

Study of Akt1 Gene Intronic Variant in Chronic Myeloid Leukemia



BASSAM ALI SACHIT AL QARHGOLI

M.Sc. (GENETICS) - University College of Science, Osmania University, Hyderabad-INDIA
- 2016

B. Sc. Degree – Biology Department, College of Science, Thi Qar University, Thi Qar,
IRAQ - 2008

ABSTRACT

Chronic myelogenous leukemia (CML) is a hematological malignancy characterized by the translocation of 9th and 22nd chromosome. It results in the formation of a fusion gene, Bcr-Abl with increased tyrosine kinase activity which interferes with cellular proliferation, cell death and activates various downstream pathways. Despite the presence of targeted drug, Imatinib mesylate, many patients tend to develop resistance, which could be because of deregulation of genes involved in various pathways. Akt is one of the most important gene being activated by Bcr-Abl. This gene is known to regulate multiple biological processes including cell survival, proliferation,

growth, and glycogen metabolism. SNPs in this gene is found to affect the normal functioning of the gene. Hence, the present study was planned to evaluate the SNP in the intronic region to understand the role of this polymorphism in the development and progression of CML.

100 blood samples (50 CML cases, from Nizams Institute of Medical Sciences and 50 age and gender matched controls from local population) was collected and analyzed. DNA was isolated by salting out method and genotyping was carried out by PCR-RFLP and analyzed on 4% gel. Odds ratio, chi-square and hardy-weinberg were calculated using online

calculators. When controls and cases were compared, we did not find any significant association with the development of the disease. However, when response (hematological, cytogenetic and molecular) to drug was considered, the genotypic and allelic frequencies increased in minor responders compared to major responders indicating that this allele is involved in the progression of the disease. However, larger sample studies are needed to draw better conclusions.

Keywords: Chronic Myeloid Leukemia ; BCR-ABL fusion genes ; PI3K/AKT pathway; Serine–threonine kinase Akt ; AKT1 Gene ; AKT1 gene polymorphisms .

INTRODUCTION

Normal cell cycle is driven by the strict regulation of protein complexes through four important check points: the gap phase (G1), the DNA synthesis phase (S), the second gap phase (G2), and mitosis (M)(47). Cancer cells arise due to defects in regulation of cell cycle. Cancer is a genetic disorder characterized by uncontrolled proliferation of cells and apoptosis. It is a multigenic (oncogenes, tumor suppressor genes, MHC genes), multifactorial (radiations, chemicals, hormones, viruses, ultraviolet, diet, etc), and multistep (transformation, promotion, overt cancer) process(60). Cancers are caused by genetic mutations. Sometimes these mutations

are found in the germline, and result in inherited cancers or a predisposition to cancer (62) . Most often these mutations are found in somatic cells. Substantial changes gather over our lifetime. Cancers are caused by various hereditary transformations. Three classes of genes are included in cancer: tumor suppressor genes, oncogenes, and DNA repair genes. Tumor suppressor genes prevent cell division(67). Oncogenes are mutated forms of these genes that result in unregulated incitement of cell division . DNA repair genes promote repair of mutations that occur during the cell cycle. Mutations in tumor suppressor genes result in the loss of this prevention of cell division (16). DNA repair genes are termed as 'caretakers' and loss of DNA repair genes results in the accumulation of many mutations within a cell. An inability to repair the damage properly due to genetic polymorphisms impairing the DNA repair capacity, can lead to genetic instability and potentially modulate individual's susceptibility to various cancers.

LEUKEMIA

Leukemias are group of haematological malignancies. The word Leukemia comes from the Greek, leukos which means "white" and aima which means "blood". It is cancer of the blood or bone marrow. In order to understand the origin of leukemia, first we have to know

about process of haematopoiesis which is regulated under strict conditions. All blood cells are developed from pluripotential stem cells. The pluripotent stem cells (HSC) give rise to multilineage progenitors (MLP). These can differentiate into hematopoietic lineages of common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). CLPs give rise to B and T cells, while CMPs give rise to megakaryocyte-erythrocyte progenitors (MEP) and granulocyte-monocyte progenitors (GMP). Common myeloid lymphoid progenitors (CMLP) can be further differentiated into B cells, T cells, and GMPs.

Many forms of leukemia reflect the stage along the pathway at which chromosome damage occurred.

- Damage in pluripotent stem cell - Acute lymphoblastic leukemia (ALL) or chronic myeloid leukemia (CML) depending on the exact location and type of damage .
- Myeloid cells – Acute myeloid leukemia
- Cells in the T-cell pathway - Acute lymphoblastic leukemia (T-ALL) or chronic lymphocytic leukemia (T-CLL).
- Early B-cells – Acute lymphoblastic leukemia

Damage in the later B-cell pathway – Non-Hodgkins lymphoma or multiple myeloma

(11).

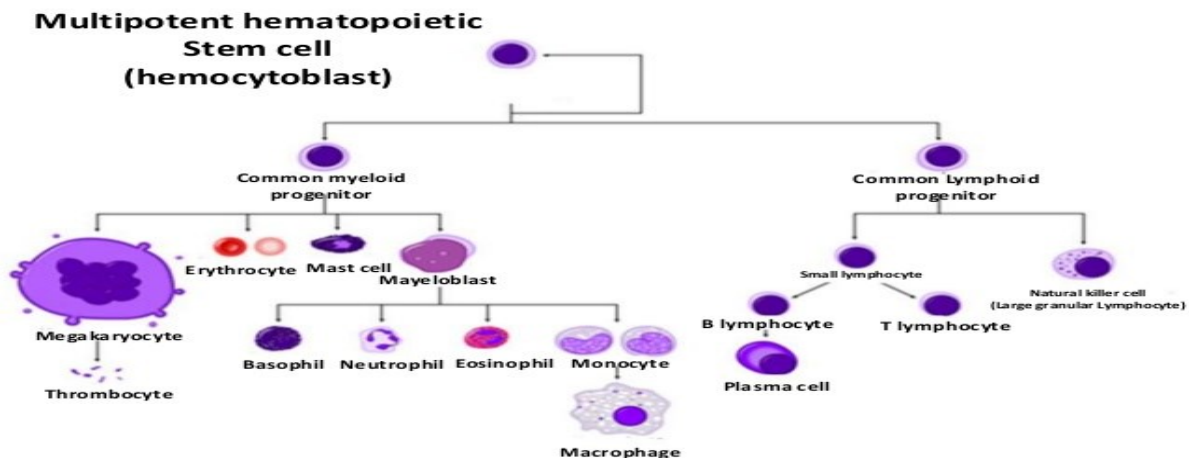


Fig 1: Hematopoietic stem cell differentiation

Leukemia is characterized by uncontrolled proliferation of the white blood cells from spongy tissue, which are grouped into four types on the basis of cell lineage (myeloid/lymphoid) and disease progression (acute/chronic) (42). They are listed as:

- Acute lymphoblastic leukemia or ALL
- Acute myelogenous leukemia or AML
- Chronic lymphocytic leukemia or CLL
- Chronic myelogenous leukemia or CML

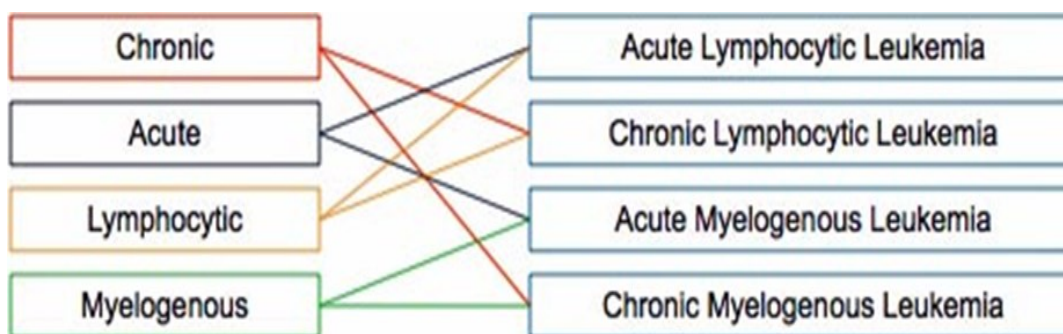


Fig 2 : Classification of Leukemia.

CHRONIC MYELOID LEUKEMIA

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disorder, characterized by the presence of Philadelphia chromosome. The Philadelphia chromosome (Ph) is a shortened chromosome 22 resulting from a reciprocal translocation between the long arms of chromosomes 9 and 22 $t(9;22)(q34;q11)$ leading to BCR-ABL fusion transcript/gene(68). BCR-ABL fusion gene CML is a triphasic disease with distinct biological and clinical features, mostly presenting as clinically benign state (chronic phase), which evolves to an incurable aggressive disease (blast crisis). The treatment of CML depends on the phase of the disease

gives rise to a chimeric protein, with elevated consistent tyrosine kinase activity and ability to transform. Philadelphia chromosome is the hallmark of CML; nearly 95% of the CML patients carry this Ph +ve chromosome(48). It is the most common hematological malignancy in Asia accounting to 15-20% of all adult leukemia's.

and the patient's overall health(9). Currently tyrosine kinase inhibitors - TKIs (Imatinib, Nilotinib, and Dasatinib) are being used as targeted drugs in the treatment of cancers. Imatinib is the most opted drug for treating all ph+ve CML cases. It specially targets the

tyrosine kinase domain and blocks the phosphorylation which is needed for kinase activation and signal transduction. An increasing problem is the occurrence of primary and development of secondary resistance in CML patients which might be due to mutations in kinase domain of BCR-ABL, candidate gene variants and/or activation of critical signaling pathways(15).

Although the t(9;22) translocation is the primary cause, findings of recent studies suggest that single nucleotide polymorphisms in various critical genes involved in the downstream signaling pathway may affect the risk of CML. Examples for polymorphic genes associated with increased risk for CML include the AKT (rs2494732- an intronic variant), MTHFR (A1298C) , FLT3 (D324N) , NQO1, p53 (codon 72) , PDCD5 and cytokine (TGF-beta; IFN-gamma,IL4) genes (24).

The phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB, AKT)/mammalian target of rapamycin (MTOR) pathway regulates various cellular functions including growth, proliferation, migration, and apoptosis(39). AKT gene is critical for cell survival and encodes an important downstream effector of the PI3K/AKT/MTOR pathway that regulates key cellular functions including glucose MTOR. AKT, alternatively known as AKT1, has five widely studied polymorphisms (rs3803300, rs1130214, rs2494732, rs2498804,

and rs1130233); the first four have been linked to the risk or prognosis of nasopharyngeal carcinoma, oral squamous cell carcinoma, and non-small cell lung cancer(38). The role of AKT1 gene polymorphisms in hematopoietic malignancies has rarely been investigated. The intention in selecting this gene is because of its association with various cancers and hence to detect its association with regard to the CML(33) .

