TO INVESTIGATE THE ROLE OF TGFβ-SMAD PATHWAY IN CHRONIC MYELOID LEUKEMIA

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CERTIFICATE

This is to certify that the research work embodied in this thesis titled ***"****To investigate the role of TGFβ-Smad pathway in Chronic Myeloid Leukemia****"*** has been carried out by Yogender Shokeen under the supervision of Prof. Dr.Neeta Raj Sharma and co-supervision of Dr. Vibha Taneja. This work is original and has not been submitted so far, in part or in full, for the award of any other degree or diploma of this or any other university.

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DECLARATION

This is to certify that the research work embodied in this thesis entitled *"To investigate the role of TGFβ-Smad pathway in Chronic Myeloid Leukemia"* has been carried out by me under the supervision of Dr. Neeta Raj Sharma, co-super vision of Dr. Vibha Taneja and mentorship of Dr. Shyam Aggarwal. This work is original and free from any form of falsification, fabrication and plagiarism. I shall be solely responsible for any such dispute arising out of my doctoral work.

Yogender Shokeen

Abbreviations

Abl	Ableson Murine Leukemia
ADAM17	A Disntegrin and Metalloproteinase-17
AEJ	Adenocarcinoma of Esophageal junction
AML	Acute Myeloid Leukemia
AP	Accelerated Phase
APOH	Apolipoprotein H
ARTS	Apoptosis Related Protein in the TGFβ Signaling
ATF-3	Activating Transcription Factor-3
ATP	Adenosine tri-phosphate
BCR	Break Point Cluster
BMP	Bone Morphogenetic proteins
BP	Blast Phase
BRGs	Bone related genes
C8A	Complement component 8-alpha
CA6	Carbonic Anhydrase 6
CCR	Complete Cytogenetic Response
CDK	Cyclin Dependent Kinase
CDKI	Cyclin Dependant Kinase Inhibitor
cDNA	Complimentary DNA
CEA	Carcino-embryonic antigen
CHR	Complete Hematological Response
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukemia
CMR	Complete Molecular Response
Co-Smad	Common Smad
CP-CML	Chronic Phase Chronic Myeloid Leukemia
CRC	Colorectal Cancer
CTGF	Connective Tissue Growth Factor
CYP2F1	Cytochrome P450 family 2 subfamily F member 1
DNA	Deoxy-ribose Nucleic Acid

ELISA	Enzyme Linked Immunosorbent Assay
ELN	European Leukemia Net
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen Receptor
FISH	Fluorescence in situ hybridization
FUT3	Fucosyltransferase 3
GIST	Gastrointestinal Stromal Tumors
HCC	Hepato-cellular carcinoma
HGMD	Human Gene Mutation Database
HLA	Human Leukocyte Antigen
HLA-DRB	Major Histocompatibility Complex Class II, DR Beta
HSC	Hematopoietic Stem Cells
HSE	Herpes Simples virus 1 Encephalitis
hTERT	Human Telomerase Reverse Transcriptase
IFNα	Interferon α
IL	Inter-leukine
IM	Imatinib Mesylate
I-Smad	Inhibitory Smad
KCNJ	Potassium Voltage-Gated Channel Subfamily J
MAC	Membrane Attack Complex
MCR	Major Cytogenetic Response
MFG-E8	Milk Fat Globule–Epidermal growth factor-8
MMP	Matrix Metalloproteas
MMR	Major Molecular Response
NGS	Next Generation Sequencing
NK	Natural Killer
NSCLC	Non-small Cell Lung Cancer
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase Chain Reaction
Ph	Philadelphia
PTH-rP	Parathyroid Hormone Related Protein

QC	Quality Control
RBC	Red Blood Cells
RCC	Renal Cell Carcinoma
RHPN2	Rhophilin Rho GTPase Binding Protein 2
RNA	Ribose Nucleic Acid
R-SMAD	Receptor Smad
RT-PCR	Real-Time Polymerase Chain Reaction
SCT	Stem Cell Transplant
SD	Standard Deviation
SNP	Single Nucleotide Polymosphism
TCF	Transcription Factor
TGF	Transforming Growth Factor
TGFβR	Transforming Growth Factor β Receptor
TKI	Tyrosine Kinase Inhibitor
UNC93B1	Unc-93 homolog B1
US	United States
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

Abstract

State of Purpose: Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder originating from bone marrow stem cells and resulting in uncontrolled proliferation of myeloid cells. The disease is diagnosed by the presence of Philadelphia (Ph) chromosome, which is formed due to translocation chromosome9 to chromosome22 bringing BCR and ABL gene in proximity, forming BCR-ABL fusion gene, responsible for uncontrolled Tyrosine kinase activity leading continuous cell proliferation. Recommended firstline treatment of CML is Tyrosine kinase inhibitor (TKI) imatinib mesylate, whose discovery has brought a paradigm shift in the cancer treatment. Despite commendable results, approximately ¼ patients show resistance to the Imatinib. Several mechanisms have been defined for drug resistance including role of BCR-ABL dependent and independent pathways. TGFβ-Smad pathway is an important BCR-ABL independent pathway, which has established role in many type of cancers, but still inconclusive in CML. There are no studies elaborating the reason of reduced tumor suppressive activity of TGFβ-Smad pathway despite elevated levels of TGFβ1 ligand, which is expected to increase the pathway function.

Hypothesis: We hypothesized that study of differential expression of key genes of this pathway along with exome sequencing of genes can provide substantial information about its activity in CML. Present study is focused on role of genetic alterations in TGFβ-Smad pathway and its affect on drug response. Apart from study of TGFβ-Smad pathway, we also emphasize on discovery of genetic variants, which can be used as susceptibility and prognostic biomarkers in CML.

Objectives:

Objective 1: To examine the differential expression of key genes of TGFβ-Smad pathway in Chronic Myeloid Leukemia

Objective 2: To discover genetic mutations in TGFB-Smad pathway genes and examine their association with Chronic Myeloid Leukemia.

Objective 3: To evaluate the role of Genetic markers in incidence of Chronic Myeloid Leukemia and response to Imatinib Mesylate.

Material and Methods: All patients (>18 years) diagnosed with CML were prospectively enrolled for a period of two years (October 2013-October 2015) at Department of Medical Oncology, Sir Ganga Ram Hospital, Delhi, India. Diagnosis was confirmed by reverse transcription polymerase chain reaction (RT-PCR) for *BCR-ABL* fusion gene and fluorescent in situ hybridization (FISH) for translocation (9; 22). Enrolled patients were segregated as responders and resistant as per European Leukemia Net, 2013 (ELN, 2013) recommendations. Healthy subjects with no known history of malignancy and above 18 years of age were enrolled as age-matched controls. The study was reviewed and approved by the Ethics Committee, Sir Ganga Ram Hospital, Delhi (EC No.: EC/11/12/439). Informed consent was signed and submitted by all subjects at the time of enrollment. The TGFβ1 serum levels between patients and healthy controls were measured using TGFB1 sandwich ELISA (DRG Instruments GmbH#EIA1864,

Marburg, Germany). The transcript level of TGFβR1, TGFβR2, SMAD4 and SMAD7 was examined by Real-Time PCR (Stratagene Mx3005P) using SYBRgreen chemistry (Applied Biosystem#43855612, Vilnius, Lithuania). ACTB (β-actin) was used as an endogenous gene. The raw data was analyzed manually by $2^{-\Delta Ct}$ method and the median of $2^{-\Delta Ct}$ was compared between patients and controls. Twenty patients and 5 controls were selected for Clinical Exome Sequencing using Next Generation Sequencing A targeted panel with probes covering all coding exons and essential splice sites for 4800 clinically significant genes including TGFβ1, TGFβR1, TGFβR2, SMAD4 and SMAD7 genes was used for sequencing these samples using Illumina's TruSight technology (Illumina, San Diego, USA). Statistical analyses were performed using SPSS, version 16.0

Results: TGFβ1 serum levels were significantly elevated ($p = 0.02$) and TGFβR2 and SMAD4 were significantly down regulated ($p = 0.012$ and $p = 0.043$ respectively) in the patients. c.69A>G in TGFβ1, c.1024+24G>A in TGFβR1 and g.46474746C>T in SMAD7 were the most important genetic variants observed with their presence in 10/20, 8/20 and 7/20 patients respectively. In addition, TGFβR1 transcript levels were reduced in CML patients with c.69A>G mutation. None of the genes differed significantly in terms of expression or genetic variants between responder and resistant patient groups.

Mutations in genes associated with cancer related functions were found in different patient groups. Four variants: rs116201358, rs4014596, rs52897880 and rs2274329 in C8A, UNC93B1, APOH and CA6 genes respectively, were present in IM responders; whereas rs4945 in MFGE8 was present in IM failures. Mutations in HLA-DRB1 (rs17878951), HLA-DRB5 (rs137863146), RPHN2 (rs193179333), CYP2F1 (rs116958555), KCNJ12 (rs76684759) and FUT3 (rs151218854) were present as susceptibility markers.

Conclusion: Our findings demonstrate the role of differential expression and genetic variants of TGFβ-Smad pathway in CML. Decreased *TGFβR2* and *SMAD4* levels observed in the present study may be responsible for reduced tumor suppressive effects of this pathway in CML. The potential genetic markers discovered in this study can help in predicting response to IM as frontline therapy and can be a potential tool for planning of personalized medicine. Susceptibility markers may also be used as panel for individuals prone to have CML.

Key Words: Chroni Myeloid Leukemia, TGFβ-Smad pathway, TGFβ1, TGFβR2, Smad4, Next Generation Sequencing, Prognostic markers, Susceptibility Markers.

Dedicated to:

MY BELOVED FATHER
AND
ALL CANCER PATIENTS

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CHAPTER 1:

INTRODUCTION

CHAPTER 1: INTRODUCTION

Cancer is a thought of fear and suffering, a burden which is carried by mankind from centuries. It is considered as lifestyle disease however genetic mutations are the main cause of cancer (Vogelstein B, 2002). Solid tumors and hematological malignancies are two major classification of cancer. Solid cancers are caused by tissue specific somatic mutations in particular organ, whereas hematological cancers are caused by mutations in bone marrow stem cells which result in continuous proliferation of particular cell type (Gavhane YS, 2011; Sant M, 2010). Leukemias are one of the well known categories of hematological malignancies, and Chronic myeloid leukemia (CML) being the best understood leukemia till date (Sant M, 2010). Prime cause of leukemogenesis in CML is formation of the Philadelphia (Ph) chromosome (Nowell PC, 2007), resulting from reciprocal translocation between long arms of chromosome9 and chromosome22 (9:22) (q34:q11) (Rowley JD, 1973). This translocation results in fusion of BCR (breakpoint cluster region) and ABL (Ableson) genes and considered as hallmark of CML (Kim DH, et al, 2009). BCR is responsible for the synthesis of Tyrosine kinase, whereas ABL is a proto-oncogene. The BCR-ABL fusion protein results in deregulated tyrosine kinase activity which activates several signal transduction pathways and leading to uncontrolled cell growth (Bollmann PW, 2011). It is a clonal myelo-proliferative disorder, characterized by enhanced proliferative capacity and prolonged survival of hematopoietic stem cells (HSCs), reduced apoptosis, and altered cell adhesion properties (Kim DH, et al.,2009). Incidence of CML in US is 1.5 per 100,000, and in India its incidence rate is 0.8-2.2 and 0.6 to 1.6 in men and women respectively (Au WY, et al, 2009; Kumar L, et al, 2006).

The standard treatment for CML is Imatinib mesylate, a 2-phenylaminopyrimidine derivative, which specifically inhibits BCR-ABL tyrosine kinase activity. Imatinib binds with ATP binding site of BCR-ABL tyrosine kinase and stabilize a non-ATP-binding form of BCR-ABL, thereby preventing tyrosine autophosphorylation. This process ultimately results in "switching-off" the downstream signaling pathways involved in leukemogenesis (Marcucci G, et al, 2003). However, emergence of resistance to this drug is a major problem (Melo JV, et al, 2007) because approximately 25% of patients, from different stages of CML, show resistance to Imatinib mesylate. The reason for resistance is a number or genetic alterations acquired by CML progenitor cells due to decreased genomic stability in the course of disease progression (Marcucci G, et al, 2003). The role of BCR-ABL dependent and independent pathways shown to equally involved in Imatinib resistance (Hamad A, et al, 2013). Study of these pathways is essential to understand the course of resistance and search for alternative therapies accordingly.

TGFβ-Smad signaling is an important BCR-ABL independent pathway, with established role in many types of cancer, but with scarce data in CML. This pathway is essential regulator of cellular proliferation, differentiation, apoptosis, extracellular matrix remodelling of the cell, angiogenesis and inflammation. The TGFβ super family consists of more than 30 related members in mammals, including three kinds of TGFβs, 4 kinds of activins and over 20 kinds of bone morphogenetic proteins (BMPs). Subversion of TGFβ family signaling has been implicated in various human diseases including autoimmune

disease, vascular disorders and cancer (Su E, et al, 2010). Main components of TGFβ-Smad pathway are TGFβ1 protein, which is a ligand, TGFβR1 and TGFβR2, are the two ligand receptors, Smad 2 and Smad3 are the receptor regulated Smad (R-Smad), Smad4 is the common mediator Smad (co-Smad), whereas Smad 6 and Smad 7 acts as inhibitory Smad (I-Smad) (Su E, et al, 2010). Binding of extracellular TGFβ1 protein to the TGFβ type2 receptor on the cell surface initiates the signaling pathway dimerize type 1 receptor, which recruits and phosphorylates Smad2 and Smad3. Activated Smad2-Smad3 complex recruits Smad4, which forms higher order complex and translocate into the nucleus where they act as transcription factors. Inhibitory smads, Smad6 and Smad7, have suppressive effects on the TGFβ-Smad signaling pathway by interrupting the actions of R-Smad and Co-Smad (Su E, et al, 2010).

Boris Pasche, et al, showed various roles of this pathway in the process of malignant progression (Pasche B, et al, 2001). There are growing evidences, that in earlier stages pathway acts as tumor suppressor, but in later stages of cancer development, it contributes to cell growth, invasion, and metastasis and decreases host tumor immune responses (Pasche B, et al, 2001). Subtle alteration of TGFβ signaling may also contribute to the development of cancer. Higher levels of TGFβ have been observed in hematological malignancies and solid tumors (Liu X, et al, 2013; Choi YJ, et al, 2015). TGFβ is known to effect development and/or proliferation of cancerous cells in genitor-urinary cancers and gynecological malignancies (Pasche B, et al., 2001). Genetic and expression alteration of essential components of this pathway are reported to be associated with different malignancies (Su E, et al, 2010). Mutations and differential expression in TGFβR1 and TGFβR2 are linked to many types of cancers. Genetic variant, c.1024+24G>A (rs334354) in intron 6 of TGFβR1 is an established genetic marker for increased susceptibility for cancer (Liu X, et al, 2013, ; Wu W, et al, 2015). Single nucleotide polymorphisms (SNPs) in these two genes are associated with susceptibility of breast cancer, colorectal cancer, gastric cancer (Scollen S, et al., 2011; Xu Y, et al., 2007; Jin G, et al., 2007). TGFβR1 gene is significantly more frequent in patients with RCC and transitional cell carcinoma of bladder (Chen T, et al, 2004). In CML, microRNAs which are targeted to TGFβR2, show differential expression as compared to the control population (Polakova KM, et al, 2011). Smad4 mutations are known to play important role in different types of solid malignancies (Miyaki M, et al., 2006; Schutte M, et al., 1999). Resistance to homeostatic effects of TGFβ are demonstrated in hematological malignancies, though in some of these malignancies, elevated levels of TGFβ are known to promote myelofibrosis and pathogenesis through their effect on the stroma and immune system (Dong M, et al., 2006). In hematological malignancies, it has been shown that absence of Smad 4 is involved in the AML (Wierenga ATJ, et al., 2002). SNPs and expression levels of this gene are found to be associated with susceptibility and prognosis of various cancers (Yin J, et al., 2011; Mangone FR, et al., 2010; Singh P, et al.,2011; Osawa H, et al., 2004; Kawate S, et al., 2001; Jeon HS, et al., 2008).

In CML, the BCR-ABL protein targets this pathway indirectly through Akt and transcription factor Foxo3 to impair the cytostatic effect of TGFβ1 (Dong M, et al., 2006). However it has also been reported that BCR-ABL expressing cells are more susceptible to TGFβ-SMAD induced growth inhibition. How TGFβ-SMAD influence CML cell proliferation in the context of BCR-ABL expression is not well established

(Dong M, et al., 2006). Lack of direct interaction between BCR-ABL and TGFβ-Smad pathway, despite being important factor in CML, makes it even more interesting for the research. Moreover, to the best of our knowledge no pertinent literature is available till date on the role of differential expression and mutations in the genes of this pathway with susceptibility or prognosis of CML.

CHAPTER 2:

REVIEW OF LITERATURE

CHAPTER 2: REVIEW OF LITERATURE

2.1. Cancer

Cancer is a disease caused due to occurrence of genetic changes called "mutations" in a set of cancer susceptibility genes, although environmental and other nongenetic factors play roles in many stages of tumorigenesis (Vogelstein B, 2002). In normal functioning, these genes are the key components of the pathways which regulate cellular growth and differentiation. Mutation in this group of genes, commonly known as oncogenes and tumor suppressor genes, can lead to genetic instability, accumulating changes in other genes that directly control cell birth and death. Equilibrium between cells birth and cell is disturbed and shifted towards uncontrolled cell proliferation in cancer (Rajgopalan, 2003; Seiber, 2003). These unwanted and uncontrolled cells encroach the spaces meant for other normal cells and hence growth of these cells results in disruption of normal functioning. Accumulation of these cells leads to the formation of malignant (cancerous) tumors (Bissel, 2001). Cancer can be broadly classified into two different categories based on tissue of origin of cancer. Malignancies arising from epithelial cells, mesenchymal cells outside bone marrow and bone, neurons, nerve, fat or other connective tissues are considered as solid tumors (Gavhane YS, 2011). As per World Health Organization indications, cancers of blood are classified into myeloid and lymphoid malignancies (Sant M, 2010).

2.2. Hematological Malignancies

Both lineage groups of Hematological malignancies are further subdivided into following subtypes:

Lymphoid malignancies are grouped into 5 major categories: Hodgkins Lymphoma (HM), mature B-cell lymphoma, mature T-cell and natural killer cell neoplasms (T-NK), lymphoblastic lymphoma or acute (precursor cell) lymphatic leukemia (LL/ALL). These groups are further subdivided according to lineage, again in accord with WHO and HAEMACARE (Sant M, 2010).

Myeloid malignancies were classified into 5 large categories: acute myeloid leukemia (AML), myeloproliferative neoplasms, myelodysplastic syndrome and myelodysplastic/ myeloproliferative leukemias. Myeloproliferative malignancies were subdivided into chronic myeloid leukemia (CML) and other morphologic subgroups (other myeloproliferative neoplasms) based on WHO recommendations (Sant M, 2010). Our study is based on chronic myeloid leukemia.

