ASXL1 and RUNX1 genes.

Certain factors are significant in the onset of disease as well its progression which includes age of the patient. The age factor is important in AML patients to assign prognostic relevance as well as signify complete remission and overall survival rates. According to statistics provided by US National Institute of Health AML most commonly occurs in people >65years old and only 6% younger adults have AML between 2003 to 2007. However, in the current study out of the cohort of 32 patients 28% were 20years old and less, 50% were 23 to 40 years old and 22% were 42 to 54 years old. Also older patients are less likely to respond to treatment as compared to younger patients. Younger patients have been observed to have faster remission rates. This study includes patients that were all younger than 65 years ($\leq$54years old) and had a normal karyotype. The explanation for finding a younger patient cohort in Pakistan can possibly be because younger

patients might be more willing to come forward and undergo treatment therapies in pursuit of better quality life whereas older AML patients (≥60years) might be reluctant to get treated in the first place with inhibitions of being unable to recover and bearing treatment costs, such being the taboo of cancer in society. Also because younger patients are more receptive to medication and have better prognosis.

In this study, overall increase in white blood cells, blasts% and platelets was observed in AML patients at time of diagnosis owing to uncontrolled numbers of immature myeloid cells however WBC counts were significantly different in all patients with a significance value of 0.014. Median WBC counts were much lower in induction groups as compared to those on long term chemotherapy with a significance value of 0.042. Clinical significance was seen in lowering of blasts percentages for both induction group and long term chemotherapy group with a value of 0.093 however no statistical significance could be marked regarding blasts percentages between induction and long-term chemotherapy groups. Platelet counts were found to be clinically significant but not statistically with a value of 0.062. Lower platelet counts were present in chemotherapy groups as compared to newly diagnosed/non-chemo. The limitation of this study is having a smaller sample size and less frequency for non-chemo and induction groups. Much better results could be obtained with relatively larger as well as individual group sample sizes.

In this study 6 FLT3/ITD mutations (18%) have been detected in CN-AML patients whereas various studies have observed FLT3/ITD mutations in 28-34% of CN-AML patients in larger cohorts worldwide (Mrózek *et al*., 2007, Schlenk *et al*., 2008). Cytogenetics is not commonly affected in AML however trisomies and other chromosomal abnormalities linked to AML can be detected through karyotyping. In the study patient 3 is a trisomy 8 case (more susceptible to RUNX1 mutation). No FLT3 mutation was observed in this patient thus trisomy 8 is on its own a

poor prognosis predictor and found in older patients with less WBCs and peripheral blasts (Wolman *et al*, 2002) making the characteristics in line with those of patient AML 3.

Our study reports *FLT3* mutations were found in M6 and M2 subtypes. Only 3 patients of 6 were mutant for *FLT3/ITD* had known subtypes (M6-Male;M2-Females). In other studies no significant link has been established between FAB types and *FLT3* mutations however lower frequency in M6 and M7 and higher frequency in M3 and M5 has been observed (Kottaridis *et al*., 2001; Noguera *et al*., 2002, Akbar *et al.*, 2014). No such significance has been observed in current study. The mutation frequency of *FLT3/ITD* ranges between 13% to 34% (Kottaridis *et al*., 2001; Noguera *et al*., 2002) which is in agreement with 18% mutation frequency in present study.

In the study *FLT3/ITD* mutant positive patients exhibited duplication sizes greater than equals to 400bp whereas only 1 patient (AML 22) had a duplication size of 200bp. Which is in line with the findings of other studies that have explored insert sizes to be from anywhere between 3 to 400 bp and more. The increase in duplication size infers long term therapy to gain remission as compared to smaller duplications but has no impact on the overall survival since *FLT3* mutations have worse prognosis unless it is accompanied with a gene mutation linked with favorable outcome (Schnittger *et al*., 2002).

Greater possibility of the co-occurrence of *ASXL1* and *RUNX1* mutations has been reported as compared to other genes i.e. *FLT3*-ITD (Gelsi-Boyer, 2012). Most studies report presence of *ASXL1* and *RUNX1* mutations in *FLT3/ITD* negative patients however in this study 1 single nucleotide variant (SNV) was observed in a patient that was *FLT3/ITD* positive and harbored an *ASXL1* mutation. No patient harbored variations in all three genes however 6 patients are *FLT3/ITD* positive of which 1 patient also had a substitution (A instead of G) in *ASXL1* exon sequence. Five more SNVs were observed in 3 different patients (*FLT3/ITD* negative). Extensive

work has been carried out on coding sequences found to impact cancer progression however only recently certain studies have been carried out to elucidate the significance of non-coding regions involved in onset and proliferation of cancer (Khurana *et al*, 2016). The question arises that SNVs found in RUNX1 could potentially effect its role in transcription as RUNX1 is an instrumental player for regulating genes in the hematopoietic process. A possible explanation could be that the variant could probably hamper RUNX1's ability as a transcription factor however its not simple to signify this without thorough analysis and without ruling out the possibility of other existing mutations to bring about the onset of AML. Similarly SNVs found in ASXL1 gene could inhibit its interaction with major players linked with epigenetics and transcription.

5.1 Conclusion

From this study it might be concluded that the presence of variants can eventually effect RUNX1 and ASXL1 genes from a functional standpoint. Also FLT3/ITD patients are inversely related with the presence of ASXL1 and RUNX1 gene mutation with the exception of one case where FLT3/ITD negative patient harbored an ASXLI nucleotide variant.

Thorough investigation of this gene signature is required in Pakistani population which would pave ways for deciphering the role of non-coding variants in AML. Larger sample size might be able to clarify the ambiguities so that the signature can also be used by clinicians to devise personalized therapies for patients.

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