DIAGNOSIS OF CML

Complete blood picture –Most patients with CML have higher WBCs in their blood with less number of red blood cells and platelets(40) .

Cytogenetic analysis – Cytogenetic analysis is used to identify the presence of ph chromosome to confirm the diagnosis and also to monitor treatment. Most of the CML cases (nearly 95%) carry philadelphia chromosome or BCR-ABL fusion gene (5).

□ **Fluorescent in situ hybridization (FISH)** – FISH is used to identify the BCR-ABL fusion gene and to monitor the minimal residual disease in patient after chemotherapy (5).

□ **Quantitative polymerase chain reaction** – Super sensitive method used for evaluating BCR-ABL gene expression (5).

Other Tests: Blood chemistry tests, Routine microscopic examination, Cytochemistry, Imaging studies, Magnetic resonance imaging

(MRI) and Ultrasound are also done. These will help in detecting the liver and kidney problems due to damage caused by the spread of leukemia cells or due to side effects of certain chemotherapeutic drugs (74,10)

GENETIC BASIS OF CHRONIC MYELOID LEUKEMIA

The root cause in the leukemogenesis of CML is formation of the BCR/ABL oncogene, which codes for a constitutively active BCR/ABL fusion tyrosine kinase (FTK) on the Philadelphia chromosome (Ph). Although BCR/ABL FTK is a key molecular marker of CML, it still remains to be understood which molecular or cellular events drive translocation of BCR/ABL gene or initiate leukemogenesis of CML (72).

Normal protein product of the ABL gene

P145 is the gene product of normal c-ABL proto-oncogene and is a nuclear protein with non-receptor tyrosine kinase activity. The p145 protein shuttles between nucleus and cytoplasm. The nuclear protein plays a role in inhibiting the cell cycle regulation, which led to notion that ABL is a tumor suppressor gene and the cytoplasmic protein is involved in the

transmission of integrin-mediated-signals from the cellular environment. Migration of this protein is linked to cellular growth control in association with several growth factor receptors like epidermal growth factor (EGF), platelet derived growth factor (PDGF), and colony stimulating factor (CSF). The p145 protein is involved in signal transduction pathways through the phosphorylation and subsequent activation of nuclear proteins.

Normal protein product of the BCR gene(p160)

The normal function of BCR protein is not known. The BCR gene normally codes for a 160 kd protein that is constitutively expressed in many cell types but with higher expression in hematopoietic cells. BCR gene with multiple break points produces fusion genes and gets translated into chimeric protein products of different lengths, which are associated with different leukemic subtypes. The functional domains of BCR and ABL are shown below(2).

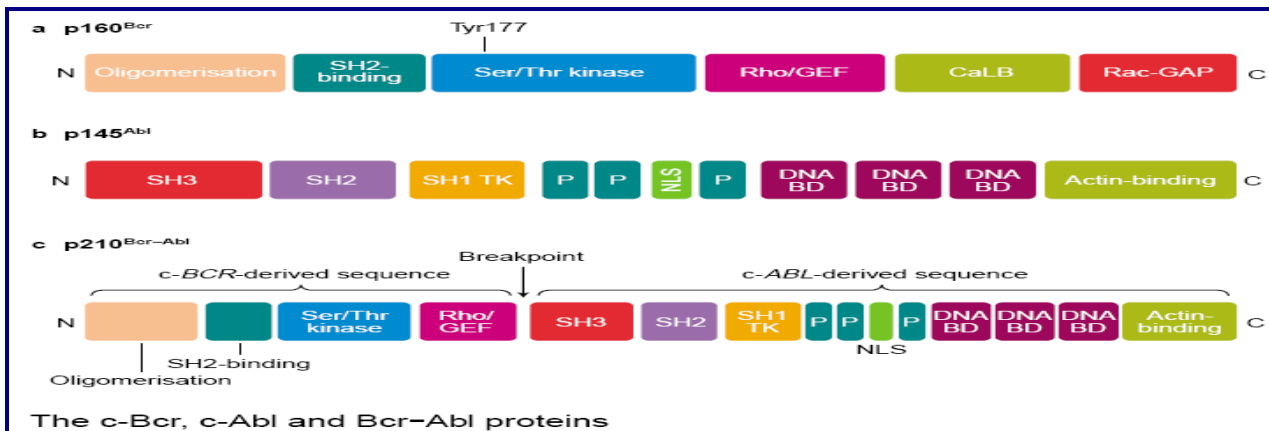


Fig 3 : BCR-ABL functional domains of BCR and ABL are shown below.

The functional domains of p145 are(76) .

1. N-terminal domain – has serine threonine kinase activity
2. Myristoylation domain - to localize the protein to the nucleus
 - SH2 domain - interacts with tyrosine-phosphorylated proteins
 - SH1 domain - performs phosphorylation function
4. C-terminal domain - which allow the interactions with other proteins

The functional domains of p160 are (76).

1. Coiled-coil motif – essential for polymerization with other proteins.
2. Dimerisation domain (DD) – facilitates in the formation protein dimmers.
3. Tyrosine residue at position 177 – essential contact point for binding the signal transduction proteins.

3. Src homology domains (SH2 & SH3)

□ SH3 domain - suppresses tyrosine kinase activity

4. Serine/threonine kinase domain – serves as a catalytic domain for phosphorylation .
5. GEF domain – serves as GDP/GTP exchange factor.
6. RAC-GAP domain – controls the rate of GTP hydrolysis.
7. RAS-GAP domain – converts active RAS proteins to inactive proteins.

BCR-ABL fusion genes

The 9; 22 translocation joins 5' portion of BCR gene with 3' end of the ABL gene. BCR-ABL translocation results for four distinct break points in the BCR gene. These four break points create three different protein

products p210^{BCR-ABL}, p190^{BCR-ABL}, and p230^{BCR-ABL} which may account for differences in

leukemogenesis (50, 2).

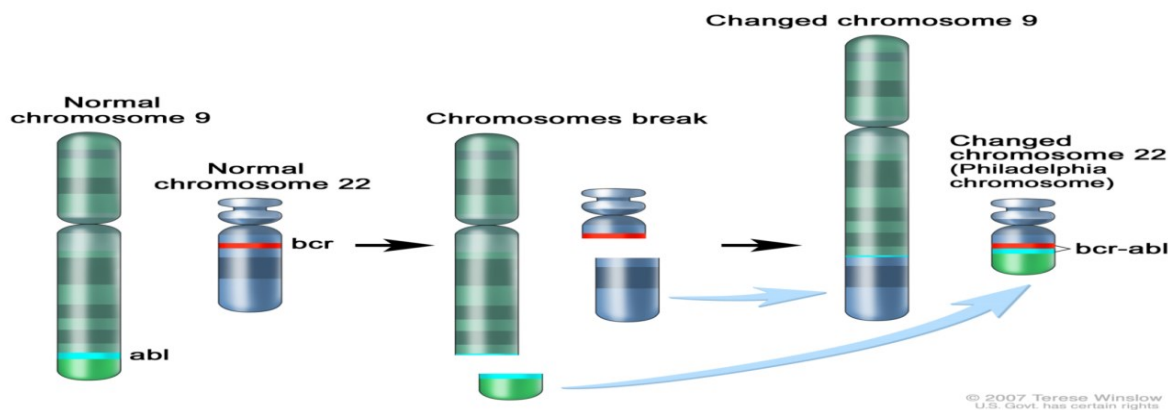


Fig 4: Reciprocal translocation between chromosomes 9 and 22 resulting in different BCR/ABL fusion gene transcripts.

Major breakpoint (M-BCR)

The breakpoint in ABL gene can occur anywhere within a >300-kb segment from the 5' end of the gene either upstream of the first alternative exon IB, between exons IB and IA, or downstream of exon IA. Regardless of the ABL breakpoint, the resulting fusion BCR-ABL transcript gives rise to two hybrid BCR-ABL mRNA molecules with a b3a2 and/or a b2a2 junction encoding a p210^{BCR-ABL} fusion protein(64,8).

Minor breakpoint (m-BCR) - It is observed in two thirds of ALLs and rarely in cases of CML and AML patients. The breakpoint in BCR is localized to the upstream, in the long (54.4kb) intron between the two alternative exons e2' and e2. The resultant fusion transcript contains

e1a2 junction and is translated into a smaller 190 -KD BCR-ABL fusion protein (p190^{BCR-ABL}(64,8).

Micro breakpoint (μ-BCR) - In this case the breakpoint in 3' end of BCR gene is located between exons e19 and e20 (c3 and c4). The resulting mRNA transcript codes for a large 230 KD BCR-ABL fusion protein (p230^{BCR-ABL}). This breakpoint is seen the cases of chronic neutrophilic leukemia (64,8).

Functions of BCR-ABL fusion gene

BCR-ABL fusion gene activates different signal transduction pathways. Three major

cellular functions of BCR-ABL gene in leukemic transformation are:

- Increased proliferation
- Reduced apoptosis

Disturbed interaction with the extra cellular matrix (58).