2.3. Chronic Myeloid Leukemia (CML)

Incidence of Chronic Myeloid Leukemia in United States is 1.6 per 100,000 of population, accounting approximately 5,430 cases annually. The estimated prevalence in US is around 25,000–30,000, which is expected to reach around 105,000 by year 2030 (Huang X, 2012). In India is incidence of CML is ranging 0.8–2.2 per 100,000 per annum (Prasad RR, 2013). According to the current estimate, world is expected to have 100,000 newly diagnosed CML patients per year, leading this chronic disorder into a serious

health problem (Leitner AA, 2011). CML is a clonal malignancy originating from hematopoietic stem cells which ultimately leads to increased numbers of myelocytes, erythrocytes and thrombocytes in the blood resulting in encroached space for other blood cells and disrupted normal functions (Sawyers, 1999; Reya T, et al, 2001). Approximately 95% of CML cases are known to have Philadelphia Chromosome (Ph) and remaining 5% have other genetic variants. Ph chromosome was first discovered by two scientists, Peter Nowell and David Hungerford in 1960 at Philadelphia, Pennysylvania, USA (Nowell PC, 2007). This chromosome was first mentioned as an unique shortened chromosome (Nowell PC, 1997), which was later described as t(9;22) (translocation from chromosome9 to chromosome22) and presently considered as molecular hallmark of CML (Rowley JD, 1973). Presence of this unique chromosome in myeloid, erythoid, B lymphoid lineages and megakaryocytic cells prove that this genetic change is not restricted to just one form of hematopoietic lineage, providing clear indication that this abnormality happens in bone marrow stem cells (Reya T, et al, 2001). Philadelphia(Ph) chromosome is a consequence of balanced translocation, bringing ABL (Abelson Murine Leukemia) gene from chromosome9 in proximity of BCR (Breakpoint Cluster Region) on chromosome22. The fusion gene so formed codes for abnormal protein having continuously activated tyrosine kinase activity responsible for the development of leukemia (Bollmann PW, 2011).

2.3a.ABL Gene

The ABL gene is the human homologue of the v-*abl* oncogene carried by the Abelson murine leukemia virus (A-MuLV). It encodes a non-receptor tyrosine kinase of 145 kd with two isoforms resulting from alternative splicing of the first exon and expressed ubiquitously. At its NH2 terminus, three SRC homology domains (SH1-SH3) are located. The main function of tyrosine kinase activity is attributed to SH1 domain, whereas interaction with other proteins takes place through SH2 and SH3 domains (Figure 1). Normally, Abl protein is involved in variety of functions including regulation of cell cycle, cellular response to genotoxic stress and information transmission through integrin signaling. As evident, ABL protein perform complicated roles to modulate cellular signals from outside and inside sources which effects apoptosis and hence cell cycle (Deninger MWN, et al, 2000).

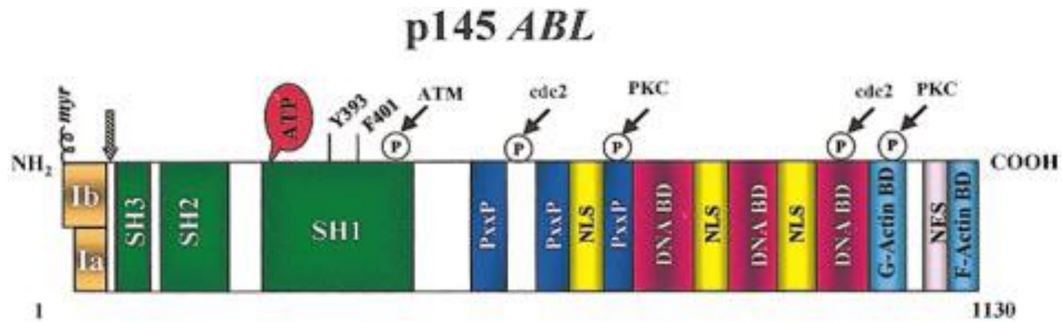


Figure 1: Structure of the Abl protein. Type Ia isoform is slightly shorter than type Ib, which contains a myristoylation (myr) site for attachment to the plasma membrane.Note the 3 SRC-homology (SH) domains situated toward the NH2 terminus.Y393 is the major site of autophosphorylation within the kinase domain, and phenylalanine 401 (F401) is highly conserved in PTKs containing SH3 domains. The middle of each protein is dominated by proline-rich regions (PxxP) capable of binding to SH3 domains, and it harbors 1 of 3 nuclear localization signals (NLS). The carboxy terminus contains DNA as well as G- and F-actin–binding domains. Phosphorylation sites by Atm, cdc2, and PKC are shown. The arrowhead indicates the position of the breakpoint in the Bcr-Abl fusion protein (Source: Deninger MWN, et al, 2000)

2.3b. BCR Gene

Similar to ABL, BCR is also expressed ubiquitously. It is 160 kd large gene, with N-terminal exon coding for serine-threonine kinase. Bap-1 is the only known substrate of this kinase. Pleckstrin-homology (PH) domain is situated at centre of the molecule, which stimulates the de-phosphorylation of GTP (Guanidine triphosphate) to GDP (Guanidine diphosphate) with assistance from Rho guanidine exchange factor, activating transcription factor like NF-kb. The GTPse activity of C-terminus regulates actin polymerization and NADPH oxidase activity in phagocytic cells through activating Rac, which is a GTPase of Ras superfamily. Another key component of Ras signaling, Grb-2 (key adapter molecule for activating Ras pathway), also binds to tyrosine 177 of phosphorylated BCR (Deninger MWN, et al, 2000) (Figure 2).

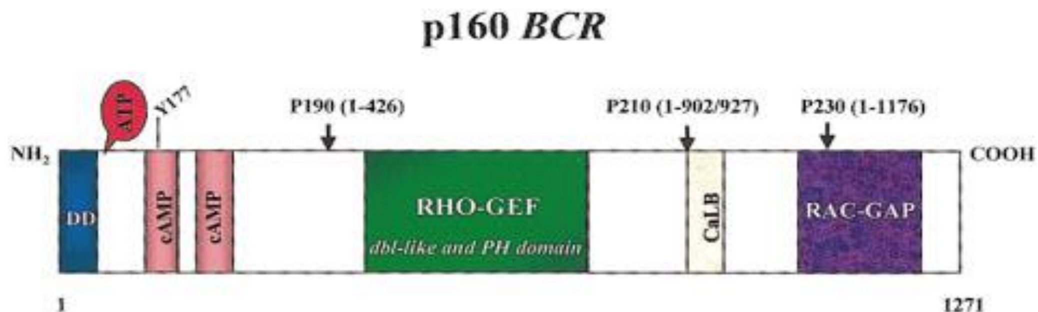


Figure 2; Structure of Bcr protein. Note the dimerization domain (DD) and the 2 cyclic adenosine monophosphate kinase homologous domains at the N terminus. Y177 is the autophosphorylation site crucial for binding to Grb-2. The center of the molecule contains a region homologous to Rho guanidine nucleotide exchange factors (Rho-GEF) as well as dbl-like and pleckstrin homology (PH) domains. Toward the C-terminus a putative site for calcium-dependent lipid binding (CaLB) and adomain with activating function for Rac-GTPase (Rac-GAP) are found. Arrowheads indicate the position of the breakpoints in the BCR-ABL fusion proteins (Source: Deninger MWN, et al, 2000).

2.3c. BCR-ABL Gene

ABL gene is located on chromosome9 at q34 locus, with the size of 300kb. For the formation of BCR-ABL gene, the breakpoints in ABL gene mostly occur between first alternative exon1a and second alternative exon1b, though it can also happen either upstream of 1b or downstream of 1a. Irrespective of the locus of breakpoint on abl gene, fusion with BCR takes places only at exon2a (Figure 3). Unlike ABL, breakpoints in BCR gene occur between exon b1and b5 (also called exon 12 and exon 16) in the so called Major breakpoint region (M-bcr), which is 5.8 kb in size.

In rare cases of CML, the breakpoint can occur between e2' and e2, in the 54.4 kb region known as misnor breakpoint cluster region (m-bcr). When the splicing takes place in M-bcr region, it forms the fusion transcript with either b2a2 or b3a2 junctions, producing 210 kd chimeric protein (P210BCR-ABL), whereas splicing in m-bcr region forms e1a2 transcript which produces 190 kd protein (P190BCR-ABL). Occasionally, other transcripts such as b2a3, b3a3, e1a3, e6a2, or e2a2, have also been observed in CML patients (Scheijen B, et al, 2002).

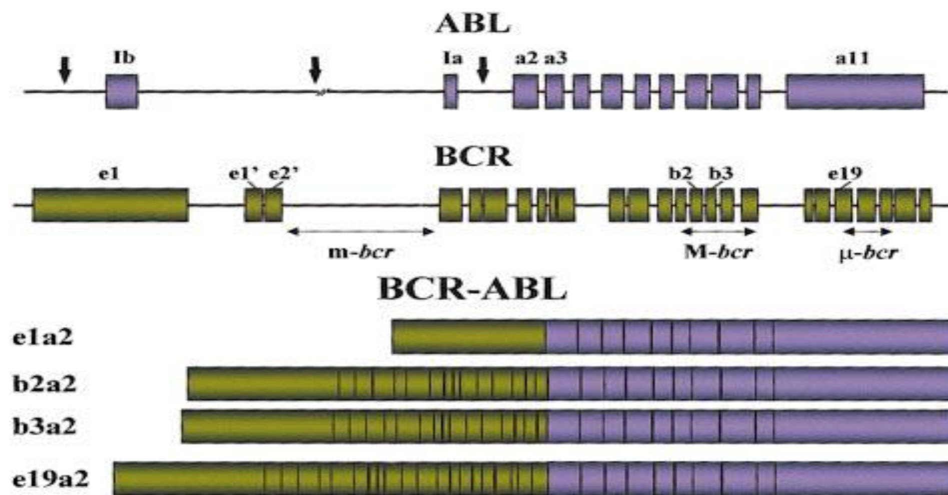


Figure 3: **Structure of BCR-ABL fusion gene:** Locus of breakpoint in ABL and BCR genes along with resultant BCR-ABL transcripts (Source: Gishizky M, et al, 1992).

The BCR-ABL gene, once formed, codes a constantly active cytoplasmic tyrosine kinase, activating growth and differentiation pathways in hematopoietic cells. It deviates RAS, RAF, MYC, JUN, STAT and PI3K129 pathways from normal functioning. BCR-ABL initiates a process to transform hematopoietic cells such that their growth and survival becomes independent of cytokines (Gishizky M, et al, 1992).

2.3d. Phases of CML

Phases of CML can be defined according to recommendation of WHO (World Health Organization) (Vardiman JW, 2008) and ELN (European Leukemia Net). Following table elaborates the criteria declared by both agencies for CML staging (Table1):

Accelerated Phase	**Definition**
ELN Criteria	Blasts in blood or marrow 15-29%, or blasts plus promyelocytes in blood or marrow >30% , with blasts < 30%, Basophils in blood ≥ 20% Persistent thrombocytopenia (Platelets < 100 x 10^9/L) unrelated to therapy Clonal chromosome abnormalities in Ph+ cells (CCA/Ph+), major route, on treatment
WHO Criteria	Blasts in blood or marrow 10-19% Basophils in blood ≥ 20% Persistent thrombocytopenia (< 100 x 10^9/L) unrelated to therapy CCA/Ph+ on treatment Thrombocytosis (>1000 x 10^9/L) unresponsive to therapy, Increasing spleen size and increasing white blood cell count unresponsive to therapy
Blast Phase	
ELN Criteria	Blasts in blood or marrow ≥ 30% Extramedullary blast proliferation, apart from Spleen
WHO Criteria	Blasts in blood or marrow ≥ 20% Extramedullary blast proliferation, apart from spleen, Large foci or clusters of blasts in the bone marrow Biopsy

Table1: Clinical and hematologic criteria for the definition of AP and BP according to WHO and ELN (Source: Baccarani M, et al, 2013).

2.3e. Symptoms and Diagnosis of CML

Symptoms for CML includes weight loss, asthenia, small fever, sweats, and malaise, though they are not specific or frequent, as approximately half of the patients are fortuitously diagnosed by abnormal blood counts accompanied by splenomegaly. Leukocytosis with basophilia and immature granulocytes, mainly metamyelocytes, myelocytes and promyelocytes, and few or occasional myeloblasts, along with frequent Thromobocytosis almost confirms the CML. Phase classification can be done by blood counts and differential counts (Table1) (Vardiman JW, et al, 2008; Baccarani M, et al, 2006; Baccarani M, et al, 2009). Final confirmation of CML is done by cytogenetics showing t(9; 22)(q34;q11) and reverse transcriptase polymerase chain reaction (RT-PCR)

showing BCR-ABL transcripts. Chromosome banding analysis (CBA) should be performed for cytogenetics of bone marrow cell metaphases, as it is must for detection of additional chromosomal abnormalities (Hook EB, 1977; Shaffer L, et al, 2009). In case of unavailability of bone marrow cells, interphase fluorescence in situ hybridization (I-FISH) of blood cells, using dual color dual fusion probes is advised for BCR-ABL+ nuclei. Qualitative RT-PCR on RNA of fresh BM of blood cells can be done for identification of BCR-ABL transcript type (Testoni N, et al, 2009).

2.3f. Treatments of CML

Recommended treatment for chronic phase CML (CP-CML) in firstline settings are the TKIs Imatinib mesylate (400 mg once daily), Nilotinib (300 mg twice daily), and Dasatinib (100 mg once daily). When the TKI fails in firstline setting, other TKIs or dose escalation of same drug are the recommended options for second or subsequent lines. Bosutinib (500 mg once daily) and Ponatinib (45 mg once daily) are also approved for patients who failed on prior TKIs medications. Omacetaxine, a non-TKI drug is also available in US for TKI failed patients (Cortes J et al, 2012; Cortes J, et al, 2013). Hydroxyurea can only be used for a brief time period before beginning a TKI to bring down elevated TLCs. rIFNα can be prescribed in a rare case where TKI should be avoided, whereas combination of TKIs and rIFNα can be potentially useful but it is still under investigation (Talpaz M, et al, 2013). Cytotoxic chemotherapies are prohibited in CML except the situation when patient has to be prepared to allogenic stem cell transplant (alloSCT). AlloSCT still remain as an option for the patients in advanced stages and has been repeatedly failed to TKIs (Jiang Q, et al, 2011).

Despite availability of all above options for CML treatment, Imatinib Mesylate (400mg once a day) is the preferred choice as firstline treatment for the clinicians throughout the world due to its availability, price, experience and proven data. Resistance to IM is still an important topic for research investigation, which is also the focus of our study.

2.3g. Imatinib Mesylate

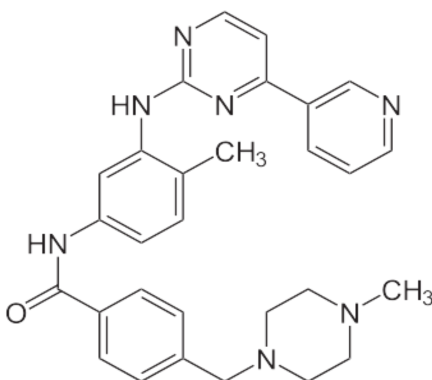


Figure 4; Molecular diagram of Imatinib mesylate (Source: Panigrahi A, et al, 2006)

Discovered in 1990s, Imatinib, is a phenylaminopyridin derivative with chemical formula C29H31N7O.CH4SO3, has specific activity for ABL, platelet derived growth factor receptor, c-kit, and Albeson-related gene. During development it was called STI571, where STI stands for signal transducer inhibitor which reflects its function as tyrosine kinase inhibitor. It is marketed in generic form called Imatinib mesylate (IM) and brand name Gleevec (USA) or Glivec (Europe/Australia). Currently IM is the first line therapy for Chronic Myeloid Leukemia and also used for treatment in Ph+ gastrointestinal stromal tumors (GISTs) and Ph+ Acute Myeloid Leukemia (AML) (Panigrahi A, 2006).

Imatinib mesylate blocks BCR-ABL tyrosine kinase ATP binding site, which stabilizes the non-ATP binding form of BCR-ABL, restricting autophosphorylation of tyrosine and its substrates (Figure 5). Its action ultimately results in stopping the signaling pathways responsible for leukemogenesis (Druker, BJ, 2003).

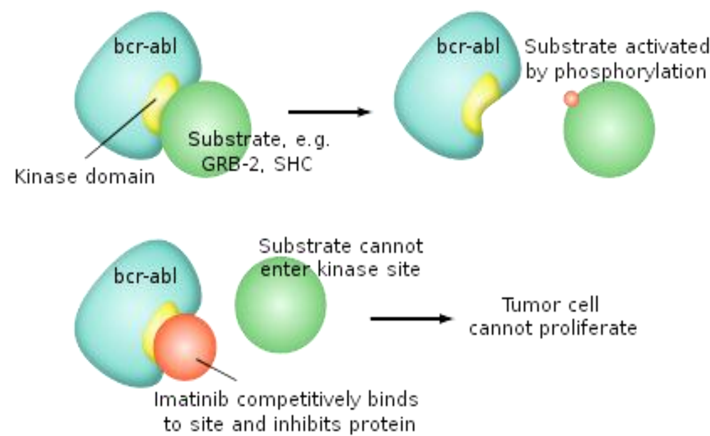


Figure 5: Mechanism of action of Imatinib (Source: Druker,BJ, 2003)

2.3h. Resistance

Imatinib mesylate has been proved an excellent therapeutic option, but still a significant proportion of patients show resistance at a rate of 80% in BP, 40–50% in AP, and 10% in CP after at 2 years of treatment (Kantarjian H, et al, 2002). IM resistant CML patients can be classified as patient showing primary resistance or secondary resistance, where primary resistant patients are most frequently in Blast phase or accelerated phase, and secondary resistant group include patients who relapse after responding to IM treatment initially, mostly in Chronic phase. The resistance process can also be described as BCR-ABL dependent or BCR-ABL independent resistance (Gorre ME, et al, 2002; Hamad A, et al,2013).

There are several BCR-ABL dependent molecular process defined for IM resistance. BCR-ABL amplification and its increased expression is reported to be associated disease progression to blast crisis, as it is important for proliferation and survival of CML blast cells. In the cells obtained from blast crisis CML patients, where high levels of BCR-ABL are observed, it suppresses the differentiation of myeloid cells and chemotherapy

induced apoptosis. This correlation of BCR-ABL amplification with disease correlation is an important factor for IM resistance (Elmaagacli AH, 2000; Perrotti D, et al, 2002). Though mechanism responsible for this overexpression is still not clear, but in some cases double Ph chromosome was found, which can be a probable reason for higher BCR-ABL levels.

BCR-ABL gene mutations, occurring during the course of CML, are another mechanism of IM resistance. Many mutations in this hallmark gene has been detected, and reported responsible for drug resistance. Most of these mutations are observed in at least 13 amino acids of the ATP-binding site or other regions of the tyrosine kinase domain (Gambacorti-Passerini CB, et al, 2003; Hochhaus A, et al, 2002). These mutations change the conformation of Imatinib binding site, resulting in prevention of continuous tyrosine kinase activity from blockade. Mutation at position 315, substituting threonine to isoleucine, is the most prominent and frequent known mutation responsible for resistance (Gorre ME, et al, 2002).

Another BCR-ABL dependent mechanism of Imatinib resistance is binding of alpha1-acid glycoprotein (AGP) present in serum with physiological concentration of drug, which restricts the ability of drug to control tyrosine kinase activity of BCR-ABL in dose dependent manner (Gambacorti-Passerini CB, et al, 2003).

