Università degli Studi di Milano Bicocca

Scuola di Medicina e Chirurgia

Dottorato in Ematologia Sperimentale, XXVII ciclo

Elucidating the oncogenic role of early genetic events in chronic myeloid leukemia

Dr. Praveen Khandelwal

Coordinator: Prof. Carlo Gambacorti-Passerini

Tutor: Dr. Luca Mologni

Academic Year: 2014-2015

Abstract

In hematological myeloid malignancies the accumulation of oncogenic events plays a significant role in disease progression. Therefore, we studied the mutational landscape in chronic myeloid leukemia (CML) patients at diagnosis through functional genomic approach. In the last few years, Next Generation Sequencing (NGS) provided an important new research technique to study the molecular pathogenesis of cancer. We conducted a mutational analysis on 23 chronic phase *BCR-ABL1*+ CML patients through NGS (exome and RNA sequencing) performed on samples collected at diagnosis. A total of 107 non-synonymous variants (range 0-11 per patient) were identified by setting a threshold of mutation frequency >25%, which corresponds to the presence of a heterozygous mutation in >50% of cells, assuming a pure tumoral sample. A positive correlation was observed between number of mutations and patient age, indicating that several events were passenger mutations, being expanded by the neoplastic transformation. However, when using a newly in-house developed tool (Oncoscore) to weigh the oncogenic potential of each mutation, a significant correlation was observed between the Sokal score and Oncoscore by using linear model statistical analysis. In long term follow-up (>2 years), 21 CML patients achieved complete cytogenetic responses (CCyR) and 2 failed to achieve any cytogenetic response with tyrosine kinase inhibitors. These two patients showed an Oncoscore value of 165.4±27.20(SEM) which was significantly higher than the one (80.59 ±12.72) in the 21 responding patients. No fusions (other than BCR/ABL1) were identified by RNA Seq, and no chromosomal alterations were observed by using the CEQer software. In conclusion, CML patients at diagnosis carry genetic alterations additional to the BCR/ABL1 fusion, which could be relevant for response to treatment and progression of the disease.

In addition, whole exome sequencing of 10 blast crisis CML patients was performed. We identified a *SETBP1* mutation, which was also frequently mutated in other hematological malignancies and considered as biologically relevant. To study biological and functional properties of Setbp1 and its role in neoplastic transformation, we set out to build a murine conditional knock-in model of Setbp1 p.G870S.

Table of Contents

1. Introduction

1.1 Hematological malignancy

Cancer in hematopoietic system was first explored in the 19th century and since then constant progresses has led to significant improvement in patient survival with early diagnosis along with improved treatment strategies [1]. The hematological malignancies can stem from different cell progenitors such as myeloid or lymphoid progenitors. They include various forms of leukemia, lymphomas and myeloma. The biology of these hematological disorders and their clinical relevance are different from one to another. Physiologically, the core component of hematological system is the hematopoietic stem cell (HSC). HSCs are defined as cells with the ability both to self-renew and to differentiate into immature progenitor cells, which in turn triggers further differentiation into mature blood cells. Generally, HSCs are sub-divided into long term (LT) and short term (ST) HSC cells that are capable of having an indefinite and limited self-renewal proliferating potential respectively [2]. A self-renewal property is essential for LT stem cells to indefinitely differentiate into mature blood cells, such as myeloid (erythrocytes, platelets, granulocytes and macrophages), lymphoid (T-cells, NK-cells and B-cells) and dendritic cells via multipotent progenitors (MPP).

John Dick *et al.* first explained the concept of leukemic stem cells (LSC) in acute myeloid leukemia; they mentioned that a small portion of leukemic cells were able to proliferate, self-renew and propagate cancer [3, 4]. Cancer stem cells were observed in many major neoplasms such as leukemia, lymphomas, breast cancer, glioblastoma, neural cancer and multiple myeloma [5, 6]. It has been observed that an even minor portion of leukemic stem cells brings disease transformation on overtime accumulation of oncogenic events.