GENETIC BASIS OF DISEASE PROGRESSION

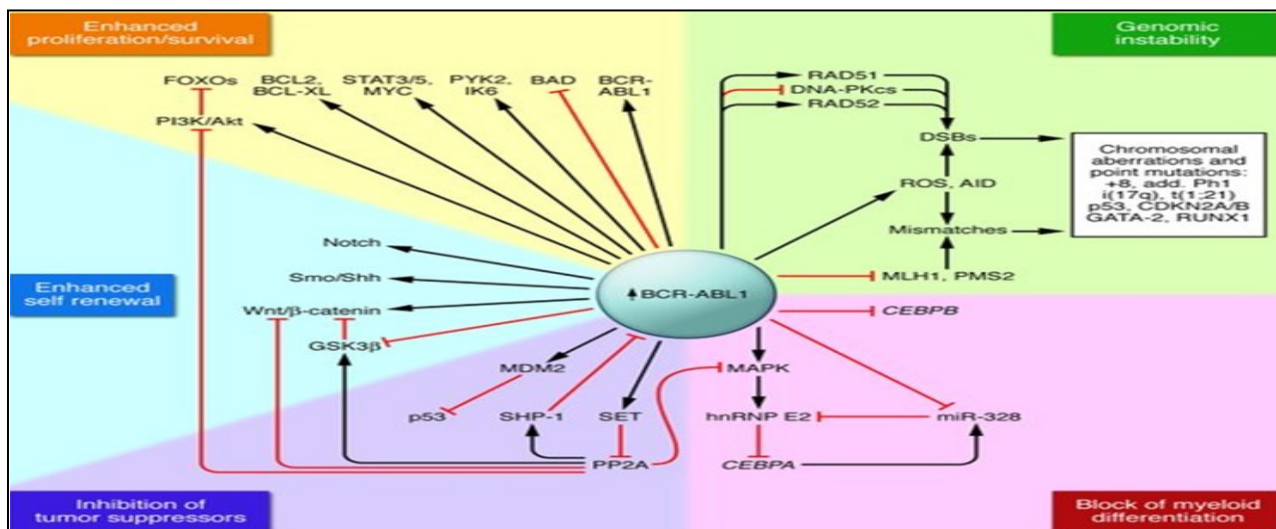
There is some indirect evidence that the formation of the BCR-ABL fusion gene may not be the initial or the sole event in the development of CML. Molecular abnormality arising due to mutations in genes controlling signal transduction, DNA repair, replication, cell differentiation, apoptosis, cytoskeleton adhesion have been implicated in the pathogenesis of CML. Specially hematopoietic progenitor cells with BCR-ABL expression are able to attain growth factor autonomy and can

withstand the noxious effects of cytotoxic drugs and ionizing radiation, in addition to activation of anti apoptotic mechanisms. Hence the understanding of gene-environment interaction and genotype-phenotype correlation especially with respect to CML susceptibility genes would enable to identify the risk genotypes and develop appropriate treatment modalities. Several mechanisms are involved in the induction of leukemogenesis in CML. According to the various research done in this criteria, certain pathways have been proposed that provoke the leukemogenesis in CML (51, 63).

1. Apoptosis-related pathway
2. Myeloid cell growth pathway
3. Angiogenesis pathway
4. Multidrug resistance pathway
5. WT1 (Wilms tumor gene) pathway
6. Interferon signaling pathway

Other than the major six pathways that are responsible in the disease progression, there

Fig 5 : Major candidate pathways.



are certain mitogenic signaling pathways that get activated by various mutations at the genomic level(70) . Such activated pathways are as follows:

1. Ras and MAP kinase pathways

Several links between BCR-ABL and Ras have been defined. Auto-phosphorylation of tyrosine provides a docking site for the adapter molecule Grb-2. Ras activation is important for the pathogenesis of Ph-positive leukemias. There is still dispute as to which mitogenic-activated protein (MAP) kinase pathway is downstream of Ras in Ph-positive cells. Stimulation of cytokine receptors such as IL-3 leads to the activation of Ras and the subsequent recruitment of the serine–threonine kinase Raf to the cell membrane. Raf initiates a signaling cascade through the serine–threonine kinases Mek1/Mek2 and Erk, which ultimately leads to the activation of gene transcription (70,2) .

2. Jak-Stat pathway

Constitutive phosphorylation of Stat transcription factors (Stat1 and Stat5) has since been reported in several BCR-ABL positive cell lines and in primary CML cells and Stat5 activation appears to contribute to malignant transformation. Although Stat5 has pleiotropic physiologic functions, its effect in BCR-ABL–transformed cells appears to be primarily anti-apoptotic and involves transcriptional activation of BCL-xL (Horita O et al., 2000;

Sillaber C et al., 2000). In contrast to the activation of the Jak-Stat pathway by physiologic stimuli, BCR-ABL may directly activate Stat1 and Stat5 without prior phosphorylation of Jak proteins. The role of the Ras and Jak-Stat pathways in the cellular response to growth factors could explain the observation that BCR-ABL renders a number of growth factor dependent cell lines to factor independent. During the chronic phase, CML progenitor cells are still dependent on external growth factors for their survival and proliferation, though less than normal progenitors(70,2) .

3. PI3 kinase pathway

PI3 kinase activity is required for the proliferation of BCR-ABL positive cells. BCR-ABL forms multimeric complexes with PI3 kinase, Cbl and the adapter molecules Crk and Crkl in which PI3 kinase is activated. The next relevant substrate in this cascade appears to be the serine–threonine kinase Akt. This kinase had previously been implicated in anti-apoptotic signaling. BCR-ABL might be able to mimic the physiologic IL-3 survival signal in a PI3 kinase-dependent manner. Ship and Ship-2, 2-inositol phosphatases with somewhat different specificities, are activated in response to growth factor signals and by BCR-ABL. Thus, BCR-ABL appears to have a profound effect on phosphoinositol metabolism, which might again shift the balance to a pattern

similar to physiologic growth factor stimulation.

4. Myc pathway

Over expression of Myc has been demonstrated in many human malignancies. It is thought to act as a transcription factor, though its target genes are largely unknown. Activation of Myc by BCR-ABL is dependent on the SH2 domain, and the over expression of Myc partially rescues transformation of defective SH2 deletion mutants whereas the over expression of a dominant negative mutant suppresses transformation . The pathway linking Myc to the SH2 domain of BCR-ABL is still unknown. Depending on the cellular

context, Myc may constitute a proliferative or an apoptotic signal. It is therefore likely that the apoptotic arm of its dual function is counter balanced in CML cells by other mechanisms, such as the PI3 kinase pathway. Multiple signals initiated by BCR-ABL have proliferative and anti-apoptotic qualities that are frequently difficult to separate. Thus, BCR-ABL may shift the balance towards the inhibition of apoptosis while simultaneously providing a proliferative stimulus. Various studies are carried out at the genome level in order to identify the genes which are responsible for the progression of chronic myeloid leukemia. Few genes are listed below.

TABLE 1- Functional annotation of the “progression” genes:

KEYWORD	EXAMPLES (Gene)
Ribosome	ROK 13A
Wnt Signaling	Cadherin, MDI1, Prickle 1, FZD2
Nucleosome	BSZ1A, HIST1H2AE
Sugar Metabolism	RP1A, ALDOC, G6PD
Myeloid Differentiation	CEBPA, CEBPE, FOXO3A
Apoptosis	GADD45G, BCL2, FOXO3A, MCL1
DNA Damage Response	GASDD45G, XRCC1, FANCG, XRN2

Three major mechanisms have been implicated in the malignant transformation by BCR-ABL, namely altered adhesion to stroma cells and extracellular matrix, constitutively active mitogenic signaling and reduced apoptosis-resulting into a malignant (70,2) .

ROLE OF AKT1 GENE IN CML

Aberrant tyrosine kinase activity plays a critical role in many hematologic disorders,

including chronic myeloid leukemia characterized by the constitutive activity of BCR-ABL. ABL therefore represents a crucial target for new therapeutic strategies. Many molecular pathways are abnormally activated by the oncoprotein. Such pathways may provide additional opportunities to develop new drugs to overcome the resistance to tyrosine kinase inhibitors. In particular, the phosphoinositide 3-kinase (PI3K)/AKT pathway can be effectively blocked by mTOR inhibitors, and several compounds can hit the RAS pathway and the resulting mitogen-activated protein (MAP) extracellular signal-

regulated kinase (ERK)1/2 (MEK) and MAP kinase activation. Furthermore, mitotic kinases can be blocked by Aurora kinase inhibitors, and Pim kinases can be blocked by selective serine-threonine kinase inhibitors. Finally, the abnormal pathways that sustain the self-renewal of leukemic stem cells are described as possible targets to completely eradicate leukemic clones. Such pathways include the Hedgehog pathway, which can be blocked by Smoothened inhibitors, and the CXCR4/SDF1 axis, which can be targeted by specific antagonists (76,48).

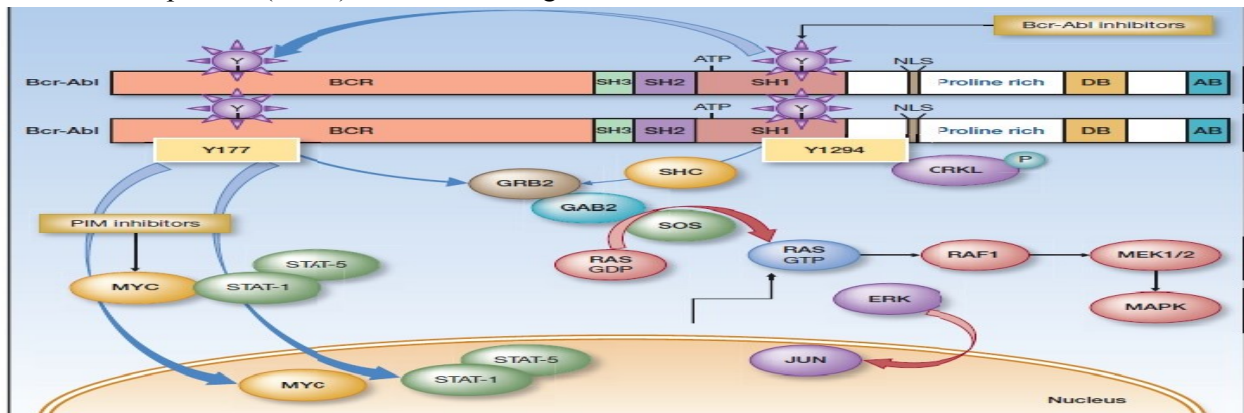


Fig 6 : molecular pathway activated by BCR-ABL.

FUNCTION OF AKT GENE

Role of Akt in Normal Cell Cycle Progression:

Activated Akt kinase modulates the function of numerous substrates related to cell cycle progression at the G1/S and G2/M transitions,

either by direct phosphorylation of the target proteins themselves or, indirectly, by regulating protein expression levels (41, 13).