Apart from BCR-ABL dependent mechanism of resistance, BCR-ABL independent mechanisms also play equally important role in the process. Acquisition of cytogenetic changes, other than formation of Ph chromosome, marks the genomic instability caused due to additional hits and in turn activates BCR-ABL independent mechanism. Patients with simpler karyotype are least expected to be resistant, whereas more complicated karyotypes signal towards high chances of IM resistance. Activation of Bcr-Abl–independent Src Family Kinases (SFK) plays important role in Bcr-Abl–independent mechanism of imatinib resistance (Donato NJ, et al, 2003; Wu J, et al, 2008; Wu J, et al, (Blood), 2008). SFK pathway, along with other cross-talk pathways, activates the networks which facilitates the resistance. Recent studies clearly demonstrate activity of antisense RNA for Lyn, significantly restoring Imatinib susceptibility (Ptasznik A, et al, 2004). Elevated levels of Lyn kinase have been linked to levels of another cytokine, TGFβ1, which are associated with Imatinib resistance in CML (Smith PG, 2012). TGFβ1 activates several pathways including an important tumor suppressor pathway TGFβ-Smad pathway. This pathway shares a controversial connection with BCR-ABL. TGFβ-Smad signaling is known to increase the hyper-responsiveness of CML cells leading to better response through BCR-ABL inhibition (Moller GM, 2007). Though, this pathway inhibits the activation of AKT, which is a downstream component of BCR-ABL pathway, leading to release of inhibitory sequestration of FOXO that promote quiescence in CML stem cells, ultimately resulting in TKI resistance (Zhu X et al, 2011; Naka K et al, 2010). We, in the present study, elaborated the relation between TGFβ-Smad signaling and CML as an example of BCR-ABL independent pathways, and evaluated its significance in Imatinib resistance as well.

Identification of the molecular basis of resistance is important, because it could provide insight into disease progression and into the design of novel therapeutic strategies to prevent and overcome treatment resistance. We, in our research, also focused on

identification of potential susceptibility markers for CML and prognostic markers for Imatinib resistance in later part of the study.

2.4. TGFβ-Smad pathway

TGFs were first discovered as growth factors in 1978, when they were extracted from murine sarcoma virus transformed cells (De Larco, et al, 1978; Roberts AB, et al, 1980). Initially they were referred to as sarcoma growth factors, but later, segregated as different family with similar properties. The family was named so as they were secreted by transformed cells and had the ability to transform untransformed cells by inducing soft agar colony growth. Later, both classes of TGFs, TGFα and TGFβ, were isolated from sarcoma virus transformed cells (Anzano MA, et al, 1983). Among TGFβ family, 3 TGFβs, namely TGFβ1, 2 and 3, are known in mammals till date. Despite being 3 members, TGFβ1 is normally referred to as TGFβ, being the most common cytokine of this family (Schmierer B, et al, 2007).

The name of TGFβ was derived from its capacity to induce growth of kidney cells in soft agar, though later it was discovered to have multifunctional activities including growth inhibition (Shipley GD, et al, 1986; Roberts AB, 1998). It is involved in various cellular functions like cell proliferation, differentiation, apoptosis, adhesion, cell migration and extracellular matrix protein production. These multifunctional properties of TGFβ are demonstrated by loss of function studies of these ligands in mice model, which proved their role during embryogenesis and in maintaining homeostasis during their adult life (Goumans MJ, et al, 2000). Immature pleuripotent cells like activated B and T cells, dendritic cells, neutrophils, macrophages, etc. either produce TGFβ or are sensitive to its response (Letterio JJ, 1998; Lebman DA, 1999)

Impaired TGFβ signaling pathway has been found to impact many human disorders such as vascular disorders, autoimmune diseases, and cancer (Blobe GC, et al, 2000). Main components of TGFβ-Smad pathway are TGFβ1 protein, which is a ligand, TGFβR1 and TGFβR2, are the two ligand receptors, Smad2 and Smad3 are the receptor regulated Smad (R-Smad), Smad4 is the common mediator Smad (co-Smad) and Smad6 and Smad7 acts as inhibitory Smads (i-Smads).

2.4a. TGFβ1

TGFβ1 is normally present in extracellular matrix, where it is present as an element of large latent complex, secreted as a dimer and present in biologically inactive form. It is attached to Latency associated protein (LAP) by non covalent bond, which is re-attached to TGFβ binding protein (Miyazono,K et al, 1988; Wakefield, L.M et al, 1988; Miyazono,K 1991). It is activated by BMP-1, which cleaves off TGFβ-binding protein from the complex and later the LAP is cleaved by metalloproteinases (MMPs)**.** The nude TGFβ, present in extracellular matrix is ready to attach to the receptor and activate the signaling pathway (Yu Q, 2000; D'Angelo M, 2001).

2.4b. TGFβ Receptors (Type I and Type II): Structure and Function

The signaling is initiated by binding of TGFβ to receptor type 2 (TGFβR2), which in turn phosphorylate and recruit type I receptor (TGFβR1). TGFβR1 later recruits further downstream components of the pathway.

As TGFβ binds to the receptor2, it initiates the formation of heterotetrameric complex of both transmembrane receptors. The receptors have cysteine residue rich N-glycosylated extracellular domain, where as intracellular domain is composed of serine/threonine kinase. Upon phosphorylation of TGFβR1, by TGFβR2, changes the confirmation of glycine and serine/threonine rich intracellular domain (GS domain) and activates its kinase. TGFβR1, acting downstream of TGFβR2, further activate intracellular Smad signaling (Figure 6) (Massagué J, 1998; Derynck R, et al, 1997).

Apart from TGFβR1 and TGFβR2, TGFβ can also bind to betaglycan, also termed as TGFβR3 or endoglin. These so called type 3 receptors do not have any intracellular enzymatic activity and how do they facilitate the flow of signaling pathway is also not well known (López-Casillas F, 1993; Cheifetz S, 1992).

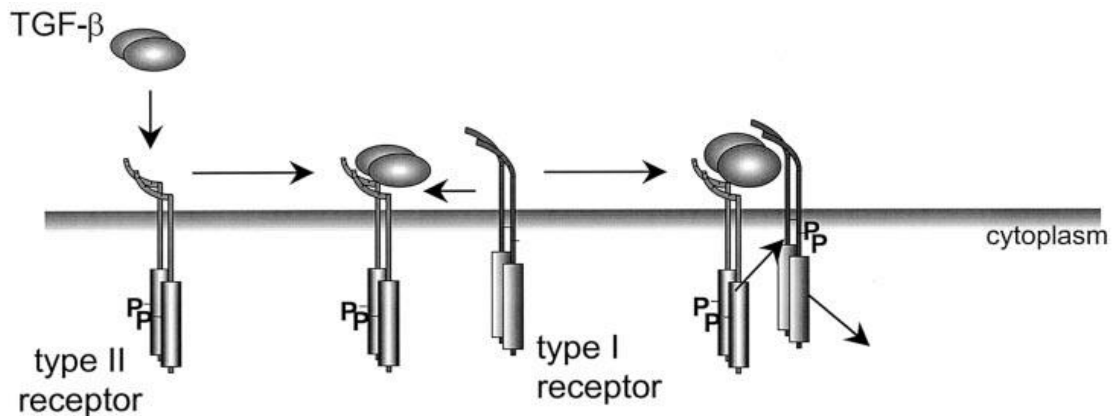


Figure 6: Activation of TGFβRs: Ligand bind initially to TGFβR2. This can be recognized by TGFβR1, which is then recruited into complex. Subsequently, the constitutively active TGFβR2 kinase phosphorylates and activates the TGFβR1, which progresses the signal downstream. (Source: Dennler S, et al, 2002)

2.4c. Intracellular Signalling: Formations and Activity of SMADs

After activation, intracellular signaling is initiated by TGFβR1 by phosporylating Smad proteins (Itoh S, et al, 2000). Smad proteins are composed of two conserved Mad homology (MH) domains 1 and 2, at N-terminal and C-terminal respectively. The domains are combined by proline rich bridge of variable length. There are 3 different division of Smad protein classified on the basis of their functions: receptor Smads (R-Smads), common Smad (Co-Smad) and inhibitory Smad (I-Smad). MH2 domain of R-Smad, which is present in cytoplasm, gets phosphorylated at C-terminal serine residues by TGFβR1 (Abdollah S, et al, 1997; Souchelnytskyi S, et al, 1997). Following

activation, it forms a hetermomeric complex with Co-Smad, Smad4, via MH2 domains, translocates and accumulates in nucleus. This complex of R-Smads and Co-Smad expresses the target genes with the help of transcription factors. I-Smads, Smad6 and 7, controls the activity of signal transduction by restricting the activation of R-Smads and Co-smad, by competitively binding to activated TGFβR-I (Figure 7) (Imamura T, et al, 1997; Nakao A, 1997).

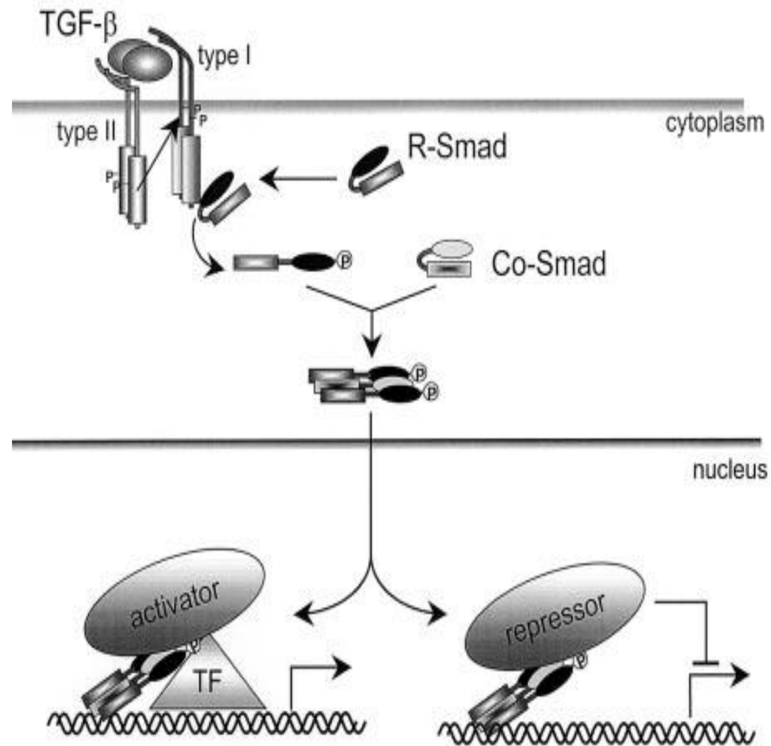


Figure 7: Smad activation: Upon ligand induced heteromeric complex formation and activation of TGFβR1 kinase, R-Smads are phosphorylated. These activated R-Smads form heteromeric complex with Co-Smad and accumulate in the nucleus. Together with coactivators (p300, CBP, P/CAF) and transcription factors and these transcription factors, these Smad complexes participate in transcriptional regulation of target genes. (Source: Dennler S, et al, 2002)

Communication between Smad proteins occur through protein-DNA and protein-protein interactions through their MH domains, as they lack intrinsic enzymatic activities. Domains of R-Smads have attraction towards each other, which is the force behind intrinsic affinity towards each other and control each other's function (Hata A, et al, 1997). C-terminal serine of R-Smads phosphorylation is triggered due to ligand activity, which allows its interaction with DNA and other proteins. MH1 domains of Smad3 and Smad4 interact directly with Smad-binding elements (SBE), which is a 5' GTCT DNA sequence (Zawel L, et al, 1998; Dennler S, et al, 1998; Shi Y, et al, 1998). Smad3 and Smad4 interact directly due to protruding hairpin loop like structure formed by combination of MH1 domain and SBE. Structure of Smad2 revealed an extra loop in close proximity to the putative DNA binding motif, which interfere and avoid its direct

interaction to Smad4 through DNA binding (Shi Y, et al, 1998; Yagi K, et al, 1999). Both Smad3 and Smad4 have the ability to bind directly GC-rich DNA sequence, which provide them opportunity to bind with ligand fused heterologous DNA binding domain and attain transcriptional activity (Liu F, et al, 1996). MH2 domains have the ability to recruit transcription factors like CBP/p300 or P/CAF in ligand dependent manner, which possess intrinsic histone acetyltransferase (HAT) activity. Lysine residues in the N-terminal tails of histones were acetylated, facilitating gene activation, which reduce histone attraction towards DNA and encourage binding of transcription factors to nucleosomal DNA. Presence of elevated levels of transcription factors are known to increase Smad-dependant transcription (Shen X, et al, 1998).

2.4d. TGFβ-Smad in Cancers: Different roles, Different ways

As TGFβ pathway is central in many key cellular functions and its alterations have been noted in human tumors. These alterations can be at genetic level, expression level or protein level, making its impact on activity of pathway. Alternatively, it can be due to the amplification or overexpression of inhibitors of the pathways. TGFβ pathway is known to play dual role in cancer, as they act as tumor suppressor in initial stages of cancer and as tumor promoter in advanced stages. Deregulation of this pathway has been involved in colon, pancreas, breast, lung and prostate.

There are numerous mechanisms by which TGFβ-Smad pathway performs its functions in cancers (Samanta D, et al, 2015). In hepatocellular carcinoma, TGFβ-Smad pathway forms a positive loop with c-kit for tumor progression, disruption of which promots tumor supressive function of TGFβ-Smad pathway, indication the loop between the two signalling pathways as a potential therapeutic target (Rojas A, et al, 2016). The process of bone metastasis in breast cancer is induced by dynamic antgonism mediated by RUNX2 between TGFβ-Smad and BMP-Smad signaling pathways through elevating expression of some bone related genes (BRGs) (Tan CC, et al, 2016), whereas in case of adenocarcinoma of Esophageal junction (AEJ), TGFβ is abnormally activated as most genes of this patheway shows either over expression or underexpression in the patient tissues (Peng D, et al, 2016). Genetic variant, c.1024+24G>A (rs334354) in intron 6 of TGFβR1 is an established genetic marker for increased susceptibility for cancer (Liu X, et al, 2013, ; Wu W, et al, 2015). Differential levels of TGFβ1 have been tumors of different origins (Liu X, et al, 2013). In colorectal cancer cells, CD51 was considered a functional marker and a potential thereupetic target, as it was found to upregulate TGFβ signalling pathway by binding to TGFβ receptors and levels of SMAD4 are considered as important prognostic marker (Wang J, et al, 2017; Liu L , et al, 2013). In hepatocellular carcinoma, TGFβ-Smad pathway forms a positive loop with c-kit for tumor progression, disruption of which promots tumor supressive function of TGFβ-Smad pathway, indication the loop between the two signalling pathways as a potential therapeutic target (Rojas A, et al, 2016). The process of bone metastasis in breast cancer is induced by dynamic antgonism mediated by RUNX2 between TGFβ-Smad and BMP-Smad signaling pathways through elevating expression of some bone related genes (BRGs) (Tan CC, et al, 2016), whereas in case of adenocarcinoma of Esophageal junction (AEJ), TGFβ is abnormally activated as most genes of this patheway shows either over expression or underexpression in the patient tissues (Peng D, et al, 2016). Smurf2, a

regulatory factor of Smad signalling, plays major role in deciding the fate of cells in pancreatic cancer cell by controlling the functions of pathways regulated by TGFβ (Wu B, et al, 2016). Elevated levels of TGFβ and low levels of SMAD4 are demonstrated in hematological malignancies. including AML and T-cell lymphomas (Choi YJ, et al, 2015; Andreef M, et al, 2013). In pedeatric B cell- ALL, activation of TGFβ-Smad pathay converts healthy natural killer (NK) cells into dysfunction NK cell in ALL culture, while abrogating the pathway reverse the effect, indicating TGFβ induced alteration helps ALL blast cells to evade innate immune survellience (Rouce RH, et al, 2016).

2.4e. Tumor Suppression

The well known role of TGFβ is its tumor suppressor effects in epithelial, endothelial, myeloid, and lymphoid cell types. Presence of TGFβR2 RNA in breast cancer cells is known to prevent tumor formation [Sun L, et al, 1994], while genetic change inactivating it or overexpression of its dominant negative increase tumorigenicity [Wang J, et al, 1995; Go C, et al, 1999; Böttinger EP, et al, 1997). Moreover, positive correlation between lower TGFβR2 levels and advanced and aggressive tumor stages clearly indicate TGFβ signaling pathway as a tumor suppressive pathway in early stages of cancer (Kim SJ, et al, 2000). TGFβ not only exerts strong cytostatic effects on its target tissues and restrict cell cycle in the G1 phase, but it also induces apoptosis and prevents cell immortalization of the cells. (Siegel PM, et al, 2003; Moses HL, et al, 1990).

2.4eI. Controlling Cell Cycle

To arrest the cell cycle in G1 phase, TGFβ express Cyclin-dependent kinase inhibitors (CDKIs) p15INK4B and/or p21KIP1, resulting in restriction of specific CDK activity (Li JM, et al, 1995; Datto MB, et al, 1995). Transcription of p15 and p21 is induced by TGFβ and mediated by association of Smad with specific transcription factors like Foxo forkhead and Sp1 (Seoane J, et al, 2004; Pardali K, et al, 2000; Ho J, et al, 2004). p15INK4B interacts with either CDK4 or CDK6 or with CDK4-cyclin D or CDK6-cyclin D complexes, while p21CIP1 interacts with CDK2-cyclin A or CDK2-cyclin E complexes. TGFβ induced expression of p15INK4B leads it to bind with CDK4 and CDK6, preventing their further binding with regulatory cyclins, blocking their functions and assuring G1 arrest (Reynisdóttir I, et al, 1995).

Oncogene c-MYC, and the ID family of helix-loop-helix transcription factors (ID1,ID2, and ID3) regulates angiogenesis, cell growth and differentiation, found upregulated in human cancer (Ho J, et al, 2005; Kang Y, et al, 2003; Kowanetz M, et al, 2004). Inhibition of these proteins by TGFβ adds on to its tumor suppressive effect. c-MYC inhibition, leading to cell growth arrest is induced by TGFβ and mediated by a complex including Smad3, Smad4 and repressor E2F4/5, and p107 (Chen CR, et al, 2002). Expression of 2 CDkIs p15INK4B and p21CIP1 is controlled by occupation of regions proximal to their promoters by c-MYC and zinc-finger MIZ1 (Seoane J, et al, 2001; Staller P, et al, 2001). Thus, TGFβ further contributes G1 arrest by inhibiting c-MYC expression leading to increased expression of p15INK4B and p21CIP1. TGFβ also induce activating transcription factor-3 (ATF-3), an important ID1 repressor, which restricts ID1 expression in a Smad-3 dependent mechanism. ID1 are implicated in tumorigenesis through interaction with retinoblastoma tumor suppressive protein (pRB)

and also delays cellular senescence in primary mammalian cells by inhibiting cell cycle regulatory protein p16INK4a (Lasorella A, et al, 2000; Kowanetz M, et al, 2004). As expression of c-MYC is reduced by TGFβ, it results in reduction of ID2 because c-MYC binds its gene promoter region and activates its expression. GULP, an engulfment protein was also found to be a key regulator of TGFβ regulated growth inhibition in ovarian cancer cells (Ma CI, et al, 2012). TGFβ was also shown to specifically inhibit expression of the tyrosine phosphatase CDC25A in normal mammary epithelial cells, by means of a Smad3/E2F4/5/p130 inhibitory complex. CDC25A normally dephosphorylates an inhibitory site on CDK4 and CDK6. Thus, TGFβ-mediated inhibition of CDC25A expression allows for sustained CDK4/6 phosphorylation on their inhibitory sites and further induces cell cycle arrest (Iavarone A, et al, 1997).