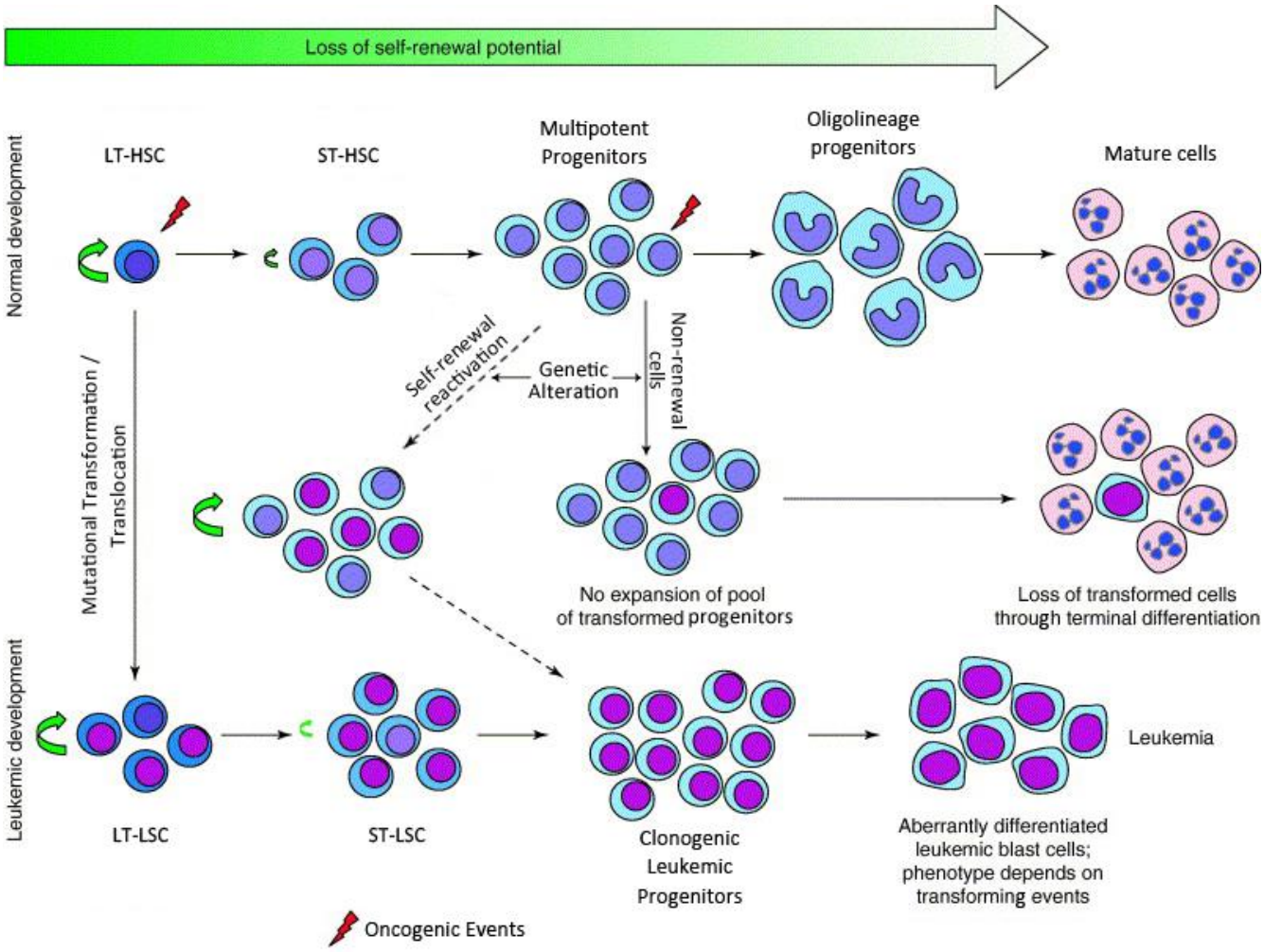

Figure 1: Systematic model of normal and cancerous stem cell hematopoietic system. In human hematopoietic stem cells (HSCs), small populations of cells are sustaining itself by self-renewal idiosyncrasy. Long term (LT) HSC and short term (ST) HSC are the progressively lineages of HSC are transformed into less self-renewal restricted progenitor cells which further produce fully functional mature blood cells. Disturbance of self-renewal and differentiation signalling pathways are possible by accumulation of different incidences such as chromosomal translocation and/or evolution of mutation in leukemic stem cells (LSCs). LSCs possess the capability of sustained self-renewal pathways impaired differentiation properties into mature hematopoietic cells and transformed into self-sufficient survival and more aggressive leukemic blast cells. Figure refer from [3].

Oncogenic events responsible for leukemic transformation may be due to one or more occurrences of chromosome rearrangement or genetic alteration during haematopoiesis [7]. On *Invivo* characterization of a humanized CML-CP mouse model, patient CD34+ cord blood cells were transplanted into NOD/SCID mice and after a few weeks growth of myeloid cells with specific population of erythroid and megakaryocytic lineages were observed. Additionally, there were also an elevation of white blood cells along with over- size spleen caused by increased infiltration of myeloid cells [8]. In figure 1, it is shown that leukemic development depends on intrinsic or extrinsic oncogenic episodes dysregulating genes associated with self-renewal pathways that can transform normal cell development into cancer forming cells.