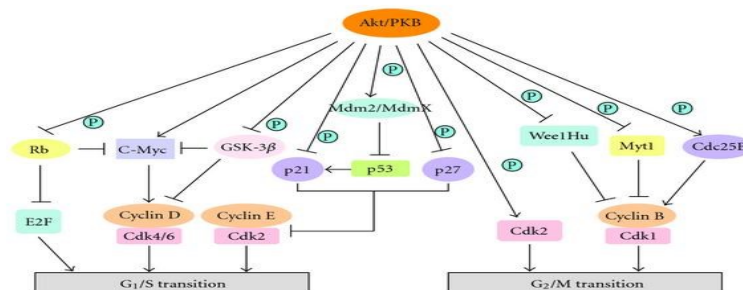


Fig 7: Role of Akt in Normal Cell Cycle Progression

Mechanisms of Akt Regulation of G1/S Progression:

Addition of mitogens to quiescent (G0) mammalian cells rapidly triggers a number of biochemical signalling cascades, including the PI3K/Akt pathway, to promote cell growth through two key enzymes mTOR and p70S6K. Growth-factor-mediated Akt activation increases the transcription of c-Myc, a strong promoter of cell cycle progression, causing cells to exit G0 both by inducing the expression of D-type cyclins and suppressing the expression of multiple negative cell cycle regulators such as p21Cip1, p27Kip1, and p15INK4b. Akt also controls the stability of c-Myc and cyclin D1 nuclear-cytoplasmic translocation of cyclin D1 leading to ubiquitin-dependent proteolysis in the cytoplasm. Furthermore, PI3K/Akt is also necessary and

sufficient to induce E2F transcriptional activity in T cells through phosphorylating and deactivating Rb. Another important function of Akt in G1/S progression is positive regulation of mid- and late-G1-phase cyclin/Cdk activity via phosphorylation and inactivation of Cdk inhibitors, including p21Cip1 and p27Kip1 (4, 41, 13,56).

Role of Akt in Genome Stability:

Akt Regulation in Response to DNA Damage:

Akt is activated not only by growth factors but also by DNA damage. The PIKK family members ATM, ATR, and DNA-PK are involved in Akt activation in response to genotoxic stresses, which may provide a prosurvival signal by triggering cell cycle arrest or inhibiting apoptosis(44,27)

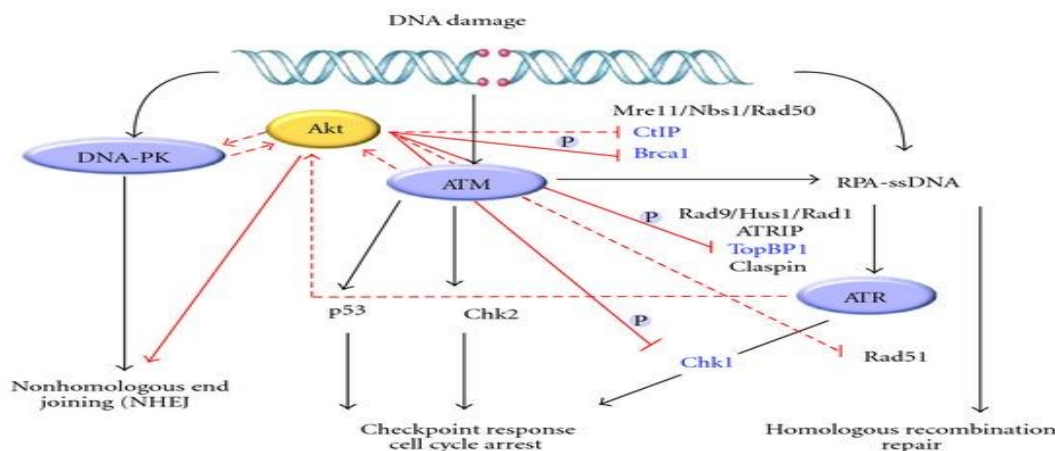


Fig 8: Akt Regulation in Response to DNA Damage . Akt Modulates DNA Damage Checkpoint Signalling and DNA Repair:

Mounting evidence implicates the role of Akt in modulating checkpoint responses and DNA repair. Activation of Akt overcomes DNA-damage-induced G2 arrest and apoptosis in a p53-independent manner. Overexpression of a constitutive active form of Akt or loss of PTEN abrogates G2 cell cycle checkpoint and Chk1 activation upon exposure to genotoxic stresses. In addition, Akt-mediated suppression of G2 arrest is associated with reduced recruitment of Chk2 to sites of DNA damage and inhibition of Chk2 activation in human glioblastoma cells. The ability of Akt to suppress G2 arrest induced by disparate agents suggests that Akt has a broad ability to override this checkpoint regardless of the pathway by which the process is initiated.

Akt Targets Involved in DNA Damage Responses:

Akt is thought to phosphorylate up to 100 substrates, some of which have been implicated in DNA damage checkpoint response and DNA repair. For instance, direct phosphorylation of cell cycle checkpoint kinase Chk1, TopBP1, Brca1, and RPS3 by Akt is important for the activation of DNA damage signalling cascade (**Figure: 14**). In addition, Akt activity is pivotal for the focal accumulation of key factors regulating DSB resection and DNA repair, including Rad51, Brca1, CtIP.

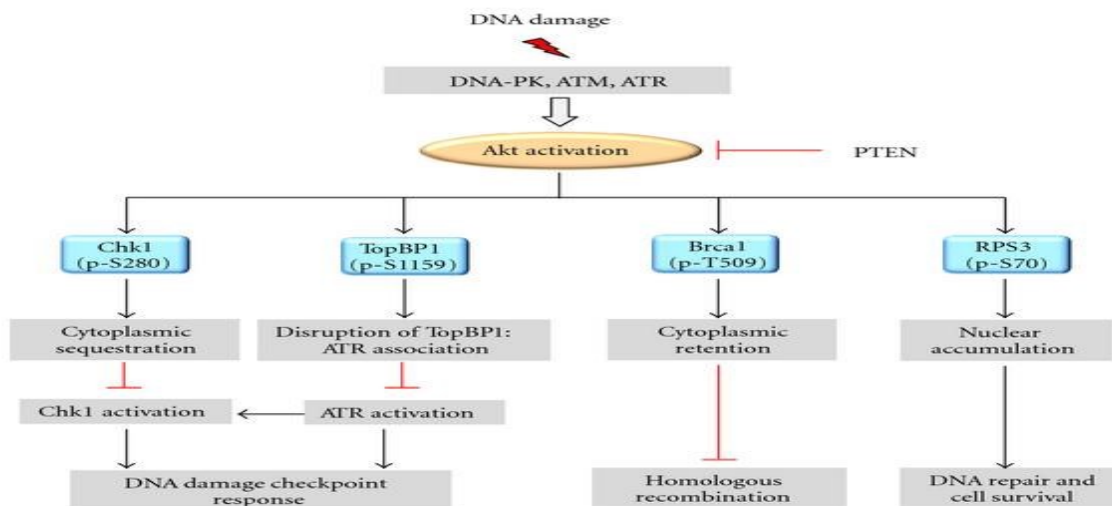


Fig 9 : Akt Targets Involved in DNA Damage Responses

MATERIALS

Source: The present study mainly deals with the association of AKT1 gene polymorphisms as well as with epidemiological parameters with Chronic Myeloid Leukemia. The results were analyzed in relation to clinical and epidemiological variables. For the present study, patients were recruited from NIMS (Nizams Institute of Medical Sciences, Hyderabad). The hospital is well equipped and all the required diagnostic tests including cytogenetic, biochemical and immuno-histochemical analysis are routinely being done here. It maintains a very good tumor registry for each patient, updating the patient's follow up and treatment modalities.

Study design:

The study was designed to work on 50 blood samples previously collected from NIMS (Nizams Institute of Medical Sciences), Hyderabad and 53 age normal healthy controls without any history of CML or any other cancers were selected randomly from different government offices, colleges, hostels and residential localities. The patients with confirmed diagnosis of CML were enrolled for the

study. For the present study cytogenetic analysis was done to identify the presence of ph chromosome which can be screened by either of the two methods cytogenetic analysis or molecular analysis through RT-PCR technique. The diagnosis of CML is also based on the other tests like complete blood picture, bone marrow biopsy, Hemoglobin percentage, LDH (lactate dehydrogenase), and Alkaline phosphatase levels. All the patients were informed about the present study and those patients with confirmed diagnosis of CML who had given their consent for participation were recruited. Epidemiological data like sex of the patient and age at onset of CML, familial history of cancer, parental consanguinity, caste, occupation, food habits was collected using prescribed proforma through personal interview of patient and family members. Detailed pedigree information was taken to identify familial incidence of cancers. The clinical information such as phase of the disease, drug history, disease progression, hematological and cytogenetic responses were noted down with the help of oncologist.

Collection of blood samples from cases and controls:

5ml of blood sample was collected in EDTA vacutainer and stored at 4⁰C till use. The EDTA blood was used for DNA isolation (isolation of the DNA within 1-2 days gives more yield). After DNA isolation, from the stock DNA samples 20-30µls of DNA sample was aliquoted into working vials (stored at 4⁰C) and the rest of the stock was stored in -20⁰ freezer. The aliquoted DNA samples were analyzed for all the molecular parameters for case vs control comparison.

Parameters: The parameters under study were classified into 3 groups. They are epidemiological, clinical, and molecular parameters.

Epidemiological parameters

- i. Age at onset
- ii. Occupation

iii. Diet

iv. Area of living

Clinical parameters

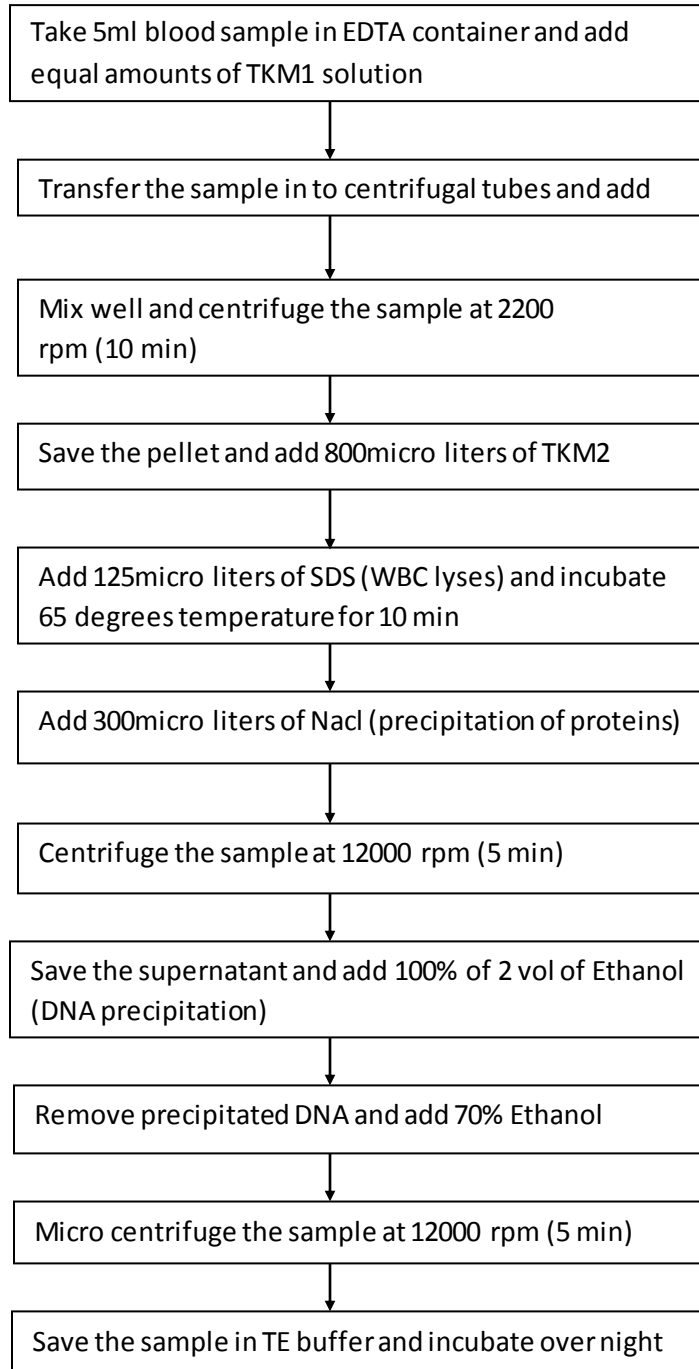
- i. Phase of the disease
- ii. Haematological response
- iii. Cytogenetic response
- iv. Molecular response