.2.4eII. Initiating Apoptosis

Cell death induced by TGFβ, is well documented to show its effect in target tissues of various organs including epithelium, immune system and liver (Oberhammer F, et al, 1992; Chaouchi N, et al, 1995; Yanagisawa K, et al, 1998). In liver cancers, the Daxx adaptor protein interacts with TGFβR2 gets stabilized and further activates JNK and Fas mediated apoptotic pathway (Perlman R, et al, 2001). Also, death-associated protein kinase DAPK, stimulated by TGFβ in liver cancer, promotes cell death by linking Smad proteins to the mitochondrial proapoptotic mechanism (Jang CW, et al, 2002). Another mitochondrial protein, ARTS (apoptosis-related protein in the TGFβ signaling pathway), has shown to potentiate apoptosis in cells resistant to TGFβ-mediated cell death (Larisch S, et al, 2000). TGFβ also activates the pro-apoptotic factor Bax through Smad and the SAPK/p38-dependent transcriptional induction of pro-apoptotic Bcl-2 family members, inducing mitochondrial release of cytochrome C and activation of the apoptosome, resulting in caspase-dependent apoptosis in hepatocytes and B-lymphocytes (Ohgushi M, et al, 2005; Wildey GM, et al, 2004). Expression of transcription factor E2F1, stimulated by a transcriptionally active E2F1-pRb-P/CAF complex on multiple TGFβ pro-apoptotic target gene promoters, leads to the formation of a transcriptionally active E2F1-pRb-P/CAF complex on multiple TGFβ pro-apoptotic target gene promoters, highlighting E2F1 as a central mediator of the TGFβ apoptotic program (Korah J, et al, 2012).

2.4eIII. Giving Cells Natural Death

After every cell cycle, the length of chromosomal ends (telomeres) get reduced as DNA polymerase is not able to completely replicate genetic material during cell division, giving every cell a limitation to which it can replicate. This process reduces the length of telomeres to a critical point resulting in genetic instability, senescence and ultimately cell death after particular number of cell cycles. This property is lost in case of cancer, giving cell immortalization due to reactivation of an enzymatic program named telomerase activity. This activity is commonly observed in cancer and now this property is being used as a prognostic marker. Telomerase is actually a reverse transcriptase enzyme that DNA repeats at the telomeres, preventing shortening of chromosomes. In humans, TGF*β* represses the expression levels of human telomerase reverse transcriptase (hTERT), a protein component of the telomerase enzyme, in cancer and normal cells (Lacerte A, et al, 2008; Rama S, et al, 2001; Yang H, et al, 2001). This mechanism of hTERT

repression, induced by TGFβ is specific for Smad3 and needs E2F1 as well as stress activated kinase and histone deacetylase activities (Lacerte A, et al, 2008)

2.4f.Tumor Promoter

When the tumor has progressed to advanced stages, escaping the tumor suppressor effect of TGFβ and other tumor suppressive effects, the pathway starts showing its paradoxical activity of tumor promoter. The key mechanisms involved in tumor progression caused by TGFβ are epithelial to mesenchymal transition (EMT), evading immune system, cancer cell proliferation promotion by modulating microenvironment, etc. (Derynck R, et al, 2007; Wakefield LM, et al, 2002).

2.4fI. Epithelial to Mesenchymal Transition (EMT)

Transition from Epithelial to Mesenchymal state of a cell initiates with the loss of polarity and contacts with other cell due to down regulation of E-cadherin and other components of the cell junction (Thiery JP, et al, 2009). Simultaneously, mesenchymal state associated transcription factors like Snail, Slug, Twist, FoxC3 and cytoskeleton associated genes such as Fibronectin, alpha smooth muscle actin and Vimentin, etc., which are essential for enhanced motility and invasiveness, show elevated expression (Padua D, et al, 2009, Xu J, et al, 2009; Moustakas A, et al, 2007). TGFβ has been shown a major regulator of the EMT process. Over expression of Smad7 and under expression of Smad3 and/or Smad4 genes, shows significantly decreased EMT and vice versa, in response to TGFβ in cells (Meulmeester E, et al, 2011; Valcourt U, et al, 2007). In cancer, EMT transformed cells are located in the invading tumor edges, areas usually rich in TGFβ and similar cytokines. Reversibility and plasticity of EMT mechanism is dependent on TGFβ and how the cell responds to TGFβ levels (Connolly EC, et al, 2012). Interestingly, along with acquiring mesenchymal characteristics, epithelial cells also acquire some stem cell properties under the influence of TGFβ (Padua D, et al, 2009, Meulmeester E, et al, 2011). When the malignant mammary epithelial cells are immortalized, EMT induction by TGFβ, Snail or Twist, stimulates expression of cancer stem cell surface markers, which are similar in homology to mesenchymal stem cells derived from bone marrow (Mani SA, et al, 2008). TGF-β/Smad signaling induced EMT is activated by a disintegrin and metalloproteinase-17 (ADAM17) in gastric carcinoma cells (Xu M, et al, 2016). In non-small cell lung cancer (NSCLC), expression of SMAD4 is supressed by miR-205 resulting in inhibition of TGFβ-Smad induced EMT, invasion and migration (Zeng Y, et al, 2016),

2.4fII.Evasion of Immunity

When immune-suppressive effects of TGFβ, dominates over its own anti-inflammatory effect, the result is disease progression towards more advanced stages in cancer (Massague J, 2008). TGFβ inhibits antigen presenting function, acting on both CD4+ and CD8+ T cells and natural killer (NK) cells, further decreasing T-cell activation and inhibiting NK cells by transcriptional repression of NKG2D and NKp30 (Arteaga CL, et al, 1993; Lee JC, et al, 2004; Castriconi R, et al, 2003). Inhibition of TGFβ has been shown to suppress metastasis in breast cancer cell lines by increasing NK cells (Arteaga CL, et al, 1993). TGFβ controls the innate immune system (neutrophils and

macrophages) along with T cells secretion to drive type I differentiated anti-tumor cells into more immature type 2 cells, which in turn release more TGFβ and IL-11 into tumor environment, resulting in further advancement of tumor (Connolly EC, et al, 2012; Flavell RA, 2010).

2.4fIII.Angiogenesis and Invasion

Angiogenesis is the term used for production of new blood vessels in for supply of nutrients through blood for growing need of developing tissues, whereas penetration of tumor in another organ or invading on another tissue system is called invasion.

TGFβ stimulates angiogenesis through its influence on angiogenic factors like vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF). Mouse model tests demonstrate impaired TGFβ signaling by removal of TGFβ receptors has discovered defected angiogenesis leading to death of mice, but elevated expression of TGFβ in tumor cells of their environment promotes regular angiogenesis (Larsson J, et al, 2001). TGFβ endorses invasion with the secretion of matrix metalloproteases MMP-2 and MMP-9, reducing the expression of protease inhibitor TIMP (Derynck R, et al, 2001). TGFβ also induces hyaluronan synthesis by increasing hyaluronan synthase 2 levels in mammary epithelial cells (Porsch H, et al, 2013).

2.4fIV. Enhance Metastasis

Clinical correlation strongly links TGFβ with metastatic process. The expression levels of TGFβR2 were inversely proportional to overall survival in Estrogen receptor negative (ER-ve) breast cancer (Buck MB, et al, 2004), as well as elevated levels of TGFβ were observed in metastatic breast cancer relative to primary tumors (Dalal BI, et al, 1993). Comparison between plasma levels of TGFβ pre and post-operative stages of prostate, breast, pancreas, colorectal and other type of cancers also indicates association between TGFβ levels and metastasis (Massague J, et al, 2008). Increased TGFβ levels and circulating tumor cells and lung metastases in breast metastatic model were detected in mouse model treated with radiotherapy and chemotherapy, but administration with anti-TGFβ neutralizing antibodies were shown to prevent increasing metastasis (Massague J, et al, 2008; Meulmeester E, et al, 2011; Biswas S, et al, 2007).

Apart from preparing the cancerous cells for distal metastasis, TGFβ also defines growth of metastasis themselves. It is a key player in invasion of metastatic cancer to bones. TGFβ is released from bone matrix due to degradation of the matrix when osteoclasts were activated by metastatic cells. Metastatic process accelerates after release of other osteolytic cytokines like parathyroid hormone related protein (PTH-rP), IL-11 and CTGF stimulated by increasing levels of TGFβ. In the intracellular downstream Smad components, Smad3 and Smad4 were central elements for bone metastasis, while Smad2 was discovered in association with lung, liver and brain metastasis of breast cancer (Massague J, et al, 2008; Meulmeester E, et al, 2011; Kang Y, et al, 2005).

2.4g.TGFβ-Smad as Therapeutic Target in Cancers

2.4gI. Antisense Molecules

As TGFβ is mostly found with elevated levels during progression of cancer, restricting its production is expected to reduce its presence in the tumor microenvironment. TGFβ2, which increased expression is associated with poor prognosis in glioblastoma and pancreatic cancer, can be controlled with and antisense molecule AP12009 (Trabedersen, Antisense, Pharma). AP11014 and AP15012 are other two antisense molecules in pre-clinical trials for treatment of non-small cell lung cancer, prostate carcinoma, colorectal cancer and multiple myeloma (Lampropoulos P, et al, 2012).

2.4gII. Trapping Cytokine

The over production of TGFβ in the tumor microenvironment can be reduced using ligand traps.

Trapping includes monoclonal neutralizing antibodies against TGFβ, soluble TGFβ receptors and TGFβ receptors antibodies. 1D11, manufactured by Genzyme Corp., Sanofi, is a monoclonal antibody that binds to TGFβ1, 2 and 3, revealed significantly reduction in lung metastasis by increasing anti-tumor response of CD8+ T cells (Nam JS, et al, 2008). It also controls Gh2 levels reducing PTHrP resulting in decreased bone loss (Connolly EC, et al, 2012). 2G7, is another monoclonal antibody, which inhibits breast cancer metastasis by increasing NK cells activity reducing radiation stimulated metastatic acceleration is also under trails (Arteaga CL, et al, 2012; Biswas S, et al, 2007; Ganapathy V, et al, 2010). GC-1008 (Fresolimumab), CAT-152 (Lerdelimimab) and CAT-192 (Metelimumab) are the three completely humanized monoclonal antibodies. GC-1008 has been tested in advanced renal cell carcinoma (RCC), multiple myeloma, glioma and breast cancer (Morris JC, et al, 2008).

Using soluble TGFβ receptors is another to block over produced TGFβ, before it can induce the signaling pathway. Soluble TGFβR2 and TGFβR3 (betaglycan) have been tested. Reduction in pancreatic and breast cancer metastasis was noted when TGFβR2 was expressed, whereas soluble TGFβR3 was observed to inhibit pulmonary metastasis when administered intraperitoneally to athymic nude mice (Bandyopadhyay A, et al, 2002).

Another monoclonal antibody, PF-03446962 is an anti-TGFβR1 molecule, which competitively binds to its ligands like BMP9 and TGFβ with high efficiency. It has the capacity to serve as an anti-angiogenesis agent and has the ability to inhibit endothelial cell sprouting (van Meeteren LA, et al, 2012).

2.4gIII. Controlling Signal Transduction

Once the ligand binds to the receptor, there are two possible strategies to still contain the signaling pathway. First, to stop receptors from phosphorylating downstream signaling components using kinase inhibitors, second targeting the intracellular signaling elements, such as Smads, with peptide aptamers (Connolly EC, et al, 2012).

SB-431542, developed by GlaxoSmithKline, is a TGFβ-Smad pathway inhibitor, used to inhibit proliferation of osteosarcoma, and proliferation, motility and angiogenesis of glioma cells, as well as transcription of collagen and fibronectin in renal carcinoma cells (Hjelmeland MD, et al, 2004; Matsuyama S, et al, 2003). It acts by preventing recruitment of Smad2 and Smad3 through competitive binding to TGFβR1. SB-505124 is another TGFβR1 inhibiting molecule, which is 5 times more efficient in its activity compared to SB-431542 (Calone I, et al, 2012; DaCosta Byfield S, et al, 2004). Limitation of these two molecules is unstability and lack of specificity, which can possibly cause unexpected events. Other TGFβR1 inhibitors at experimental stage are Ki26894, LY364937 and SD-208. These molecules have shown impressive results in breast and gastric cancer, glioma and metastatic multiple myeloma (Ehata S, et al, 2007; Shinto O, et al, 2010; Bandyopadhyay A, et al, 2006; Uhl M, et al, 2004; Mohammad KS, et al, 2011).

LY2109761 is an kinase inhibitor of TGFβR1 and TGFβR2, is used to control metastasis in breast cancer, CRC, and pancreatic cancer (Melisi D, et al, 2008; Korpal M, et al, 2009; Zhang B, et al, 2009), though in skin cancer its use has been associated with resistance and disease progression (Connolly EC, et al, 2011). LY2157299, produced by Eli-lilly and Co., is another TGFβR1 inhibitor, presently under clinical trials in lung and breast cancer (Bueno L, et al, 2008).

Nobiletin, a polymethoxy flavonoid, inhibits TGFβ1-induced migration, invasion and adhesion, accompanied by attenuation of MMP-2, MMP-9, p-Src, p-FAK, p-paxillin, Snail, Slug, Twist and ZEB1 expression in NSCLC cells. It also inhibited the transcriptional activity of Smads without changing the phosphorylation status or translocation of Smads induced by TGFβ resulting in reducing tumor growth and restricting EMT (Da C, et al, 2016) .

Oxymaterine, an alakaloid, inhibits the growth of various cancer cells including CRC by alleviating EMT via inhibtion of TGFβ-Smad activation by reducing p38 dependant elevation of PAI-1 levels (Wang X, et al, 2017).

2.4gIV. Another Approach: Enhancing Tumor Suppression through TGFβ

Signalling pathways playing key roles in stem cell differentiation and proliferation are potential targets for cancer treatments. Reciprocal cross talk between TGFβ and Wnt signaling in intestinal tissues results in accelerated cell cycle arrest and differentiation. Transcription factor (TCF)-4, which is affected by Wnt signaling and Smad4, constitute a transition between proliferative progenitor and differentiated epithelial cells. This switch is permanently reversed in colorectal cancer, as TGFβ signaling is inhibited and TCF-4 is continuously activated due to genetic variations in Wnt cascade, resulting in abnormal crupt foci and formation of adenomatous polyps. These findings indicate TGFβ and Wnt signaling pathways as probable therapeutic targets in Gastro-intestinal cancers (Shah S, et al, 2006; Larriba MJ, et al, 2007).

Interlink between JAK-STAT and TGFβ signaling pathway is also important in cancer as TGFβ has the ability to downregulate IL-6 stimulated phosphorylation of STAT3 (Walia B, et al, 2003). In hepatocellular carcinoma, disturbed TGFβ signaling has been shown to

significantly elevate the levels of STAT3 (Tang Y, et al, 2008). Also, a STAT3-specific inhibitor, NSC-74859, remarkably inhibits STAT3 phosphorylation in HCC with restricting TGFβ pathway, indicating IL6/STAT3 can provide a potential target for HCC treatment (Lin L, et al, 2009).

Carcinoembryonic antigen (CEA) is observed to play key role in CRC metastasis by inhibiting TGFβ pathway through binding directly to TGFβR1. The use of anti CEA specific antibody or siRNA induced CEA silencing can possibly restore TGFβ signaling resulting in tumor suppression (Li Y, et al, 2010).

Bliomycin, pulmonary fibrosis causing agent, induces EMT via TGFβ-Smad pathway, which was evident from expression of EMT phenotypic markers E-cadherin and Vimentin in alveolar epithelial cells (Chen KJ, et al, 2016)

Above literature elaborates the significance of TGFβ pathway cancer. It explains how this pathway effect the carcinogenesis and targeting it with several different strategies can provide us impressive advancement in cancer therapeutics.

HYPOTHESIS

Knowledge Gap

Literature about role of TGFβ-Smad pathway in CML indicates inconsistent and confusing results due to lack of studies designed to explore its components in direction of flow of the signaling pathway. Present literature is limited to inconclusive explanation of elevated TGFβ1 expression, reduced TGFβ receptor expression and mutations in related genes of the pathway in different studies. These studies present scattered data and none of the studies demonstrated reason behind loss of tumor suppressive effect of this pathway in CML. Moreover, there is an immense need to discover the genetic mutations, which can be probable reason behind altered expression of candidate genes of the pathway.

Apart from TGFβ-Smad pathway, the genetic biomarkers required for controlling increasing incidence of CML in high risk population, and markers needed to estimate the prognosis in patients being treated with Imatinib Mesylate. Only few studies are available, with a least number of subjects. There is a urgent need for more studies with different patient cohorts to conquer more genetic markers, which can provide us the more reliable information in molecular mechanism of CML.

Hypothesis

In the search of solution to the TKI resistance in CML, scientists and healthcare professionals are exploring all possible aspects throughout the world. TKI resistance is caused due to several factors, as discussed in previous section, affecting approximately ¼ of patients. One of prime factors, which is required to be explored more, is the role of BCR-ABL independent pathway, as it is imperative to the process. One of the BCR-ABL independent pathways is TGFβ-Smad pathway, which plays central role in differentiation and proliferation of mesenchymal stem cells in normal conditions. Bone marrow stem cells, originating source of CML, are also mesenchymal stem cells, therefore, the pathway is expected to have a key role in CML. We hypothesize that investigation of expression levels along with genetic mutations in key genes of this pathway, and correlation between mutations and differential expression can have the potential to provide us the clue to missing link between CML and loss of cancer suppressive property despite elevated levels of TGFβ1. Comparing the generated results in CML patients with healthy controls can provide us the hint about the role of pathway in pathogenesis of CML, whereas relative comparison between patient cohorts, giving different response to the therapy, can explain its role in prognosis of the disease.

Along with study of TGFβ-Smad pathway, discovery of new and more credible genetic biomarkers can provide us the insight into affected pathways in CML and can have the capacity to elaborate the the molecular mechanism of pathogenesis and progression. Newly discovered markers could be used helpful in controlling incidence as well as progression of CML, and can be used a convincing tool for planning of personalized medicines in CML.

OBJECTIVES

OBJECTIVES

Objective 1: To examine the differential expression of key genes of TGFβ-Smad pathway in Chronic Myeloid Leukemia.

Objective 2: To discover genetic mutations in TGFB-Smad pathway genes and examine their association with Chronic Myeloid Leukemia.

Objective 3: To evaluate the role of Genetic markers in incidence of Chronic Myeloid Leukemia and response to Imatinib Mesylate.

CHAPTER 3:

MATERIALS AND METHODS

CHAPTER 3: MATERIALS AND METHODS.

3.1. Subjects

All patients (>18 years) diagnosed with CML were prospectively enrolled for a period of two years (October 2013-October 2015) at Department of Medical Oncology, Sir Ganga Ram Hospital, Delhi, India. Diagnosis was confirmed by reverse transcription polymerase chain reaction (RT-PCR) for *BCR-ABL* fusion gene and fluorescent in situ hybridization (FISH) for translocation (9; 22). Enrolled patients were segregated as responders and resistant as per European Leukemia Net, 2013 (ELN, 2013) recommendations. According ELN, 2013 criteria, CML patients with ≤10% BCR-ABL or 35% Ph chromosome are considered as responders after 3 months of TKI treatment, whereas patients without complete hematological response or 95% Ph chromosome are considered as resistant. After 6 months of treatment, the criteria changes to presence of <1% BCR-ABL or 0% Ph chromosome for response and for resistance >10% BCR-ABL or 35% Ph chromosome. Patients harboring <0.1% BCR-ABL or are referred as responders and patients with >1% BCR-ABL as resistant after 12 months of treatment. Beyond 12 months of TKI treatment, loss of CHR, CMR or MMR is considered as resistance, whereas presence of <1% BCR-ABL is considered as optimal response (Baccarani M, et al, 2013). At the time of enrollment, patients' clinical and demographic data were obtained (Table 1). Healthy subjects with no known history of malignancy and above 18 years of age were enrolled as age-matched controls. The study was reviewed and approved by the Ethics Committee, Sir Ganga Ram Hospital, Delhi (EC No.: EC/11/12/439). Informed consent was signed and submitted by all subjects at the time of enrollment.

Peripheral blood sample in EDTA vials and plain vials (for serum) was obtained from patients and controls as well. Serum was collected to compare TGFβ1 levels and stored at -80°C. Peripheral blood RNA was immediately extracted (Nucleospin RNA#740200, Macheley-Nager, Duren, Germany) and stored at -80°C for further use.

3.2. TGFβ1 Serum Levels Evaluation

3.2a. Serum Separation

Serum was separated with High speed centrifugation. Blood sample was applied for centrifugation at speed of 4500 rpm for 15 minutes. Separated serum was collected in 1.5ml of 2ml eppendorf tubes for further use.

3.2b. Estimation of serum TGFβ1 levels: Enzyme Linked Immunosorbent Assay (ELISA)

The TGFβ1 serum levels between patients and healthy controls were measured using TGFB1 sandwich ELISA (DRG Diagnostics GmbH#EIA1864, Marburg, Germany). Briefly, standard and serum samples were diluted in assay buffer and acidified with hydrochloric acid (HCl) and then neutralized samples were added to the antibody coated microtiter wells. The unbound serum was washed and a biotinylated anti TGFβ1 IgG antibody was added followed by incubation with streptavidin-HRP Enzyme complex and then unbound conjugate was washed off. Substrate solution was added and absorbance

(OD) of each well at 450 nm was taken with a microtiter plate reader (Infinite M200). The intensity of developed color in standard was considered as proportional concentration and the TGFβ1 serum levels was calculated using standard curve in the patients and control samples. Median was calculated to evaluate the relative difference in the TGFβ1 levels of patients and controls.

3.2c. Calculation of ELISA Results

Average absorbance values for each set of standards, controls and patients were calculated. A standard curve was constructed, on semi-logarithem graph paper, by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (X) axis and concentration on the horizontal (Y) axis. Corresponding concentration for each sample was determined by using mean absorbance value (Figure8). Results were calculated automatically using 4-parameter curve fit. Concentration of samples was derived directly from this standard curve and multiplied with initial dilution factor i.e. 300. Samples with concentrations higher than that of the highest standard were further diluted or reported as such. For calculation of the concentrations, this dilution factor was taken into account.

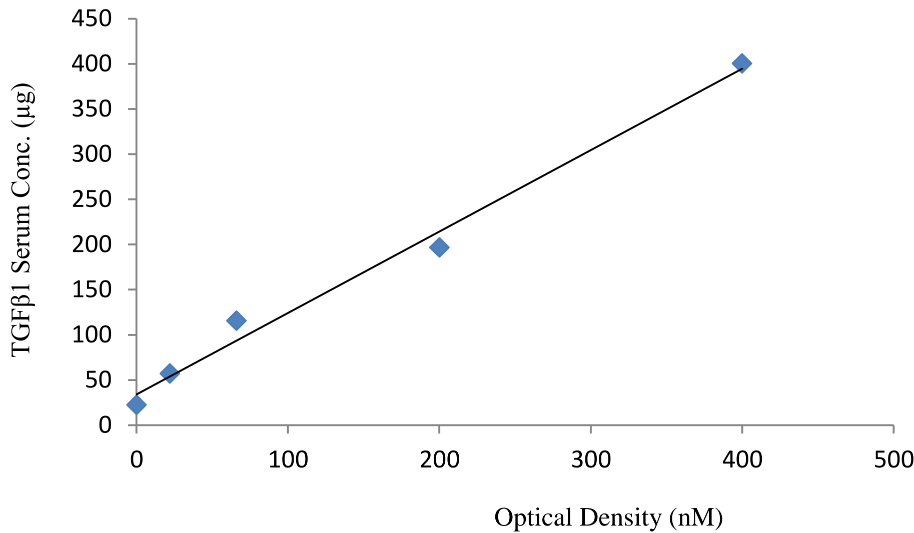


Figure 8: Standard curve for calculation of TGFβ1 serum levels.

3.3. RNA Level Evaluation

3.3a. RNA Extraction

RNA was extracted from whole blood using kit method (Nucleospin RNA#740200: Macheley-Nager, Duren, Germany). Manufacturer's protocol for RNA extraction from 200µl was strictly followed. Important steps of the protocol are mentioned below:

Lysate buffer is added to lyse cell membrane, followed by Proteanase K enzyme to disintegrate the protein with recommended incubation at room temperature for approx

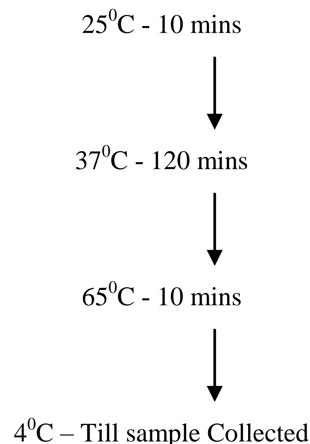
10-15 min and centrifugation for settling down of debris. Ethanol (70%) is added to precipitate RNA present in the solution. Further, the lysate was transferred to column having filter silica membrane. After filtering the lysate through membrane, the nucleic acids were supposed to bind to the membrane, whereas all other components of the lysate are discarded. Membrane is then washed with Membrane desalting buffer. rDNAse is then added to disintegrate DNA present on the membrane, followed by incubation at room temperature. 3-4 washes are then recommended with different washing buffers for purification of the RNA. RNA is finally eluted in the RNAse free water and stored at -80° temperature.

3.3b. RNA Quantification

Extracted RNA is then subjected to 1.5% Agrose gel and quantified on the NanoDrop for verify the quality. RNA with approximate concentration of 120μg/ml is considered as good quality and converted to cDNA.

3.3c. cDNA Preperation

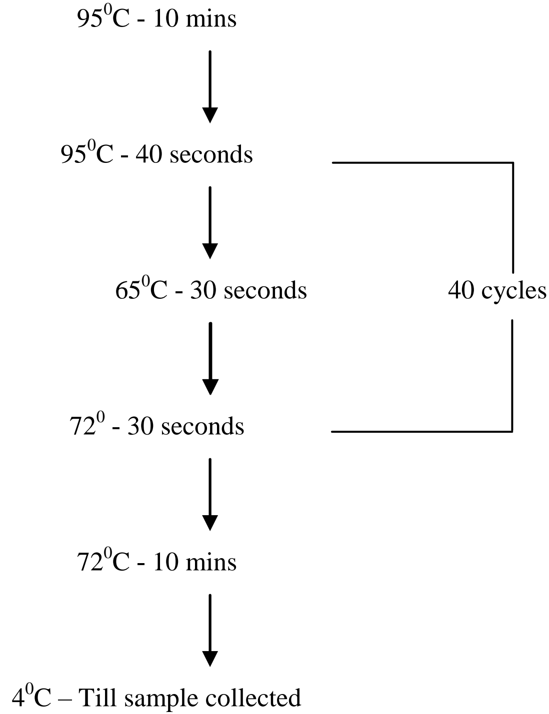
Total 1μg of RNA was converted to cDNA in a 20 μl of reaction having random primers, dNTP's, reaction buffer and reverse transcriptase enzyme using high capacity cDNA Reverse Transcription Kit (Applied Biosystem#4368814, Vilnius, Lithuania).Standardized thermal profile for reverse transcription is as follows:



3.3d. Polymerase Chain Reaction (PCR)

After preparation of cDNA, it was subjected to Polymerase Chain Reaction for confirmation. Forward and reverse primers (Manufactured by Sigma Diagnostics), meant for expression analysis, were used with Taq Polymerase (Applied Biosystem, Vilnius, Lithuania). Other components used in PCR were Taq Buffer, MgCl2, dNTPs, cDNA and H2O.

Thermal profile standardized for PCR was as follows:



PCR product was then subjected to 1.5% Agrose gel for verify the formation of bands resulting for amplification of cDNA.

3.3e. Primer Designing

Primers were designed with the help of Primer3 and Reverse Compliment bioinformatics tool. Sequence was taken from NCBI, Pubmed reference sequence. Accession no. for the sequence of standardized primer of respective gene is provided in Table2.

Gene	Accession No.	Primers
TGFβR1	NM_001130916	F-5'- AAGCCAGCCATTGCTCATAG -3' R-5'- AACATCGTCGAGCAATTTCC-3'
TGFβR2	NM_001024847	F-5'- GGGGAAACAATACTGGCTGA-3' R-5'- TCACACAGGCAGCAGGTTAG- 3'
SMAD4	NM_0053592	F- 5'- GATACGTGGACCCTTCTGGA- 3' R- 5'-ACGCCCAGCTTCTCTGTCTA-3'
SMAD7	NC_001190823	F- 5'-GGGGCTTTCAGATTCCCAAC-3' R-5'- CAAAAGCCATTCCCCTGAGG-3'

Table2: Sequence of standardized primers with accession numbers (NCBI).

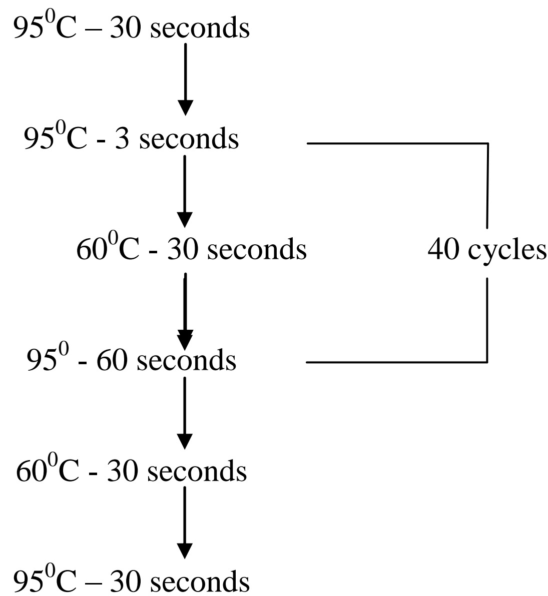
3.3f. Primers Standardization

Initially, at least 4 forward and reverse primers were designed for every study gene. All the primers were standardized using conventional PCR and RT-PCR. Primers were subjected to PCR to check formation of clear bands. Primers showing duplicate bands or primer dimmers were not considered for the use. All the primers showing clear and prominent bands were then standardized on RT-PCR. Here, selection of primers was done on the relative Threshold Cycle (Ct) demonstrated by every primer. Primers showing Ct between 20 and 25, compared to endogenous gene showing around Ct of 20, were selected. Other parameters for selection of primers were clarity of amplification curve and single and prominent peak of dissociation curve. Primers observed showing best results with above parameters were selected to study expression levels on RT-PCR (Table2).

3.3g. Examining transcript levels using Real-Time Polymerase Chain Reaction (RT-PCR)

The transcript level of TGFβR1, TGFβR2, SMAD4 and SMAD7 was examined by Real-Time PCR (Stratagene Mx3005P) using SYBRgreen chemistry (Applied Biosystem#43855612, Vilnius, Lithuania). Briefly, 25 ng of cDNA was used to prepare 10 µl of reaction containing respective primers and SYBRgreen .ACTB (β-actin) was used as an endogenous gene. The raw data was analyzed manually by $2^{-\Delta Ct}$ method and the median of $2^{-\Delta Ct}$ was compared between patients and controls (Schmittgen TD, et al, 2008).

Thermal Profile used in RT-PCR is given below:



3.4. Identification of Genetic Mutations

3.4a. DNA Extraction

Peripheral blood DNA was extracted using kit Nucleospin DNA#740951 Macheley-Nager, Duren, Germany. Manufacturer's protocol was followed, which is described briefly below:

DNA extraction procedure begins with taking Proteinase K in eppendorf having peripheral blood at room temperature (25 µl for 200µl of blood). Lysis buffer is then added to lyse cell membrane, followed by incubation at 70°C for 10 to 15 minutes. After incubation, ethanol (100%) is put into the mixture for precipitation of DNA. The mixture is transferred to collection tube having DNA binding membrane and centrifuged for DNA binding. Residue solution is discarded and membrane column is transferred to another collection tube. The membrane is then given 2-3 washes with different wash buffers. Later, the residual ethanol is dried from the membrane by dry spinning. Finally, membrane bound DNA is eluted into elution buffer. The buffer is preheated at 70°C.

3.4b. Verification and Quantification of DNA

Extracted DNA is then subjected to 2% Agrose gel (Figure16) and quantified on the NanoDrop for verify the quality. DNA with approximate concentration of 100µg/ml is considered as good quality and stored for sequencing.

3.4c. Next Generation Sequencing

A targeted panel (TruSight One, Illumina, USA) with probes covering all coding exons and essesntial splice sites of >4800 clinically relevant genes was used for sequencing the patients and controls DNA samples using Illumina's TruSight technology (Illumina, USA). The target size for this is panel 12 Mb and the designed is based on the information in the Human Gene Mutation Database (HGMD), the Online Mendelian Inheritance in Man (OMIM) catalogue, GeneTests.org, Illumina TuSight sequencing panels and other commercially available sequencing panels.

Genomic DNA isolated from blood was quantified using Qubit (ThermoFisher Scientific, USA) and 50 ng is taken for library preparation. TruSight One library preparation uses transposon based shearing of the genomic DNA. The protocol allows the DNA to be 'tagmented' (fragmented and tagged simultaneously in the same tube). A limited cycle PCR step allows the incorporation of adaptors, platform-specific tags and barcodes to prepare the DNA sequencing libraries. The tagged and amplified sample libraries were analyzed for quality using BioAnalyzer (Agilent, USA) and quantified using Qubit. 500 ng of each library was pooled and hybridized to biotinylated probes. The hybridized target DNA fragments were pulled down using streptavidin beads and two successive enrichment steps were performed to optimize the pull down of the regions of interest. Target libraries were amplified using limited PCR steps and loaded for sequencing on the MiSeq or HiSeq (Illumina, USA) to obtain ~3 GB per sample.

3.4d. Sequence Analysis

The trimmed FASTQ files were generated using MiSeq Reporter from Illumina for MiSeq data and Casava software for HiSeq data. The read were aligned against the whole genome build hg19 using STRAND® NGS V2.1.6 (Strand LifeSciences Pvt. Ltd., Bangalore). Five base pairs from the 3' end of the read were trimmed, as were 3' end bases with quality below 10. Reads which had length less than 25 bp after trimming were not considered for alignment. A maximum of 5 matches of alignment score at least 90% were computed. Reads that failed QC (quality control), reads with average quality less than 20, reads with ambiguous characters were all filtered out. The STRAND® NGS variant caller was used to detect variants at locations in the target regions covered by a minimum of 10 reads with at least 2 variant reads. Variants with a decibel score of at least 50 were reported.

3.4e. Interpretation

Interpretation of the variant data was done using the StrandOmics software, V1.9. StrandOmics is a clinical genomics interpretation and reporting platform developed at Strand Life Sciences, Bangalore. The variant annotation engine includes different algorithms to identify variant impact on gene using public content (HGMD, ClinVar, OMIM, HPO, links to dbSNP, 1000 Genomes, Exome Variant Server) and proprietary content (curated variant records). The Interpretation interface in StrandOmics allows quick filtering and evaluation of variants along with capture of justification for inclusion/exclusion.

3.5. Statistical Analysis

Statistical analyses were performed using SPSS for Windows 16 (SPSS, Chicago IL, USA). Non-parametric data is presented as median with standard deviation (SD). Statistical significance of differences of quantitative data was determined using Mann-Whitney U test. P-value of less than 0.05 was considered to be significant.

CHAPTER 4:

RESULTS AND DISCUSSION

CHAPTER 4: RESULTS AND DISCUSSION

RESULTS

4.1. Subject

CML patients (n=83) enrolled Healthy Controls (n=91) with similar age and sex distribution were enrolled. Enrolled patients were distributed as IM responder and IM resistant according to ELN, 2013 recommendations (Baccarani M, et al, 2013). Clinical and demographic details of patients and controls are provided in (Table3).

Group	Demographic/Clinical Variables	CML Patients (n = 83)	Healthy Controls (n = 91)	IM Intake Period (months)
Sex	Male	69	76	N/A
	Female	14	15	N/A
Age (years)	Range	19-71	19-68	N/A
	Median	41	40	N/A
Groups	Resistant	20	N/A	65.5
	Responders	57	N/A	58

Table3: Demographic and clinical variables of CML patients and controls enrolled in the study.

Twenty patients and five healthy controls were selected for sequencing. Mean age of shortlisted patients was 42.11 (SD = $\pm$ 12.76) years, whereas that of controls was 38.40 (SD = $\pm$ 12.76) years ($p = 0.570$). All the patients were in chronic phase at the time of diagnosis. IM responder group had 12 patients (55.55%), while 8 patients (44.45%) were in IM failure group. Mean age of responder group was 35.1 (SD = $\pm$6.74) years, whereas failure was 50.88 ($\pm$ 13.36) years ($p = 0.005$).

Objective 1: To examine the differential expression of key genes of TGFβ-Smad pathway in Chronic Myeloid Leukemia.

4.2. Expression of TGFβ-Smad Pathway genes

4.2a. Differential levels of TGFβ1 and TGFβR2 in CML

To see if TGFβ1 was differentially expressed in CML patients compared to healthy individuals, serum levels of TGFβ1 were evaluated in CML patients and healthy controls. The levels were significantly elevated (1.2 fold) in patients as compared to healthy controls ($p=0.020$) (Figure9a). Observed data was segregated and evaluated according to the IM response: patients responding to IM treatment did not show any significant change in comparison to the resistant group (Figure9b). The data indicates a trend of increased TGFβ1 levels in the serum of CML patients.

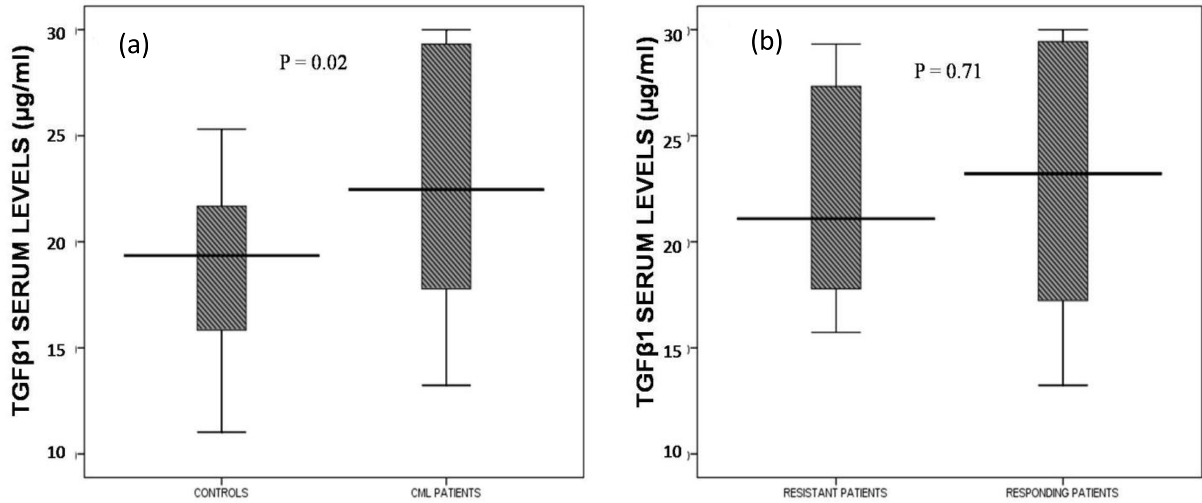


Figure9: Box-plot representation of (a) TGFβ1 serum levels in CML patients and healthy controls (b) Comparison of TGFβ1 serum levels in Resistant CML patient with Responders In the graph, central line represents median, boxes represent 25th-75th percentile and whiskers indicate minimum and maximum values. p values <0.05 considered significant.