METHODOLOGY

ISOLATION OF DNA

Principle: DNA was extracted from peripheral blood leucocytes by rapid non-enzymatic method by salting out the cellular proteins by dehydration and precipitation with saturated sodium chloride solution. Then the DNA was precipitated with 100% ethanol .

DNA ISOLATION FLOW CHART



POLYMERASE CHAIN REACTION

Principle:

Normal cells duplicate their DNA by the Replication, in the replication the double stranded DNA is separated and the Primase is the enzyme which synthesizes the primers and the DNA polymerase assembles nucleotides to form two new pairs with the guanine(G). Because of this base pairing, the newly synthesized strands have the same sequence of original parental strands and finally the replication results in the duplication of the original Parental DNA.

The same replication principle is applicable in the PCR. The PCR is also called as invitro amplification of the particular DNA sequence; it undergoes 30 to 40 rounds of replication cycles in the PCR tubes. In each replication cycle, the number of molecules of the target sequence doubles, because the products of the one cycle act as a template for the next round & finally for 'n' rounds of replication 2^n copies of specific target DNA strands are synthesized. The main enzyme of the PCR technique is DNA polymerase, due to this enzyme property gives name as Polymerase Chain Reaction.

strands using parental DNA as a template. The nucleotides adenine (A) and guanine (G) are purines and thiamine (T) and cytosine(C) are pyrimidines. The adenine(A) always pairs with the thiamine(T), and cytosine(C) always pairs with the guanine(G). The PCR mainly involves three major steps:

1. Denaturation
2. Annealing
3. Extension

□ Denaturation:

In the denaturation step, the double stranded DNA was denatured into single stranded DNA and also all the enzymatic reaction were not carried out.

□ Annealing:

The annealing temperature is very critical for the PCR amplification. Hydrogen bonds constantly form and break between the single stranded DNA and single stranded templates. But the hydrogen bonds between the template and the primer are now stable. The amplification depends upon annealing temperature which is 5°C below or above the melting temperature of the amplification primers. The annealing

temperature ranges from 55°C to 72°C generally yields the best results. The annealing temperature is a critical factor for sequence specific amplification.

□ **Extension:**

The nucleotide bases are added to the primer at the 3' hydroxyl group. Taq DNA

polymerase adds the dNTPs from 5' to 3' direction of the primer. The bases are added complimentary to the template DNA. The extension time depends on the length and concentrations of the template DNA strand. The optimum temperature for the extension is 72°C for 30seconds.

TABLE-3- REACTION MIXTURE CONDITIONS

Reagents	Normal quantity, for 10µl of reaction mixture	Optimization quantity, for 50 µl of reaction mixture	Optimization quantity, for 100µl of reaction mixture
Template DNA	0.1-1	5µl	10 µl
10x PCR buffer	variable	5 µl	10 µl
dNTPs(10mm)	0.6 µl	3 µl	6 µl
Forward primer	variable	3 µl	6 µl
Reverse primer	variable	3 µl	6 µl
Taq DNA polymerase	0.25-0.5(5U/0.1µl)	0.5 µl	1 µl
Water	variable	30.5 µl	61 µl
Total		50 µl(5 samples)	100 µl(10 samples)

AKT1 gene	PCR Product Size	PCR CONDITIONS		Primer Sequence
		For 35 Cycles		
INTRONIC VARIANT	495bp	Initial Denaturation	95°C – 5 min	FP:CCCAAGCACGTCACACCTC RP:GGGACAGAGGCCCAACTG AC
		Denaturation	95°C – 1 min	
		Annealing	64.2°C – 30 sec	
		Extension	72°C – 45 sec(Repeat 34 times)	
		Final Extension	72°C – 7 mins	

TABLE - 4 -PCR Conditions

AGAROSE GEL ELECTROPHORESIS

Requirements

An electrophoresis chamber and power supply .

□ Gel casting tray

Which are available in a variety of sizes and composed of UV-transparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis.

□ **Sample combs:** Around which molten agarose is poured to form sample wells in the gel.

□ **Agarose:** Is the linear polysaccharide made up of the basic repeat unit of agarobiose

□ **Electrophoresis buffer:** Usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE) is used.

□ **Loading buffer:** Which contains something dense (e.g. glycerol, Xylen xynol, Bromophenol blue, sucrose, etc) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual

monitoring or how far the electrophoresis has proceeded.

- **Ethidium bromide**, A fluorescent dye used for staining nucleic acids.
- **Transilluminator**: Which is used to visualize ethidium bromide-stained DNA in gels.

Principle:

Electrophoresis is a technique used to separate and sometimes purify macromolecules - especially proteins and nucleic acids - that differ in size, charge or conformation. Negatively charged molecules move towards the anode and the positively charged molecules move towards the cathode. The smaller molecules move faster than the larger molecules it is depending upon the size and shape of the molecules.

Procedure:

- ▶ Agarose powder is mixed with 0.5X TAE buffer to the desired concentration, and then heated in a microwave oven until completely melted.
- ▶ Ethidium bromide is added to the gel at this point to facilitate visualization of DNA after electrophoresis.
- ▶ After cooling the solution to about 60C, it is poured into a casting tray containing a

sample comb and allowed to solidify at room temperature.

▶ After solidifying the gel the comb is removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber and just covered with buffer.

▶ Samples containing DNA mixed with loading buffer are then pipetted into the sample wells, and a current is applied.

▶ After some time DNA will migrate towards the positive electrode.

▶ Switch of the power supply and the gel see under the transilluminator.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

It is a molecular technique used to identify individuals, based on unique patterns of restriction enzyme cutting in specific regions of DNA.

Principle:

It employs endonucleases, which recognizes a specific nucleotide sequence and digests them which can be identified on the gel by the presence of fragments of different lengths, revealing a unique

banding pattern characteristic to a specific genotype. Most RFLP markers are co-dominant and highly locus-specific.

Methodology:

1. 8 μ l of PCR master-mix was taken.
2. To each PCR tube, 2U of enzyme (Pst1) was added.

3. These tubes were subjected to overnight digestion for 16 hours in water-bath at 37 $^{\circ}$ C.

4. Then, genotyping was carried out by 3.5% Agarose Gel electrophoresis.

5. The bands patterns were noted down by observing the bands under UV trans-illuminator.

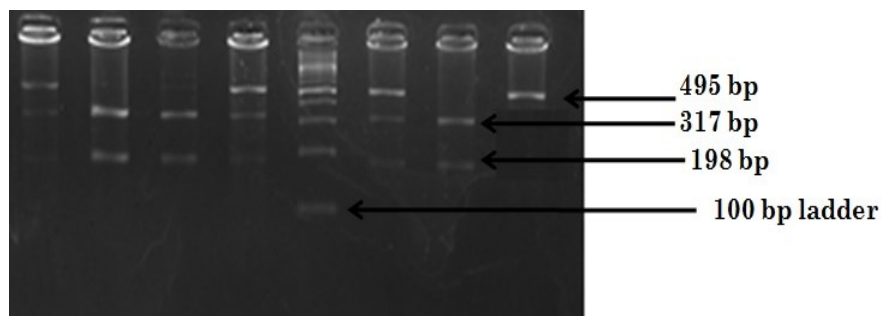


Fig 10 : Gel picture:

Lane 1,4,6 : CT(495bp,317bp,198bp)

Lane 2,3,7 : CC(317bp,198bp)

Lane 8 : TT (495bp)

Lane 5 : 100bp ladder

RESULTS AND DISCUSSION

TABLE 5- DISTRIBUTION OF EPIDEMIOLOGICAL PARAMETERS UNDER STUDY

SL NO.	EPIDEMIOLOGICAL VARIABLES		CASES
1.	Sex ratio (50)	MALES	31(62%)
		FEMALES	19(38%)
2.	AGE AT DIAGNOSIS (50)	<=30 YEARS	15(30%)
		>30 YEARS	35(70%)
3.	AREA OF LIVING (50)	RURAL	28(56%)
		URBAN	22(44%)
4.	HABITS (SMOKING AND/OR ALCOHOLIC) (50)	SMOKING AND/OR ALCOHOLIC	9(18%)
		NO SMOKING	41(82%)
5.	DIET(50)	VEGETARIAN	0(0%)
		NON-VEGETARIAN	50(100%)
6	OCCUPATION(50)	Agr&labors	19(38%)
		Students	2(4%)
		Housewife	11(22%)
		Others	18(36%)

The study of incidence of the disease and its association with epidemiological parameters might provide insight into genetic diversity of the population and interacting environmental factors.

- Sex of proband:** More number of males (62%) are affected with CML which indicate that males are at greater risk to develop CML when compared to females. This might be due to

inherent immune deficiency, effect of androgen, etc. among the males.

- Age at onset:** 70% of the CML patients developed disease after 30 years which implies that the people above 30 are at greater risk to develop CML when compared to people below 30 years.
- Area of living:** 56% of the CML patients residing in the rural areas are at greater risk to develop CML .It

might be due to possible exposure of pesticides and other chemicals.

4. **Habits:** CML was more prominent in non smokers and non-alcoholics (82%) which show that smoking and alcoholic status does not play any role

in the development of the disease.