In order to determine if TGFβ1 receptors are differentially expressed in the blood cells of CML patients, we evaluated RNA expression of TGFβR1 and TGFβR2 in CML patients and compared with healthy individuals. TGFβR1 transcript levels did not show any difference in CML patients as compared to controls (Figure10a, 10b).TGFβR2 expression was significantly low in CML patients, compared to healthy controls (p=0.012) (Figure10c). To see if there is any difference in the levels of TGFβR2 in response to IM, we further compared the levels in responders and resistant patients. However, we did not observe any significant difference of TGFβR2 levels in the two groups (Figure10d).

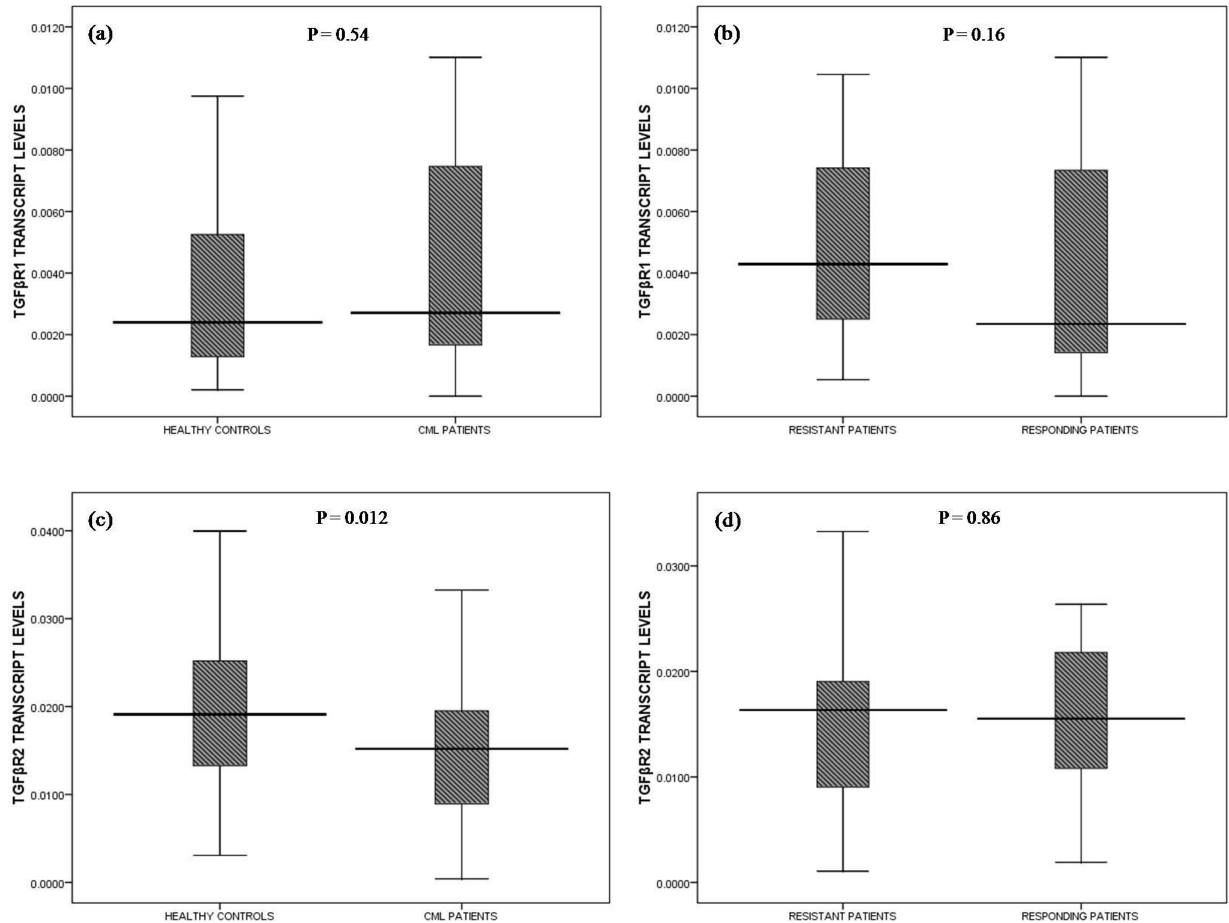


Figure10: (a) Comparison of TGFβR1 transcript levels in CML patient with healthy controls and (b) between resistant and responding CML patients (c) Transcript levels of TGFβR2 in CML patients with healthy controls and (d) between resistant and responding CML patients. In the graph, central line represents median, boxes represent 25th-75th percentile and whiskers indicate minimum and maximum values. p values <0.05 considered significant.

4.2b. Reduced levels of SMAD4 in CML

Since SMAD4 is a co-SMAD downstream of TGFβR2, we examined the transcript levels of SMAD4 in CML patients along with healthy individuals. SMAD4 RNA transcript levels were significantly reduced as compared to controls (p=0.043) (Figue11a). However, no major difference was observed in SMAD4 expression levels between IM resistant and responding patients (Figure11b).

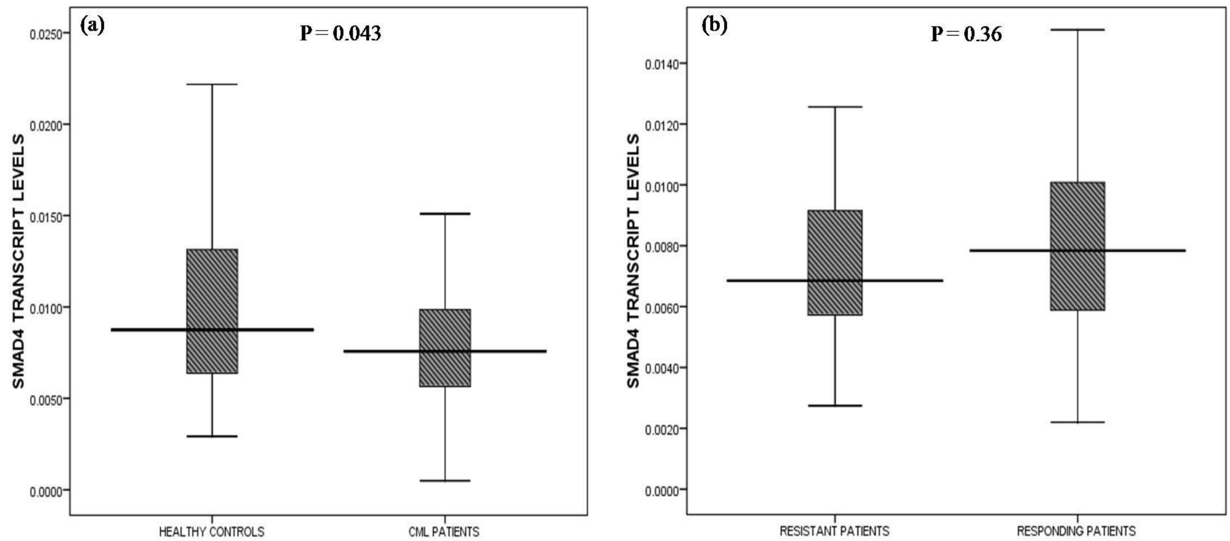


Figure11: Transcript levels comparison of SMAD4 gene between (a) CML patients and healthy controls, (b) IM resistant and responding patients. In the graph, central line represents median, boxes represent 25th-75th percentile, and whiskers indicate minimum and maximum values. p values <0.05 considered significant.

We further examined expression levels of inhibitory SMAD7 to observe its impact in CML. We evaluated RNA expression in controls and CML patients. SMAD7 RNA levels in CML patients showed slightly higher expression, but did not reach statistical significance. Comparison between responding and resistant patients did not reveal any significant difference in transcript levels (Figure12a, b).

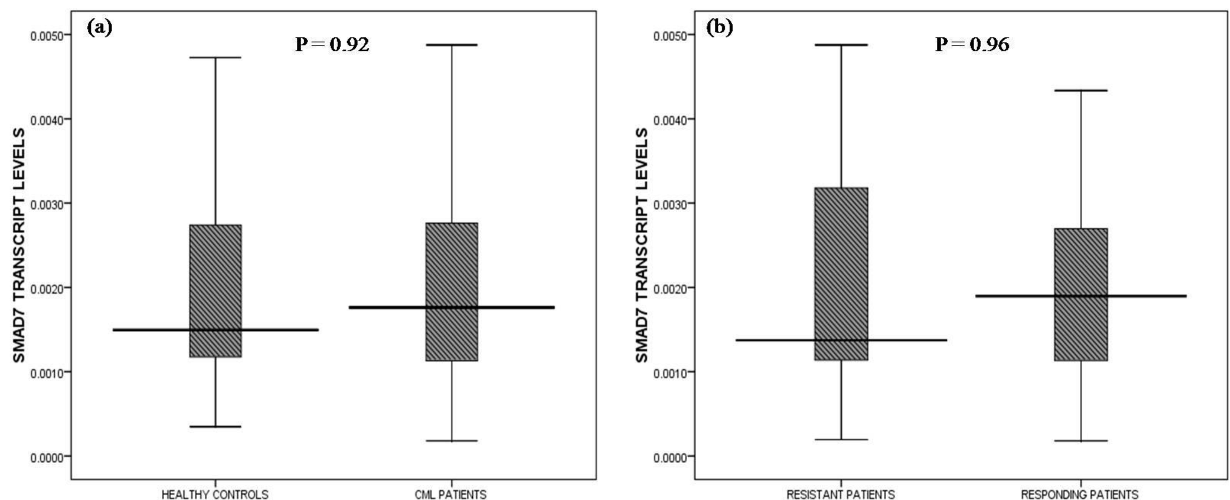


Figure12: Transcript levels comparison of SMAD7 gene between (a) CML patients and healthy controls, (b) IM resistant and responding patients. In the graph, central line represents median, boxes represent 25th-75th percentile, and whiskers indicate minimum and maximum values. p values <0.05 considered significant.

Objective 2: To discover genetic mutations in TGFB-Smad pathway genes and examine their association with Chronic Myeloid Leukemia.

4.3. Identification of genetic variations in TGFB-SMAD signaling pathway

We analyzed association of genetic variations in TGFβ1, TGFβR1, TGFβR2, SMAD4 and SMAD7 genes with CML. We sequenced exons and exon-intron boundaries of these genes in patients and healthy controls. In total 52 variants were identified. After filtering common variants among patients and controls, 33 variants were present only in the patient group. In these genetic variations, 14 (42.5%) were intronic, while 11 (33.33%) were in coding region and 8 (24.24%) were in the untranslated region (UTR). Among intronic variations 11 (34%) were single base substitution, while 2 (6%) were deletion and 1 (3%) was insertion. Coding region variations included 7 (21%) non-synonymous, 2 (6%) synonymous variations and 2 (6%) deletion (Figure13).

4.3a. Variations in TGFβ1 and TGFβ receptors

In TGFβ1 gene, 10 genetic variations were identified (Table4). A non-synonymous variation in exon 1, c.29C>T (rs1800470), was present in 10 out of 20 patients. This variation causes proline to leucine substitution (P10L) in exon1 of TGFβ1 gene. The variation lies in the conserved region and was predicted to be damaging (SIFT score 0.00, Grantham score: 98, Phastcons score: 98, GERP score 1.45). We reanalyzed the TGFβ1 serum levels in 3 patients for whom both mutation analysis and serum studies were available. Two patients harboring 29C>T mutation had elevated TGFβ1 levels (26.1 and 22.5μg/ml respectively) as compared to controls (median=19.3 μg/ml) and to the other patient harboring c.74G>C (rs1800471) in exon 1 (21.1μg/ml).

In TGFβR1, 5 genetic variations were identified. An intronic variation, c.1024+24G>A (rs334354) was present in 8 patients. This variation has shown to be an important susceptibility and prognostic marker in various cancers (20, 21). Another variation c.69A>G (rs868), identified in 4 patients, lie in the 3' UTR. Since 3’ UTR is known to play role in post transcriptional regulation of gene expression, we reviewed the TGFβR1 expression levels in patients with this mutation. We observed reduced transcript levels in patients with this mutation (Fig 13b), whereas the overall expression was not significantly altered in CML patients groups relative to healthy controls. In silico analysis of this variant shows the mutation site to be the target for miRNA Let7f/miRNA98 (Figure 13c).

In TGFβR2, 5 genetic variations were observed (Table4). Each two genetic variations (c.169+99T>C ; rs117998227, c.1242C>T ; rs2228048) were present in 2 different patients, whereas c.458delA; rs79375991 was present in 3. None of the variations could be correlated with expression due to low frequency observed in patient group.

4.3b.Variations in SMAD4 and SMAD7

Seven genetic variations were found in SMAD4 with 3 of them being intronic, 3 in UTR and 1 variation was exon 2. None of the variations were present in more than one patient, making it impossible to relate them to change in SMAD4 transcript levels or to associate with the disease (Table4).

Six variations were observed in SMAD7. Among the variations an intronic variation g.46474746C>T (rs3736242) was present in 8 out of 20 CML patients (Table4).

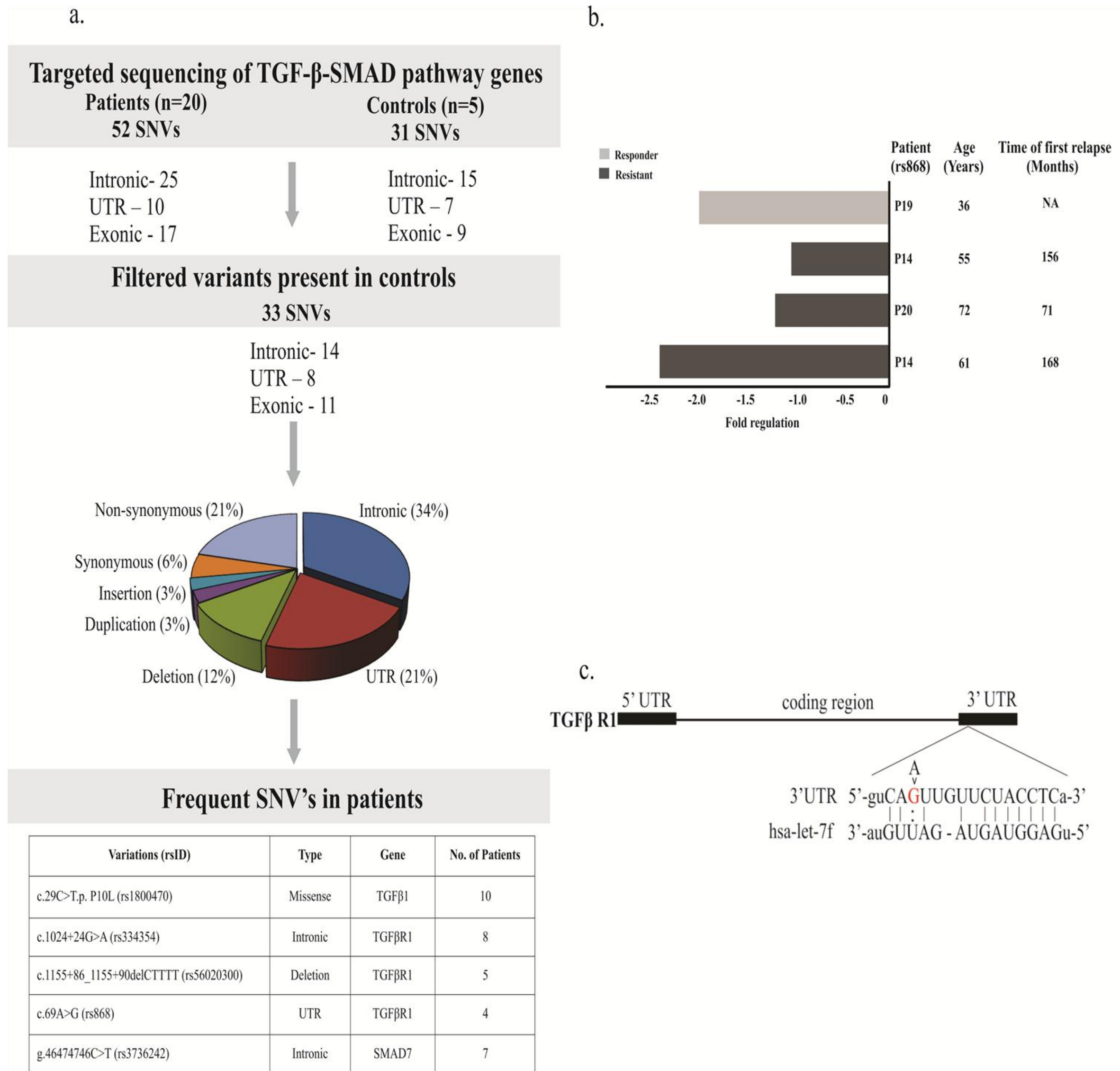


Figure13: (a) Data analysis work flow of exome of TGF-β-Smad signaling pathway candidate genes. The pie chart represents the distribution of genetic variations as per their chromosomal regions and types. Table at the bottom represents conclusive genetic variations, focused due to their frequency and significance. (b) Fold regulation of TGFβR1 transcript in the patients harboring mutation c.69A>G (rs868) along with age of patients and time period of first relapse from time of diagnosis. (c) Schematic of hsa-let7f/miR98 binding site in 3'UTR of human TGFβR1 (NM_001130916) in 5'-3' direction aligned with hsa-let7f/miR98 sequence. Fractured line between G in the UTR sequence and U in the miR sequence represents the site of mutations (A>G).

Genetic Variations	rsID	Variation Type	Intron/ Exon / UTR	No. Of Patients
TGFβ1 (NM_000660)				
c.250A>T. p. T84S	-	Missense	Exon1	1
c.74G>C.p. R25P	rs1800471	Missense	Exon1	3
c.29C>T.p. P10L	rs1800470	Missense	Exon1	10
c.635-93_635-92insA	-	Insertion	Intron3	1
c.861-20C>T	-	Intronic	Intron5	4
c.1014G>C.p. K338N	-	Missense	Exon6	1
c.*58G>C	-	UTR	3'UTR	1
c.*52G>C	-	UTR	3'UTR	2
c.*47G>C	-	UTR	3'UTR	4
c.*26dupC	-	Duplication	3'UTR	1
TGFβR1 (NM_001130916)				
c.574+39A>G	rs11568778	Intronic	Intron3	5
c.1024+24G>A	rs334354	Intronic	Intron6	8
c.1155+86_1155+90delCTTTT	rs56020300	Deletion	Intron7	5
c.1156-15delT	-	Deletion	Intron7	2
c.69A>G	rs868	UTR	3'UTR	4
TGFβR2 (NM_001024847)				
c.169+99T>C	rs117998227	Intronic	Intron2	2
c.458delA.p.K153S fsTer35	rs79375991	Deletion	Exon4	3
c.1242C>T	rs2228048	Synonymous	Exon5	2
c.1156-15delT	-	Intronic	Intron7	1
c.1599+62A>G	rs192590842	Intronic	Intron7	1
SMAD4 (NM_005359c)				
c.604G>T.p. A202S	-	Missense	Exon5	1
c.905-52A>G	rs948589	Intronic	Intron7	1
c.955+58C>T	rs948588	Intronic	Intron8	1
c.1448-49G>C	rs375313666	Intronic	Intron11	1
c.7T>A	-	UTR	3'UTR	1
c.1G>T	-	UTR	3'UTR	1
c.12G>C	-	UTR	3'UTR	1
SMAD7 (NM_001190823)				
c.179-106C>T	rs76886865	Intronic	Intron1	1
c.608C>T.p.T203M	-	Missense	Exon2	1
c.393C>G.p. C131W	-	Missense	Exon2	1
c.330C>T.p. L110L	rs3809922	Synonymous	Exon2	1
g.46474795delG	-	Deletion	Exon2	3
g.46474746C>T	rs3736242	Intronic	Intron2	7

Table4: Description of TGFβ-Smad pathway genetic variations observed in CML patients.