5. **Diet:** Most of the CML patients were found to have non-vegetarian (100%).
6. **Occupation:** 38% of CML patients were found to be agricultural and other labourers

Sample status	Genotype frequencies	Allelic Frequencies
---------------	----------------------	---------------------

TABLE 6 - DISTRIBUTION OF CLINICAL PARAMETERS UNDER STUDY

SL NO.	RESPONSE		
1.	PHASE (50)	CHRONIC	49(98%)
		ADVANCED	1(2%)
2.	HEMATOLOGICAL RESPONSE (50)	COMPLETE	34(68%)
		PARTIAL/ NON- RESPONDERS	16(32%)
3.	CYTOGENETIC RESPONSE (50)	COMPLETE	33(66%)
		PARTIAL/ NON- RESPONDERS	17(34%)
4.	MOLECULAR RESPONSE (50)	COMPLETE	26(52%)
		PARTIAL/NON- RESPONDERS	24(48%)

1. **Phase of CML:** As the duration for chronic phase varies from several months to years, most of the CML patients were diagnosed in chronic phase (98%) as compared to advanced phases (2%).

2. **Response to drug (Hematological, Cytogenetic and Molecular responses):** Most of the patients had hematological, cytogenetic and molecular response (68%, 66% and 52% respectively) which was of clinical importance as most of the patients were Ph+ve and were treated with Imatinib mesylate.

	W CC		H CT		M TT		Total	C	T
	N	%	N	%	N	%			
CONTROLS	21	42	12	24	17	34	50	0.54	0.46
CASES	22	44	18	36	10	20	50	0.62	0.38

Chi-square: 3.038,df-2,p-value-0.21 Hardy-Weinberg equilibrium: controls x^2 -13.36,cases x^2 -2.78
 Odds ratio : CC Vs CT-OR-0.70(0.27 to 1.80) CC Vs TT-OR-1.78(0.67- 4.76)
 CT Vs TT-OR-2.55(0.88 – 7.43)

Table 7- Distribution of Genotype and allelic frequency with samples status

INFERENCE: The variant genotypic and allelic frequencies increased in controls (34% and 0.46) when compared with cases (TT-20%,T-0.38) which indicated that this allele may not play a role in the development of CML .

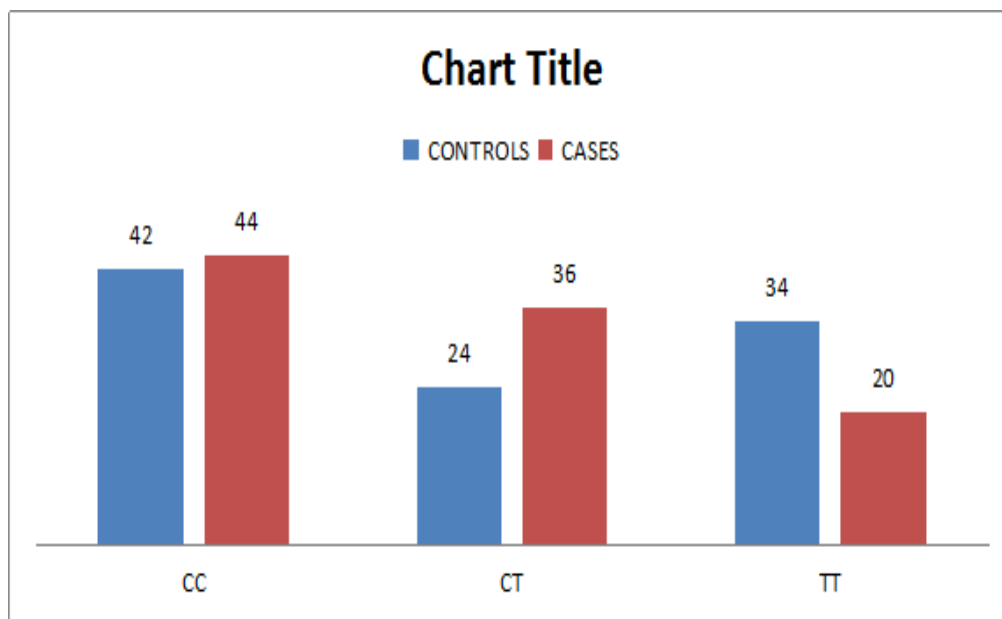


Figure 11 : Genotype frequency of samples status

TABLE 8 - Distribution of Genotype and allelic frequency with age status

AGE STATUS	Genotype frequencies						Allelic Frequencies		
	W CC		H CT		M TT		Total	C	T
	n	%	n	%	n	%			
<=30	6	40	5	33.3	4	26.7	15	0.56	0.44
>30	16	45.7	13	37.1	6	17.1	35	0.64	0.36

Chisquare-0.596,df-2,p-value-0.74
 Odds ratio: CC Vs CT-0.975(0.24 to 3.93)
 CC Vs TT-0.5625(0.11 to 2.71)
 CT Vs TT-0.5769(0.11 to 2.95)

Inference: T allele frequency increased in individuals who were in the age group <=30 (0.44) compared to the individuals whose age was >30 (0.36), similar trend was observed when the genotypic frequencies were calculated (<=30 (26.7%) and >30 (17.1%)), suggesting that the individuals who are less than 30 are more susceptible to develop the disease.

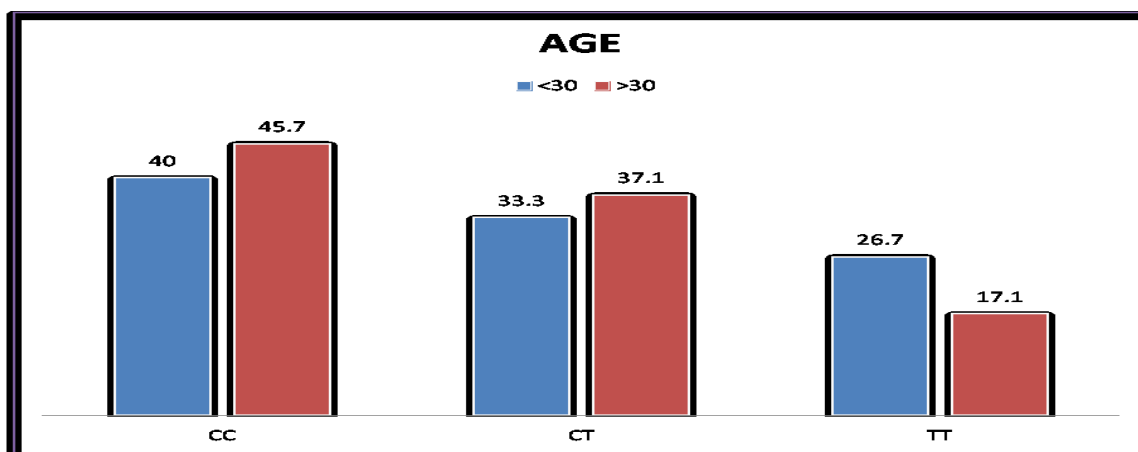


Figure 12 : Genotype frequency of age status

TABLE 9 - Distribution of Genotype and allelic frequency with gender status

GENDER STATUS	Genotype frequencies							Allelic frequencies	
	CC		CT		TT		Total	C	T
	n	%	n	%	n	%			
MALE	15	48.4	8	25.8	8	25.8	31	0.61	0.39
FEMALE	7	36.8	10	52.6	2	10.5	19	0.63	0.37

Chi square-4.087,df-2,p-value-0.13
 Odds ratio: CC Vs CT-2.6786(0.73 to 9.75)
 CC Vs TT-0.5357(0.08 to 3.21)
 CT Vs TT-0.2(0.03 to 1.21)

Inference: Males (25.8%) carrying TT genotype are prone to CML compared to females (10.5%) while the allelic frequencies did not show much variation in males(0.39) and females (0.37). The increase in the frequency suggest that the males are more prone to CML.

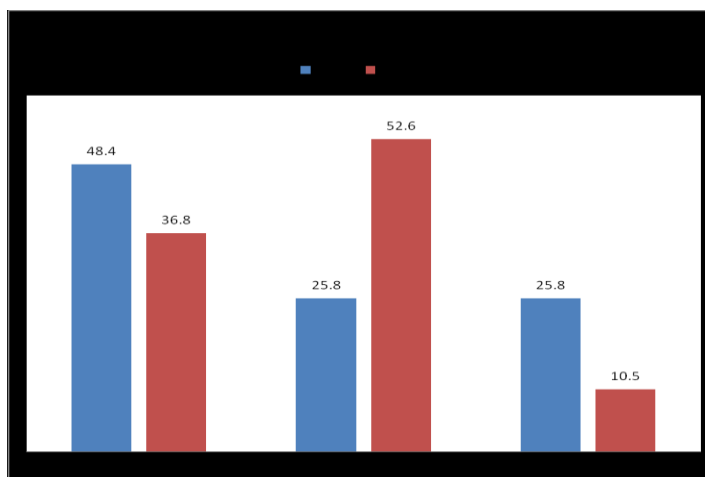


Figure 13: Genotype frequency of gender statu

TABLE 10 - Distribution of Genotype and allelic frequency with area of living status

AREA OF LIVING STATUS	Genotype frequencies						Allelic Frequencies		
	W CC		H CT		M TT		Total	C	T
	N	%	n	%	n	%			
RURAL	15	53.6	8	28.6	5	17.9	28	0.68	0.32
URBAN	7	31.8	10	45.5	5	22.7	22	0.55	0.45
Chi-square-2.447,df-2,p-value-0.29 Odds ratio: CC Vs CT-2.6786(0.73 to 9.75) CC Vs TT-2.1429(0.46 to 9.89) CT Vs TT-0.8(0.16 to 3.76)									

Inference: The variant genotype and allelic frequencies increased in patients from urban area (22.7% and 0.45) compared to that of rural area (17.9% and 0.45). This could be because of the possible exposure of pollutants.

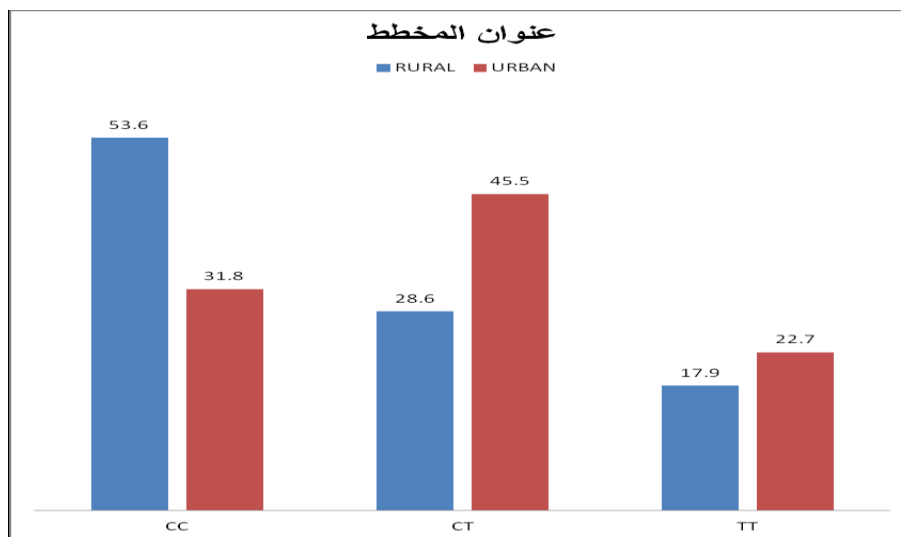


Figure 14 : Genotype frequency of area of living status

TABLE 11 - Distribution of Genotype and allelic frequency with habit status

HABIT STATUS	Genotype frequencies							Allelic Frequencies	
	W CC		H CT		M TT		Total	C	T
	n	%	n	%	n	%			
SMOKE/ALCOHOL	5	55.6	3	33.3	1	11.1	9	0.72	0.28
NO SMOKE/ALCOHOL	17	41.5	15	36.6	9	22	41	0.60	0.40

Chisquare-0.788,df-2,p-value-0.67
 Odds ratio: CC Vs CT-1.4706(0.29 to 7.21)
 CC Vs TT-2.6471(0.26 to 26.24)
 CT Vs TT-1.8(0.16 to 20.02)

Inference: TT genotypic (22%) and T allele frequencies (0.40) increased in non-smokers and non-alcoholics compared to that of smokers and alcoholics (TT 11.1%, T-0.28) showing that the smoking and alcoholic status of an individual does not have any effect on the risk to develop CML.

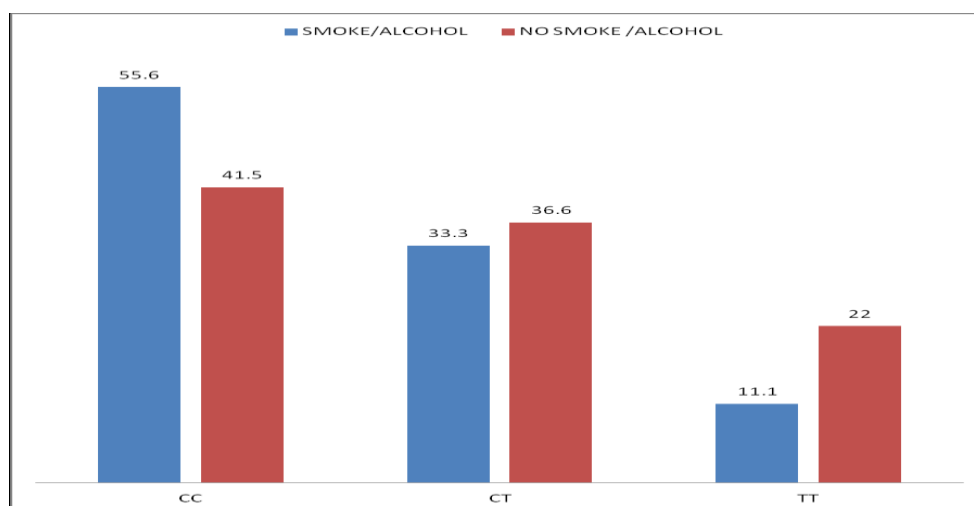


Figure 15 : Genotype frequency of habit status

Table 12- Distribution of Genotype and allelic frequency with occupation status

OCCUPATION STATUS	Genotype frequencies						Allelic Frequencies		
	CC		CT		TT		Total	C	T
	n	%	n	%	n	%			
AGRICULTURE & LABOR	11	57.9	6	31.6	2	10.5	19	0.74	0.26
STUDENT	1	50	0	0	1	50	2	0.50	0.50
HOUSEWIFE	4	36.4	6	54.6	1	9.1	11	0.64	0.36
OTHERS	6	33.3	6	33.3	6	33.3	18	0.50	0.50

Chisquare-7.378,df-6,p-value-0.28,yates chisquare-3.369,yates p-value-0.76

Inference: The allelic and genotypic frequencies increased in students (TT-50%,T-0.50) and in individuals who were involved in doing other jobs (TT-33.3%, T-0.50). This observation could be because of very few numbers of patients in this group compared to other groups.

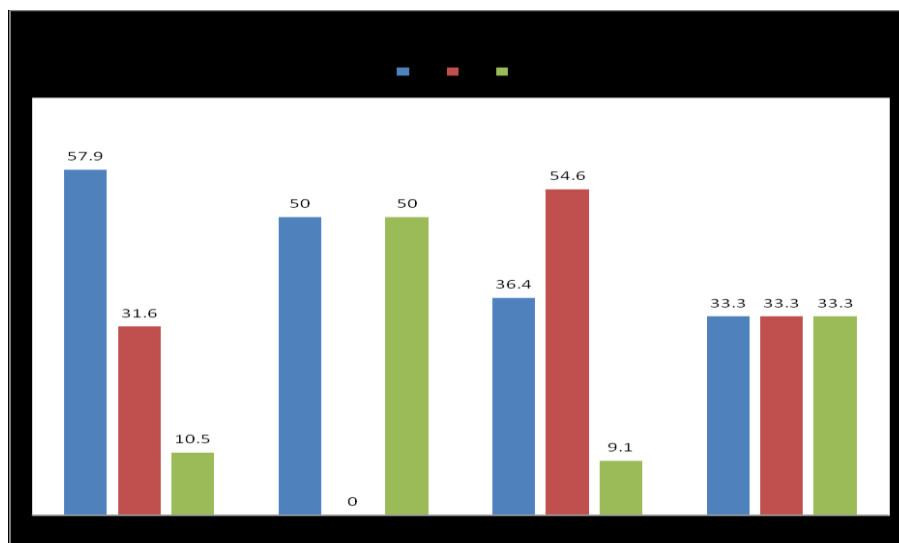


Figure 16 : Genotype frequency of occupation status

TABLE 13 - Distribution of Genotype and allelic frequency with phase status

PHASE STATUS	Genotype frequencies						Allelic frequencies		
	W CC		H CT		M TT		Total	C	T
	n	%	n	%	n	%			
CHRONIC	22	44.9	18	36.7	9	18.4	49	0.63	0.37
ADVANCED	0	0	0	0	1	100	1	0	1
Chi-square-4.082,df-2,p-value-0.12,yates chi-square-0.523,yates p-value-0.76									

Inference: More number of individuals were found to be in Chronic phase compared to that of advanced phase and found to respond well to Imatinib mesylate. The increase in the genotypic frequency in the advanced phase (100%) compared to that of chronic phase (18.4%), could be explained by the presence of very few individuals.

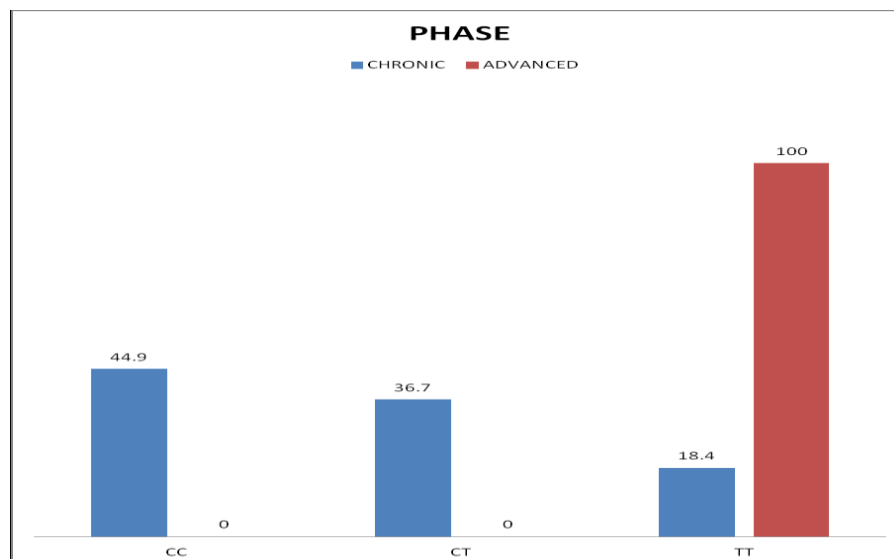


Figure 17 : Genotype frequency of phase status

Table 14- Distribution of Genotype and allelic frequency with hematological response

HEMATOLOGICAL RESPONSE	Genotype frequencies						Allelic Frequencies		
	W CC		H CT		M TT		Total	C	T
	n	%	n	%	n	%			
MAJOR RESPONSE	16	47.1	12	35.3	6	17.7	34	0.65	0.35
MINOR RESPONSE	6	37.5	6	37.5	4	25	16	0.56	0.44

Chi square-0.535,df-2,p-value-0.76
 Odds ratio: CC Vs CT-1.3333(0.34 to 5.17)
 CC Vs TT-1.7778(0.36 to 8.58) CT Vs TT-1.3333(0.26 to 6.60)

Inference: Both genotypic and allelic frequency increased in minor responders (25% and 0.44) compared to major responders (17.7% and 0.35), indicating that this allele is involved in the progression of the disease.