Objective 3: To evaluate the role of genetic markers in incidence of Chronic Myeloid Leukemia and response to Imatinib Mesylate.

4.4. Identification of Genetic Biomarkers

4.4a. Subjects

Eighteen patients and five healthy controls were selected. Mean age of patients was 42.11 (SD = $\pm$ 12.76) years, whereas that of controls was 38.40 (SD = $\pm$ 12.76) years (p = 0.570). All the patients were in chronic phase at the time of diagnosis. Patients were distributed as IM responder and IM resistant as per ELN, 2013 recommendations (Baccarani M, et al, 2013). IM responder group had 10 patients (55.55%), while 8 patients (44.45%) were in IM failure group. Mean age of responder group was 35.1 (SD = $\pm$6.74) years, whereas failure was 50.88 ($\pm$ 13.36) years (p = 0.005). Clinical and demographic details of individual patients are provided in Table5.

4.4b. Clinical Exome Analysis

Data generated by sequencing was uploaded to SeatttleSeq Annotation 141 (http://snp.gs.washington.edu/SeattleSeqAnnotation141) for annotation of genetic variations. Analysis of annotated data for patients and controls was done separately. All the variants present in control group were aligned in one table and all variants of patients group were aligned in another table. In total, 97,462 variants in patients and 65,546 variants in controls were found. After filtration of common variants, 45,950 variants were selected exclusively in patient group. Distribution of genetic variants in patient group included 30,525 intronic, 2,837 variants were in untranslated regions (3'UTR = 1,737 and 5' UTR = 1,100), 5,132 variations were synonymous and 7,494 variants were non-synonymous. Non-synonymous mutations were further segregated as 6,524 missense mutations, 766 frame-shift and 166 stop gained/stop lost mutations (Figure14). We targeted only missense mutations, as these mutations play central role in altering phenotype of the gene at protein level.

4.4c. Variant Analysis of significant genes

Total 2,033 missense variants were common in atleast 2 patients and only 273 variants were left after considering the variants present in more than 25% ($\geq$5) patients. These variants were further analyzed for molecular pathway and functional correlation with CML. All the mutations observed were subjected to Kyoto Encyclopedia for Genes and Genomics (Kegg) pathways and GeneCards to identify the cellular pathway and functions associated with the mutated genes. We focused on the genes which were most frequently mutated in this group.

HLA-DRB1 had 5 variants (rs17885382, rs1778951, rs9269951, rs11554462 and c.239G>C) and HLA-A had 4 variants (rs1059455, rs199474430, rs1136949 and rs1137160). HYDIN gene also had 5 variations (rs147399921, rs201356436, rs202145987, rs1179222041 and rs148896798), which were maximum number of variants among all the genes. Apart from these candidate genes, CPZ, NSD1, PER3, SDHA and TAS2R43 had 3 mutations each (Table6). All remaining genes had either 1or2 variation.

Pt.No	Age at Dx (years)	BCR-ABL^{IS}%	First TKI (FT) Therapy	Revised TKI Therapy
Responders				
1	40	0.00%	Imatinib 400mg	-
2	41	0.8%	Imatinib 400mg	-
3	34	0.6%	Imatinib 400mg	-
4	30	1.3%	Imatinib 400mg	-
5	31	1.0%	Imatinib 400mg	-
6	21	0.00%	Imatinib 400mg	-
7	36	0.8%	Imatinib 400mg	-
8	45	0.20%	Imatinib 400mg	-
9	38	0.00%	Imatinib 400mg	-
10	35	0.07%	Imatinib 400mg	-
Failures				
11	52	2.1%	Imatinib 400mg	Nilotinib 300mg
12	55	1.4%	Imatinib 400mg	Imatinib 800mg
13	49	3.8%	Imatinib 400mg	Nilotinib 100mg
14	72	2%	Imatinib 400mg	Nilotinib 300 mg
15	53	2.4%	Imatinib 400mg	Imatinib 600 mg
16	32	3%	Imatinib 400mg	Dasatinib 100mg
17	33	4%	Imatinib 400mg	Nilotinib 400mg
18	61	2.2%	Imatinib 400mg	Dasatinib 200mg

Pt. no = Patient number, **Dx**= Diagnosis, **BCR-ABLIS% =** percentage of BCR/ABL expression in international score (Responders:at time of sample collection, Failures: After relapse, before initiating next regime), **TKI**= Tyrosine kinase inhibitor, **FT** = Firstline TKI therapy, FT Period for responders = Date of first dose to date of sample collection, FT Period for responders = Date of first dose to first relapse..

Table5: Demographic and clinical features of patients enrolled.

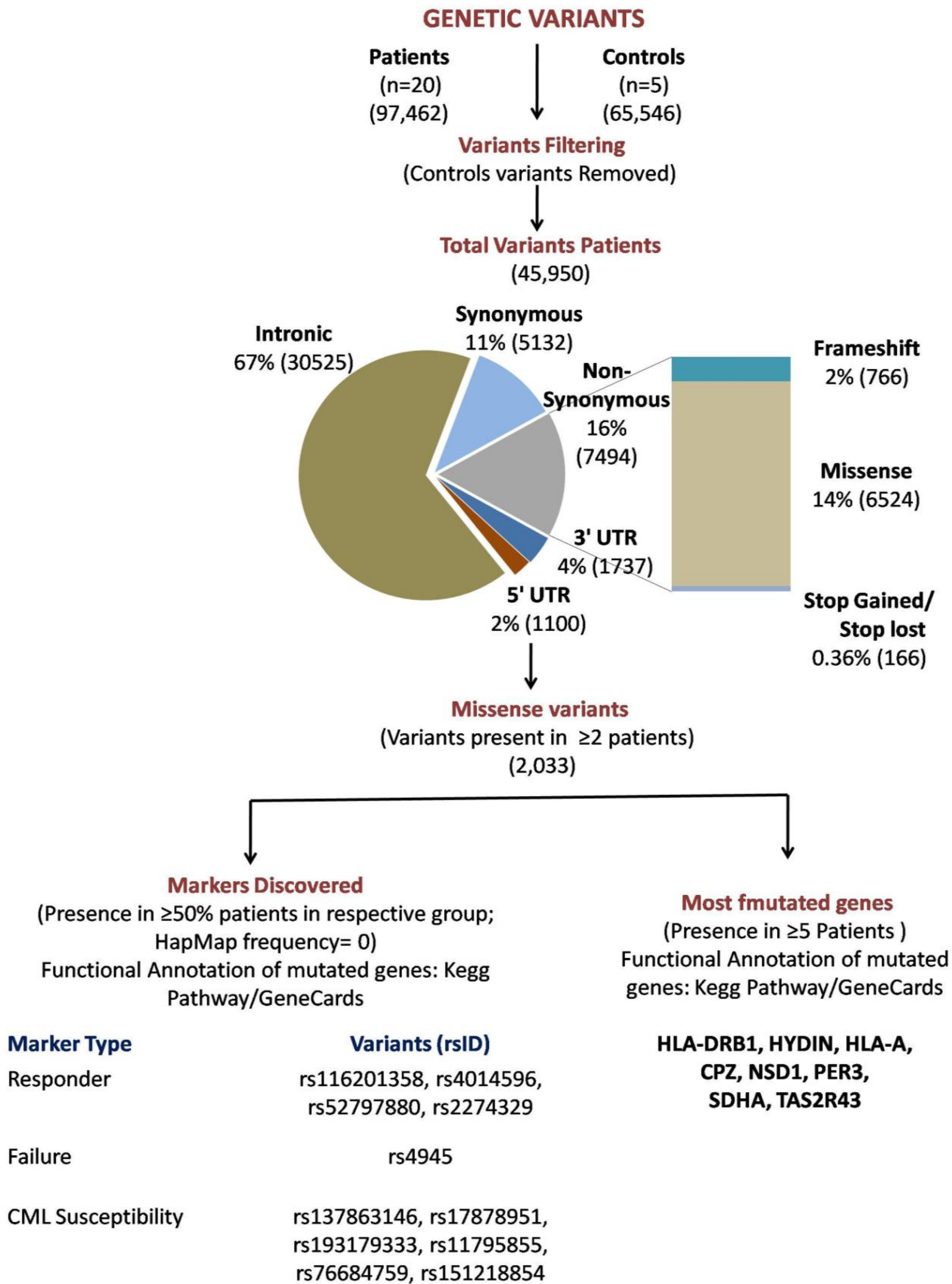


Figure14: Work flow of clinical exome data interpretation to define potential prognostic and susceptibility markers in CML cohort.

Genes	Mutations (rsID▲)	No. of Patients (n€=18)	Associated Pathways/ Functions
HLA-DRB1	rs17885382	6	hsa04514:CellAdhesionMolecules(CAMs);hsa04612:AntigenProcessingAndPresentation;hsa04640:HematopoieticCellLineage;hsa04672:IntestinalImmuneNetworkForIgAProduction;hsa04940:TypeIDiabetesMellitus;hsa05140:Leishmaniasis;hsa05310:Asthma; hsa05320:AutoimmuneThyroidDisease; hsa05322:SystemicLupusErythematosus; hsa05330:AllograftRejection; hsa05332:Graft-versus-hostDisease;hsa05416:ViralMyocarditis (Kegg Pathways)
	rs17878951	15	
	rs9269951	8	
	rs11554462	8	
	c.239C>G	8	
HYDIN	rs147394921	8	Involved in cilia motility. Mutations in this gene cause of autosomal recessive primary ciliary dyskinesia-5. (GeneCards)
	rs201356436	7	
	rs202145987	5	
	rs117922041	6	
	rs148896798	7	
HLA-A	rs1059455	6	hsa04144:Endocytosis;hsa04514:CellAdhesionMolecules(CAMs);hsa04612:AntigenProcessingAndPresentation;hsa04650:NaturalKillerCellMediatedCytotoxicity;hsa04940:TypeIDiabetesMellitus;hsa05320:AutoimmuneThyroidDisease;hsa05330:AllograftRejection;hsa05332:Graft-versus-hostDisease; hsa05416:Viral Myocarditis (Kegg Pathways)
	rs199474430	8	
	rs1136949	8	
	rs1137160	8	
CPZ	rs79736750	6	Encodes Carboxypeptidase Z,. has carboxypeptidase activity towards substrates with basic C-terminal residues (GeneCards).
	rs35993494	6	
	rs34964084	5	
NSD1	rs35848863	7	hsa00310:LysineDegradation (Kegg Pathways)
	rs34165241	7	
	78247455	5	
PER3	rs10462020	7	hsa04710:CircadianRhythm-Mammal (Kegg Pathways)
	rs10462021	7	
	rs2640909	10	
SDHA	rs1042052	5	hsa00020:CitrateCycle(TCACycle);hsa00190:OxidativePhosphorylation; hsa01100:MetabolicPathways;hsa05010:Alzheimer'sDisease;hsa05012:Parkinson'sDisease; hsa05016:Huntington'sDisease (Kegg Pathways)
	rs10426960	6	
	rs10426962	6	
TAS2R43	rs111846092	10	hsa04742:TasteTransduction (Kegg Pathways)
	rs200533679	7	
	rs201618803	9	

▲**rsID** = rsiD provided in DBSNP (NCBI), €**n** = total number of patients enrolled.

Table 6: Summary of most mutated genes (in ≥5 CML patients) revealing affected pathway and functions in CML

Gene	Patients▲	Associated Pathways/Functions
Genes of Responder Markers		
C8A	P2, P3, P4, P5, P8	hsa04610:ComplementAndCoagulationCascades; hsa05020:PrionDiseases; hsa05322:SystemicLupusErythematosus (Kegg Pathways)
UNC93B1	P2, P3, P4, P5, P7	Regulates toll-like receptor Signaling. Protein deficiency associated with herpes simplex encephalitis. (GeneCard)
APOH	P4, P5, P6, P8, P10	Associated with lipoprotein metabolism, coagulation, and the production of antiphospholipid autoantibodies. (GeneCard)
CA6	P3, P4, P5, P7, 10	hsa00910:NitrogenMetabolism. (Kegg Pathways)
Gene of Failure Marker		
MFGE8	P11, P13, P16, P18	Encodes Lectadherin, involved in phagocytosis of apoptotic cells. Implicated in healing, autoimmune disease and cancer. (GeneCard)
Genes of Susceptibility Markers		
HLA-DRB5	hsa04514:CellAdhesionMolecules(CAMs); hsa04612:AntigenProcessingAndPresentation; hsa04640:HematopoieticCellLineage; hsa04672:IntestinalImmuneNetworkForIgAProduction; hsa04940:TypeIDiabetesMellitus; hsa05140:Leishmaniasis; hsa05310:Asthma; hsa05320:AutoimmuneThyroidDisease; hsa05322:SystemicLupusErythematosus; hsa05330:AllograftRejection; hsa05332:Graft-versus-hostDisease; hsa05416:ViralMyocarditis. (Kegg Pathways)	
HLA-DRB1	hsa04514:CellAdhesionMolecules(CAMs); hsa04612:AntigenProcessingAndPresentation; hsa04640:HematopoieticCellLineage; hsa04672:IntestinalImmuneNetworkForIgAProduction; hsa04940:TypeIDiabetesMellitus; hsa05140:Leishmaniasis; hsa05310:Asthma; hsa05320:AutoimmuneThyroidDisease; hsa05322:SystemicLupusErythematosus; hsa05330:AllograftRejection; hsa05332:Graft-versus-hostDisease; hsa05416:ViralMyocarditis (Kegg Pathways)	
RHPN2	Encodes Rho-GTPase binding protein. Involved in actin cytoskeleton. (GeneCard).	
CYP2F1	hsa00980:MetabolismOfXenobioticsByCytochromeP450 (Kegg Pathways)	
KCNJ12	Encodes K+ channels. (GeneCard)	
FUT3	hsa00601:GlycosphingolipidBiosynthesisLactoAndNeolactoSeries; hsa01100:MetabolicPathways (Kegg Pathways)	

▲**Patients**= patients having mutation of respective gene,

Table7: Functional annotation of genes having potential markers using Kegg pathways and GeneCards

4.4d. Genetic markers

Our prime objective was to detect prognostic and susceptibility markers. Criterion for genetic variants to be considered as marker was presence in at least 50% patients of one group and absence in all patients of opposite group whereas mutations present in more than 50% (≥9) of CML patients were considered as susceptibility markers. Only rare mutations, which were not present in healthy population of Asia, Europe and Africa as per HapMap project, were considered. The shortlisted mutations were annotated manually with GeneCards and Kegg pathways for their functions (Table7). Important genetic information about discovered mutations is given in Table8.

Mutation	Gene	Allele	Accession No.	Chromosomal Position[€]	Codon	Amino Acid change
Markers for IM Responder						
c.107C>A (rs116201358)	C8A	A/C	NM_000562.2	1; 57333311	GCA-GAA	p.36Ala>Glu
c.1494G>A (rs4014596)	UNC93B1	C/T	NM_030930.2	11; 67759316	GTG-ATG	p.499Val>Met
c.422T>C (rs52797880)	APOH	A/G	NM_000042.2	17; 64216854	ATC-ACC	p.141Ile>Thr
c.209G>C (rs2274329)	CA6	C/G	NM_001215.3	1; 9009451	GGC-GCC	p.70Gly>Ala
Marker for IM Failures						
c.7C>A (rs4945)	MFGE8	G/T	NM_001114614.1	15; 89456544	CGC-AGC	p.3Arg>Ser
CML Susceptibility Marker						
c.181T>C (rs137863146)	HLA-DRB5	G/G	NM_002125.3	6; 32489871	TAT-CAT	p.61Tyr>His
c.200T>C (rs17878951)	HLA-DRB1	A/G	NM_002124.3	6; 32552056	GTG-GCG	p.67Val>Ala
c.1070A>T (rs193179333)	RHPN2	A/T	NM_033103.4	19; 33493188	CAC-CTC	p.357His>Leu
c.798G>C (rs117958555)	CYP2F1	C/G	NM_000774.3	19; 41628014	CAG-CAC	p.266Gln>His
c.785T>G (rs76684759)	KCNJ12	G/T	NM_021012.4	17; 21319439	ATC-AGC	p.262Ile>Ser
c.1007A>C (rs151218854)	FUT3	G/T	NM_000149.3	19; 5843844	GAT-GCT	p.336Asp>Ala

[€]**Chromosomal Position** = Chromosome no.; nucleotide position, ▲**CpG** = Cytosine phosphate Guanine

Table8: Genetic information of potential markers discovered in CML patients.

4.4dI. Prognostic markers

We discovered rs116201358 (c.107C>A) in C8A, rs4014596 (c.149G>A) in UNC93B1, rs52797880 (c.422T>C) in APOH and rs2274329 (c.209G>C) in CA6 as markers for good response as mutations were present in responding patients only. Amongst these mutations, rs116201358 (c.107C>A) and rs2274329 (c.209G>C) are expected to be most damaging (Polyphan score: 0.994 and 1 respectively), while rs4014596 (c.149G>A) was moderately damaging (Table9).

In treatment failure group, rs4945 (c.7C>A) in MFGE8 was discovered. The mutation lies in conserved region and expected to be moderately damaging (SIFT: 0.00, Polyphan score: 0.65) (Table9).

Mutation	Gene	Allele	MAF[▲] (dbsnp)	Mutation scoring				Patients (n[µ])		
				SIFT[€]	Polyphan	Grantham	GERP[α]	Responders (n=10)	Failures (n=8)	Total (n=18)
Markers for IM Responder										
c.107C>A (rs116201358)	C8A	A/C	0.021	0.00	0.994	107	5.09	5	0	5
c.1494G>A (rs4014596)	UNC93B1	C/T	0.048	0.00	0.744	21	2.8	5	0	5
c.422T>C (rs52797880)	APOH	A/G	0.066	0.00	0	89	3.37	5	0	5
c.209G>C (rs2274329)	CA6	C/G	0.0004	0.00	1	60	3.39	5	0	5
Marker for IM Failures										
c.7C>A (rs4945)	MFGE8	G/T	0.435	0.00	0.065	110	-0.479	0	4	4
CML Susceptibility Marker										
c.181T>C (rs137863146)	HLA-DRB5	G/G	0.011	0.00	0.021	83	1.02	7	5	12
c.200T>C (rs17878951)	HLA-DRB1	A/G	NA	0.00	0	64	-0.433	9	6	15
c.1070A>T (rs193179333)	RHPN2	A/T	0.031	0.00	0.989	99	4.61	6	4	10
c.798G>C (rs117958555)	CYP2F1	C/G	0.008	0.00	0.994	24	-4.65	7	2	9
c.785T>G (rs76684759)	KCNJ12	G/T	0.197	0.00	0.999	142	5.43	7	5	12
c.1007A>C (rs151218854)	FUT3	G/T	0.002	0.00	0	126	-4.57	6	5	11

[▲]**MAF** = Minor allele frequency of variant according to dbsnp (NCBI), [€]**SIFT** = Sorting Intolerant From Tolerant, [α]**GERP** = Genomic Evolutionary Rate Profiling' [µ]**n** = total number of patients in respective

Table9: Damaging potential of candidate variants related to CML prognosis and susceptibility

4.4dII. Susceptibility marker

HLA family genes HLA-DRB1 had rs17878951 (c.200T>C) and HLA-DRB5 had rs137863146. Other mutations observed in our cohort as CML susceptibility markers were rs193179333 in RHPN2, rs117958555 in CYP2F1, rs76684759 in KCNJ12 and rs151218854 in FUT3. Among these mutations, rs193179333, rs117958555 and rs76684759 were most deleterious as per SIFT, Polyphan, Grantham, PhastCons, and GERP scoring (Table9).

DISCUSSION

4.5. TGFβ-Smad pathway in CML

CML is diagnosed by the presence of BCR-ABL gene and treated by Imatinib mesylate (TKI) in first line setting. Alterations in BCR-ABL dependent and independent pathways are the cause of resistance to IM in CML (Hamad A, et al, 2013). TGFβ-Smad is one of the key BCR-ABL independent pathways, which has been extensively studied in normal and abnormal hematopoiesis (Kim SJ, et al, 2003). Alterations in this pathway have been implicated in lymphocytic (DeCoteau, JF, et al, 1997) and myeloid leukemias (Le Bousse-Kerdiles MC, et al, 1996) but its role in CML is not well established so far. TGFβ-Smad signaling is known to increase the hyper-responsiveness of CML cells leading to better response through BCR-ABL inhibition (Møller GM, et al, 2007). Though, this pathway inhibits the activation of AKT, which is a downstream component of BCR-ABL pathway, leading to release of inhibitory sequestration of FOXO that promote quiescence in CML stem cells, ultimately resulting in TKI resistance (Zhu X, et al, 2011; Naka K, et al, 2010). Present study attempted to explore more direct links between alterations in TGFβ-Smad signaling pathway and CML patients.

TGFβ1, cytokine, is a strong inhibitor of progenitor cell growth and differentiation, and its autocrine production maintains immature hematopoietic progenitor cells in quiescent state. Significant elevation was observed in TGFβ1 serum levels in CML patient group as compared to controls group. Higher levels of TGFβ1 have been observed in hematological malignancies (Liu X, et al, 2013) and solid tumors (Ciftci R, et al, 2014; Choi YJ, et al, 2015), which corroborate with our findings. Circulating TGFβ1 protein concentration levels were associated with mutation c.29C>T (rs1800470) in exon 1 of TGFβ1 gene (Wong TY, et al, 2003; Singh P, et al, 2013). We discovered this mutation in 50% of patients of the cohort selected for sequencing. Interestingly, elevated TGFβ1 levels were observed in 3 patients (serum levels available) harboring this mutation, though due to small number, the correlation between serum levels and 29C>T mutation couldn't be clearly demonstrated in our study. It lies in the conserved region and expected to be damaging by in silico analysis. It is speculated that Proline to Leucine (P10L) change modifies the peptide polarity, leading to change in protein transport rate (Wood NA, et al, 2000). We are the first to report this mutation in CML to the best of our knowledge.

A recent in-vitro study suggest BCR-ABL expression enhance TGFβ1 levels and TGFβ signaling activity in CML cell lines (Smith PG, et al, 2012), which prompted us to inquire whether increased serum levels in our cohort are also leading to increased signaling activity. Evaluation of TGFβ1 receptor transcript levels showed significantly reduced TGFβR2 expression, which probably hamper tumor suppressive effect of TGFβ1 in CML patients. The finding was similar with an earlier study, where decreased TGFβR2 levels were reported in CML patients compared to healthy individuals (Rooke HM, et al, 1999). The attempt to correlate the reduced transcript levels with genetic mutations in our cohort couldn't reveal significant observation as no mutation was present in enough number of patients to suggest such association. However, some important genetic variants were observed in TGFβR1 gene. Genetic variant, c.69A>G (rs868), present in 3' UTR of

TGFβR1, was found in 20% (4/20) patients. In silico analysis of this variant shows the mutation site to be the target for miRNA Let7f/miRNA98 (Figure13c). The Let7f/miR98 family is known to reduce TGFβR1 expression during embryogenesis and mutation in the binding region of this miRNA further reduces expression of gene (Tzur G, et al, 2009). Analysis of transcript levels in 4 patients having this mutation demonstrated reduced TGFBR1 transcript level, however no significant change in expression was observed in overall patient group (Figure13b). Out of these 4 patients, 3 were IM resistant and showed first relapse after consuming standard dose (400mg O.D.) of Imatinib Mesylate for 6 years or more. The fourth patient harboring this variant completed sixth year of standard IM treatment and was a good responder till the time of sample collection (Figure13b). Correlating this finding with clinico-demographic characteristics, this variant may play a role in late relapse. Though this claim requires concrete evidence in a larger cohort, the hint is worth attracting the attention. Another variant, c.1024+24G>A (rs334354) in intron 6 of TGFβR1, discovered in 40% (8/20) of our patients is an established genetic marker for increased susceptibility for cancer (Liu X, et al, 2013; Wu W, et al, 2015).

SMAD4, is key component of TGFβ-Smad signaling and an important marker in Colorectal cancer (CRC). Down regulation of SMAD4 in CRC is due to increased miRNA responsible for its controlled expression (Liu L, et al, 2013). SMAD4 deficient cells were observed in malignancies of diverse origins like oral epithelial cells, keratinocytes, mammary cells, bile duct, and odntoblasts (Bornstein S, et al, 2008; Qiao W, et al, 2006; Yang L, et al, 2005; Li W, et al, 2003; Xu X et al, 2006; Gao Y, et al, 2009) and leukemic cells of Chinese patients (Zhang Y, et al, 2006). Our study findings also revealed significantly reduced SMAD4 levels along with low TGFBR2 levels. SMAD4 is essential for the formation of heterologous complex with SMAD2 and SMAD3 and its translocation into the nucleus for expression of target genes. Its low expression can be another potential reason for containment of this tumor suppressor pathway.

In conclusion, CML patients have elevated TGFβ1 serum levels and c.29C>T is the major genetic variant among TGFβ1 gene mutations. Lower transcript levels of TGFβR2 can be the possible reason of decreased signaling activity that abolishes the tumor suppressor effect of the increased TGFB1 levels. Though no significant change in the transcript levels of TGFBR1 was observed in patients compared to control, TGFBR1 levels were reduced in the patients with c.69A>G variant. We also reported low levels of SMAD4 in CML. Previous studies have also reported similar findings in various other cancers including hematological malignancies such as acute myeloid leukemia and T-cell lymphoma (Singh P, et al, 2011; Go JH, et al, 2008).

Although our results are encouraging but being a single centric study sample size was the limitation. Multi-centric studies with more number of patients and detailed research on TGFβ - SMAD signaling pathway in different CML models is required to substantiate our findings.

4.6. Genetic Biomarkers

Discovery of Imatinib Mesylate in 1996 drastically changed the scenario of CML treatment. Despite the paradigm shift in prognosis, around 20% of patients fail to achieve expected remission (Quintás-Cardama A, et al, 2009). Increasing incidence of CML will soon convert it into a serious medical problem; therefore discovery of more prognostic markers which will help in proper outcome estimation and planning for personalized CML treatment is required. Susceptibility markers are also needed to control increasing incidence of CML. In search of potential prognostic and susceptibility markers, we performed NGS in 18 CML patients, classified as responder and failures. The key objective of the study was to find missense genetic variants, capable of predicting the outcome of patients treated with Imatinib Mesylate as frontline therapy and potential markers to detect healthy individuals prone to have CML. The susceptibility markers identified have been reported first time in the present study. Therefore, these findings are required to be validated in larger cohort of CML patients. Once validated, these markers can be tested in high risk population with selected criteria such as excessive medical or professional exposure to ionization radiations, exposure to chemicals like formaldehyde, benzene and dioxins and heavy cigarette smoking, which is more prone to develop CML. As per criterion for prognostic markers, we demonstrated mutations in 5 genes as markers. Out of these 5 variations observed, mutation in MFG-E8 was in failure group, whereas 4 mutations, discovered in responders, were in genes C8A, UNC93B1, APOH and CA6.

4.6a. Prognostic Markers

MFG-E8 (Milk Fat Globule–Epidermal growth factor-8), is primarily responsible for production of membrane glycoprotein lactadherin, which promotes phagocytosis of apoptotic cells. In melanoma cells MEG-E8, promotes progression by triggering epithelial to mesenchymal transition, stimulated invasion and immune suppression, whereas the high expression is linked with disease progression in oral cancer, breast cancer, prostate cancer and colon cancer (Jinushi M, et al, 2008; Yamazaki M, et al, 2014; Carrascosa C, et al, 2012; Soki FN, et al, 2014; Kusunoki R, et al, 2015). As this gene was found to be involved in progression of many cancers, rs4945, discovered in our study was also expected to be associated with IM treatment failure in CML.

Complement component 8-alpha (C8A), encodes alpha subunit of C8. C8A participate in the formation of membrane attack complex (MAC). Elevated RNA levels of C8A gene were associated with better prognosis of Hepatocellular carcinoma (Awan FM, et al, 2015) and in breast cancer patients, treated with trastuzumab (Willis S, et al, 2015). We found a deleterious mutation, rs116201358 in responders, which seems to be related with better treatment outcome similar to elevated expression in earlier studies.

UNC93B1 (Unc-93 homolog B1) is involved in regulation of toll like receptor signaling. The encoded protein traffics nucleotide sensing receptors to endolysosomes from endoplasmic receptor. It is an IFN1 signature gene, induced by chemotherapy cyclophosphoamide. IFN1 has recently been found involved in inducing anti-tumor

immunity (Moschella F, et al, 2013). Impaired UNC93B1 dependent immunity is also associated herpes simples virus 1 encephalitis (HSE) pathogenesis in children (Lafaille FG, et al, 2012). We observed that presence of mutation rs4014596 in majority of responder patients associates it with better prognosis, due to potential role in anti-tumor immunity.

APOH (Apolipoprotein H) is linked with several pathways including lipoprotein metabolism, coagulation and the production of antiphospholipid antibodies. Its elevated serum levels found to be associated with incidence of colorectal cancer (Ma Y, et al, 2012), better prognosis in Estrogen Receptor negative (ER-ve) breast cancer and acute myeloid leukemia (Chung L, et al, 2014; Lee SW, et al, 2012). We observed mutation rs52797880 in IM responding cohort, which corroborated with the previous findings correlating elevated serum levels with better prognosis in breast cancer and AML.

Carbonic Anhydrase 6 (CA6), involved in reversible hydration of carbon-di-oxide in saliva. Strong expression of this gene is associated with serous adenocarcinomas of ovary and endometrium (Smith NL, et al, 2001; Kearse KP, et al, 2000), in carcinomas of breast, pancreas, urothelium and cancers of renal and pulmonary origin (Smith NL, et al, 2002). In our cohort 60% of the responding patients had mutation rs2274329 which makes it a potential candidate as marker for better prognosis.

Co-occurance of different genetic variants in an individual or a group of patients is considered as haplotype marker (The International HapMap Consortium, 2005). In this sudy, we observed the co-existence of rs116201358 with rs4014596 and rs4014596 with rs2274329 in 4 and 3 IM responding patients respectively (Table7). This observation suggests coexistence of mentioned variants can be potential haplotypes for good prognosis in CML patients.

4.6b.Susceptibility Markers

In our search for CML susceptibility markers, we observed rs17878951 and rs137863146 in HLA-DRB1 (Major Histocompatibility Complex Class II, DR Beta1) and HLA-DRB5 in CML patients. Both of the genes are participants of Interferon-gamma and CXCR4 signaling and play key role in antigen presentation. Variants of HLA-DRB1 were found to be associated with incidence of CML in different populations throughout the world (Barion LA, et al, 2007; Yasukawa M, et al, 2000; Naugler C, et al, 2009; Rivera-Pirela SE, et al, 2016; Wang XJ, et al, 2014) and over expression of HLA-DRB5 is a potential susceptibility marker of Lung cancer (Li ZH, et al, 2016), whereas the genetic variants are markers for CLL (Hojjat-Farsangi M, et al, 2008) and breast cancer (Yang XX, et al, 2011). Another variant (rs193179333) of RHPN2 (Rhophilin Rho GTPase Binding Protein 2) gene was also observed in 10 CML patients of our cohort. It codes for Ras Homologous (Rho)-GTPase binding protein, which binds to GDP and GTP bound RhoA and RhoB, which are involved in organization of actin cytoskeleton. Mutations of RHNP2 are linked with suspicion of colorectal cancer incidence (Carvajal-Carmona LG, et al, 2011), prognosis of lung adenocarcinoma and malignant glioma (Wu K, et al, 2015; Danussi C, et al, 2013). The polymorphism of CYP2F1 (Cytochrome P450 family 2

subfamily F member 1) gene linked to lung cancer in different ethnicities (Tournel G, et al, 2007), whereas differential expression are associated with prognosis of ovarian and breast cancer (Kumarakulasingham M, et al, 2005; Downie D, et al, 2005; Calaf GM, et al, 2007). In this study rs117958555 was founds in majority of CML patients which hints towards being a susceptibility marker. In normal circumstances it involved in drug metabolism, hence genetic change in the gene can be possibly related to pathogenesis of CML. However, FUT3 (Fucosyltransferase 3) is involved in embryogenesis, tissue differentiation tumor metastasis and inflammation. Incidence of gastro-intestinal and breast cancer are linked to FUT3 genetic variations (Duell EJ, et al, 2015; do Nescimento JC, et al, 2015). Similarly, we also discovered rs151218854 in FUT3 related to incidence of CML. Another gene, KCNJ12 (Potassium Voltage-Gated Channel Subfamily J Member 12), contributes to cardiac inward rectifier current (IK1) is not reported in association with cancer to the best of our knowledge. We noticed rs76684759 in KCNJ12 in more than 70% of our patients. The mutation is present in conserved region (SWIFT Score: 0) and highly damaging (Polyphan: 0.999), which makes it a possible candidate to be a susceptibility marker of CML.

Over all, we discovered 11 missense mutations, which are potential prognostic and susceptibility markers. Variants in C8A, UNC93B1, APOH and C6A genes were found in IM responders whereas variant of MFGE8 was present in IM failures. Susceptibility markers were discovered in HLA-DRB1, HLA-DRB5, RHPN2, CYP2F1, KCNJ12 and FUT3. All the genes were associated with cancer or cancer related functions. The study is a step forward to discover more reliable and accurate markers for selecting individuals prone to CML and estimate the outcome of treatment. Findings of the study can be useful in individualizing cancer treatment after validation of all the discovered mutations in larger cohort at genetic level and at functional level.

To conclude, **w**e discovered 11 missense mutations, which are potential prognostic and susceptibility markers. Variants in C8A, UNC93B1, APOH and C6A genes were found in IM responders whereas variant of MFGE8 was present in IM failures. Susceptibility markers were discovered in HLA-DRB1, HLA-DRB5, RHPN2, CYP2F1, KCNJ12 and FUT3. All the genes were associated with cancer or cancer related functions. The study is a step forward to discover more reliable and accurate markers for selecting individuals prone to CML and estimate the outcome of treatment. Findings of the study can be useful in individualizing cancer treatment after validation of all the discovered mutations in larger cohort at genetic level and at functional level.

CHAPTER 5:

SUMMARY AND CONCLUSION

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The present study evaluate the role of TGFβ-Smad signaling pathway in CML. Results of study demonstrate significantly elevated serum levels of TGFβ1 in CML patients relative to healthy controls, although no such difference was observed between Imatinib resistant and responder groups. This indicates that levels of TGFβ1 might have role in pathogenesis of CML, but not related to prognosis. The question of loss of tumor suppressive effect of the pathway, despite increased expression of TGFβ1, was answered in investigation its downstream components. In the expression analysis of TGFβ receptors, decreased levels of TGFβR2 were observed in CML patients, whereas no noticeable change was presented by expression levels TGFβR1. Further downstream of receptors, SMAD4 was also significantly down regulated. The findings clearly indicate that even if TGFβ1 levels were elevated, its effect will not be incorporated in expression of target genes as lower expression of TGFβR2 will diminish the effect. Moreover decreased expressions of SMAD4, do not allow the hetrologous complex involving SMAD2 and SMAD3 to translocate to nucleus in sufficient quantity required for expression of target genes. We also evaluated expression of Inhibitor Smad, Smad 7, to inquire if its expression also had any impact of TGFβ-Smad pathway in CML patients. No significant different was observed between patients and controls or between resistant and responding patient groups. So, we concluded that despite increased serum levels of TGFβ1, TGFβ-Smad pathway do not show its tumor suppressive property in CML due to lower expression TGFβR2 and SMAD4.

To inquire whether the differential expression of these genes was due to any genetic alteration, we sequenced exomes of the study genes, whose expression was analyzed in CML patients. We observed several mutations in different genes, as described in previous chapters, but few of them very important and were related to expression. In TGFβ1, c.29C>T (rs1800470) was present in 50% of patients and was associated with elevated serum levels. In TGFβR1 gene, c.69A>G (rs868) mutation, present in 3' UTR, was target site of miRNA Let7f/miRNA98 and hence was found related with depleted levels of the gene. There were several other mutations observed in other genes, some of which were present in majority of patients, but unfortunately they were not related with the altered expression levels of the respective genes.

Along with study of TGFβ-Smad pathway, with also searched for potential susceptible and prognostic markers by performing Clinical Exome sequencing, a panel of 4800 clinically significant genes in CML patients using NGS. Results were encouraging as we discovered new markers related to resistant, good response and susceptibility of CML. Variants in C8A, UNC93B1, APOH and C6A genes were found in IM responders whereas variant of MFGE8 was present in IM failures. Susceptibility markers were discovered in HLA-DRB1, HLA-DRB5, RHPN2, CYP2F1, KCNJ12 and FUT3. All the genes were associated with cancer or cancer related functions.

The study is a step forward enhancement of knowledge about the role of TGFβ-Smad pathway in CML, though the results are required to be validated in bigger sample size and different CML model. After validation, we suggest, molecules capable of boosting

the expression of receptors and common smad, can be used in combination with other therapies in the initial stages, when the pathway acts as tumor suppressor, whereas inhibitors of same can be used in advanced stages when it get converted to tumor proliferator. Our findings have the potential of attracting the attention of researchers towards a new direction in CML therapy as most of the treatment strategies are based on BCR-ABL and its downstream components, but our results demonstrate the significance of BCR-ABL indepedant pathways.

Susceptibility and prognostic markers discovered in the study have been reported first time. Therefore, these findings are also needed to be further validated in larger cohort of CML patients. Once validated, these markers can be tested in high risk population which is more prone to develop CML compared to population. These markers have the capability to detect healthy individuals who are more prone to have CML in their lifetime. These markers can be recommended to high risk population with selected criteria such as excessive medical or professional exposure to ionization radiations, exposure to chemicals like formaldehyde, benzene and dioxins and heavy cigarette smoking. These recommendations will reduce the probability of having CML in such population. Moreover, prognostic markers, after validation, will be useful in predicting the reponse on Imatinib mesylate as firsline therapy and hence could be used to plan personalized therapies for CML patients.

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