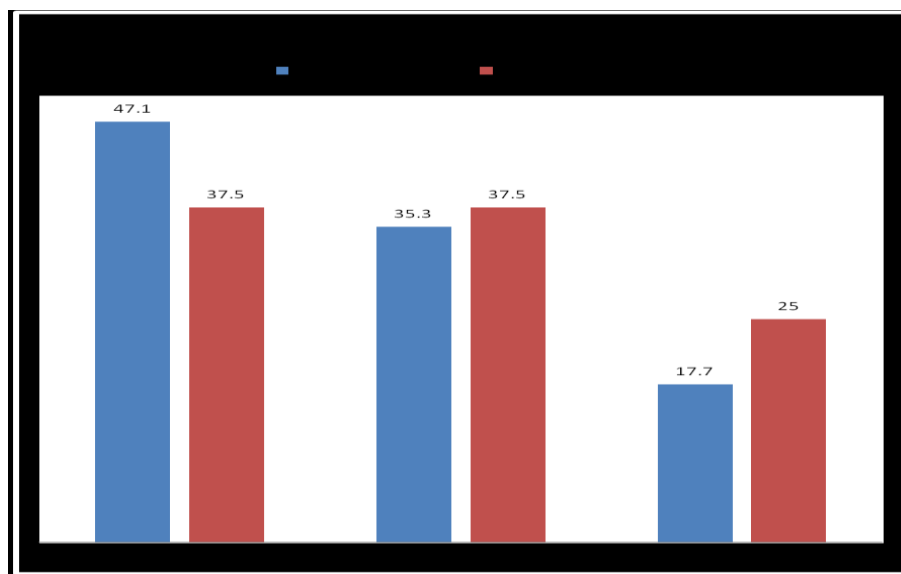


Figure 18 : Genotype frequency of hematological response

TABLE -15 - Distribution of Genotype and allelic frequency with cytogenetic response

CYTOGENETIC RESPONSE	Genotype frequencies						Allelic Frequencies		
	W CC		H CT		M TT		Total	C	T
	n	%	n	%	n	%			
MAJOR RESPONSE	15	45.5	12	36.4	6	18.2	33	0.64	0.36
MINOR RESPONSE	7	41.2	6	35.3	4	23.5	17	0.59	0.41

Chi-square-0.211,df-2,p-value-0.89
 Odds ratio: CC Vs CT- 1.0714(0.28 to 4.04)
 CC Vs TT-1.4286(0.30 to 6.73)
 CT Vs TT-1.3333(0.26 to 6.60)

Inference: Genotypic and allelic frequency increased in minor responders (23.5% and 0.41) compared to major responders (18.2% and 0.36) , indicating that this allele is involved in the progression of the disease.

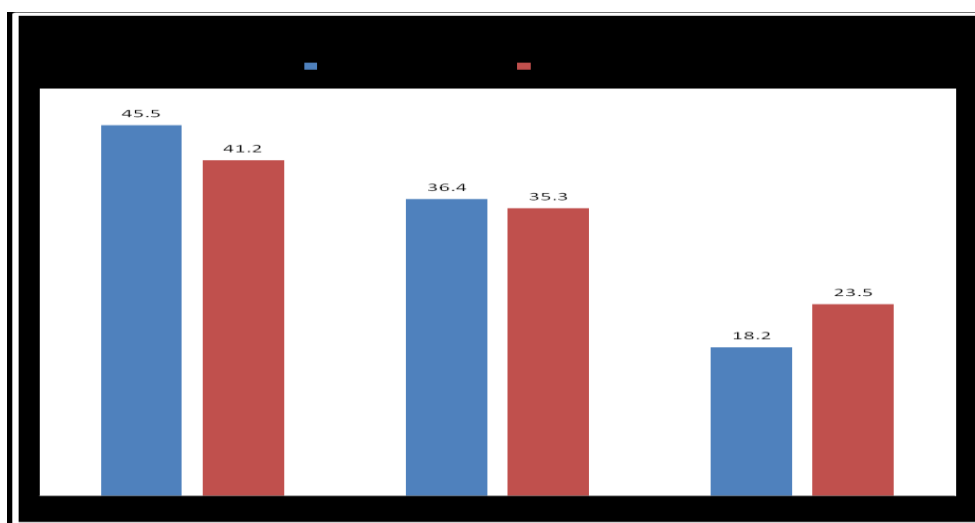


Figure 19 : Genotype frequency of cytogenetic response status

Table -16 - Distribution of Genotype and allelic frequency with molecular response

MOLECULAR RESPONSE	Genotype frequencies							Allelic Frequencies	
	CC		CT		TT		Total	C	T
	n	%	n	%	n	%			
MAJOR RESPONSE	10	38.5	11	42.3	5	19.2	26	0.60	0.40
MINOR RESPONSE	12	50	7	29.2	5	20.8	24	0.65	0.35

Chi-square-0.992,df-2,p-value-0.60
 Odds Ratio: CC Vs CT-0.5303 (0.14 to 1.88)
 CC Vs TT-0.8333(0.18 to 3.72)
 CT Vs TT-1.5714(0.33 to 7.48)

Inference: Variant genotypic frequency increased in minor responders (20.8%) compared to major responders (19.2%) while contrasting results were obtained when allelic frequencies were calculated, where it is showed an increase in major responders (0.40) compared to minor responders (0.35).

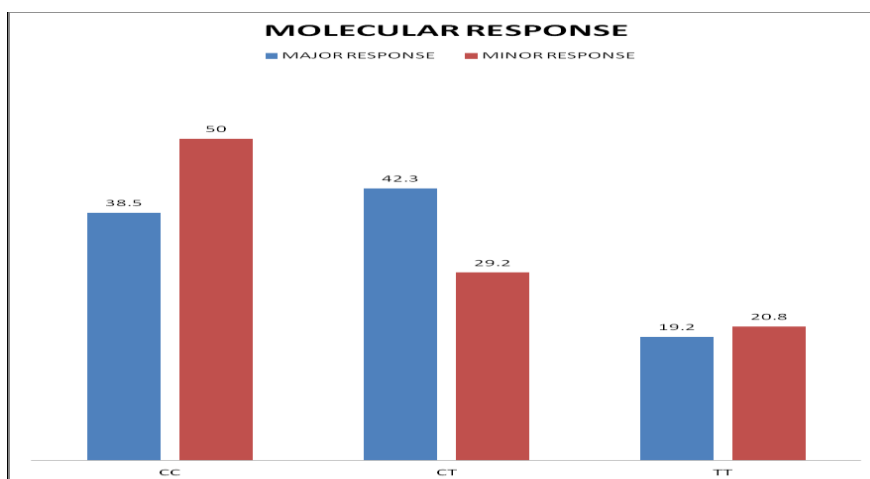


Figure 20: Genotype frequency of molecular response

CONCLUSION

- Case- control study did not reveal any significant association of the variant allele with the development of the disease. However, study of large sample may reveal any significance.
- The variant genotype and allelic frequencies increased in patients from urban area compared to that of rural area which could be because of the possible exposure of pollutants.
- Patients whose age was less than 30 were found to be more susceptible compared to the ones whose age group was more than 30 years.
- Smoking and alcoholic status of an individual did not reveal any significant association with the development of the disease.
- Males were more prone to CML compared to that of females.
- More number of individuals were found to be in Chronic phase compared to that of advanced phase and responded well to Imatinib mesylate.
- When response (hematological, cytogenetic and molecular) to drug

was observed, the genotypic and allelic frequencies increased in minor responders compared to major responders indicating that this allele is involved in the progression of the disease.

SUMMARY

Chronic myelogenous leukemia (CML) is a cancer of the white blood cells, characterized by the increased and unregulated growth of predominantly myeloid cells in the bone marrow and the accumulation of these cells in the blood. Chronic myeloid leukemia (CML) is probably the most extensively studied human malignancy. The main pathophysiologic cause for chronic myeloid leukemia is the action of BCR-ABL fusion protein which is formed as a result of the translocation between 9 and 22 chromosomes. This fusion protein is a tyrosine kinase which inhibits DNA repair causing genomic instability (Moore JK, et al. 1996). BCR-ABL, a constitutively activated tyrosine kinase, is the oncogene that causes Philadelphia-chromosome-positive (Ph+) leukemia. Although the t(9;22) translocation is the primary cause, various studies have shown that the SNPs in critical regions of the genes were found to increase the risk of CML. Hence, the study was planned to analyze the role of intron variant in the AKT1 gene in the development and progression of CML through PCR-RFLP analysis. The present case-control

study was conducted on 50 CML and 50 normal healthy controls for which all the essential data on variables was recorded. Case-control study did not reveal any significant association of the variant allele with the development of the disease. The frequency distribution showed that the sex ratio was found to be more in males than in females. People residing in urban areas were more affected. The disease was found to be more common in younger age group (≤ 30 years). Smoking and alcoholic status of an individual did not reveal any significant association with the development of the disease. When response (hematological, cytogenetic and molecular) to drug was observed, the genotypic and allelic frequencies increased in minor responders compared to major responders indicating that this allele is involved in the progression of the disease.

REFERENCES

1. Ahmed, Khalil, Olaf-Georg Issinger, and Ryszard Szyszka. 2015. 12 Protein Kinase CK2 Cellular Function in Normal and Disease States. Springer.
2. Bain, Barbara J. 2003. Chronic Myeloproliferative Disorders: Cytogenetic and Molecular Genetic Abnormalities. Karger Medical and Scientific Publishers.
3. Bain, Barbara J. *Leukaemia diagnosis*. John Wiley & Sons, 2010.
4. Bar-Eli, Menashe. 2008. Regulation of Gene Expression in the Tumor Environment. Springer.
5. Boulton, Jacqueline, and Carrie Fidler. 2002. 68 Molecular Analysis of Cancer. Springer Science & Business Media.
6. Brandt, J T. 1996. "Hematology: Basic Principles and Practice, Ronald Hoffman, Edward J. Benz, Sanford J. Shattil, Bruce Furie, Harvey J. Cohen, Leslie E. Silberstein." ARCHIVES OF PATHOLOGY AND LABORATORY MEDICINE 120: 1073.
7. Brown, H Alex. 2007. 434 Lipidomics and Bioactive Lipids: Lipids and Cell Signaling: Lipids and Cell Signaling. Academic Press.
8. Cavalli, Franco et al. 2009. Textbook of Medical Oncology. CRC Press.
9. Chawdhary, Ayesha. 2009. Delays in Diagnosis and Referral and Treatment for Hematologic Malignancies. ProQuest.
10. Chou, David. 2007. "Henry's Clinical Diagnosis and Management by Laboratory Methods." JAMA 297(16): 1827-33.
11. Cortes, Jorge, and Michael Deininger. 2006. Chronic Myeloid Leukemia. CRC Press.
12. Dennis, Edward A, and Ralph A Bradshaw. 2011. Intercellular Signaling in Development and Disease. Academic Press.
13. Divita Jr, Vincent T, Theodore S Lawrence, and Steven A Rosenberg. 2015. "Cancer: Principles & Practice of Oncology: Primer of the Molecular Biology of Cancer."
14. Dufour, Jean-Francois, Pierre-Alain Clavien, C Trautwein, and R Graf.

2015. Signaling Pathways in Liver Diseases. Springer.
15. Faderl, Stefan H, and Hagop Kantarjian. 2011. Leukemias: Principles and Practice of Therapy. Wiley Online Library.