The self-renewal signalling pathways WNT and BMI1 significantly drive the development of normal stem cell and also similarly regulate cancer stem cells [9, 10]. In cancer, deregulation of their function could be caused by genomic instability that significantly disturbs the normal self-renewal system and turns them into highly dysregulated self-renewal pathways. Genetic abnormality constitutively activates several transcriptional responses that increase self-renewal process in cancer stem cells. For example, loss of function mutations in APC stabilizes the cytoplasmic fraction of β-catenin protein. β-catenin is a transcriptional co-activator and translocate into the nucleus where it activates the Tcf/LEF family of transcription factors, thus driving transcription of Tcf target genes, including c-MYC and Cyclin D1 (Figure 2) that profoundly increase cell proliferation [11].

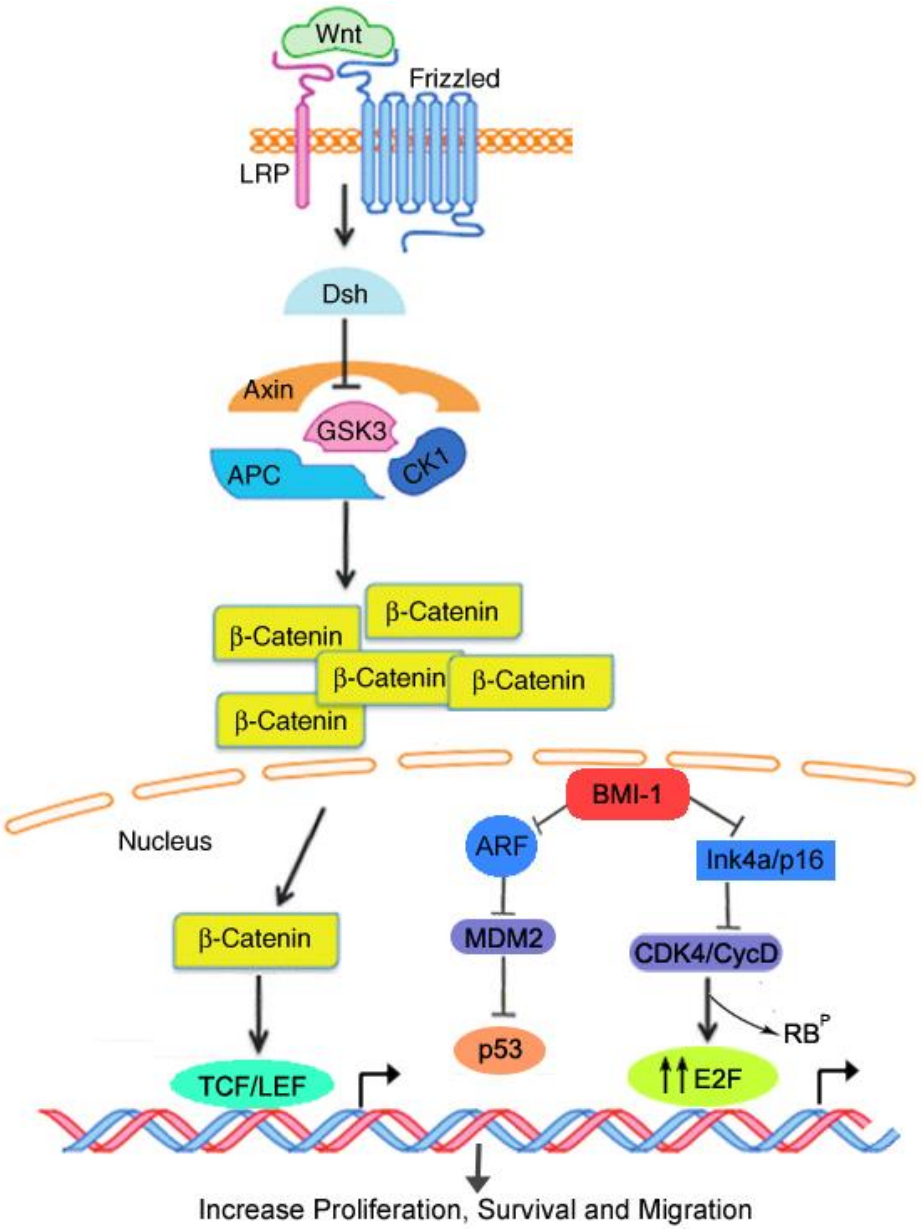

Figure 2: WNT and BMI1 signalling pathways in cancer stem cell. Extrinsic binding of Wnt ligand to extracellular frizzled receptor that activates cytoplasmic Dishevelled (Dsh) in turn inhibit the GSK3β, CK1, APC and Axin complex formation and thus inhibit the degradation of β-catenin and transport into nucleus and initiates several transcription factors. On the other-side, BMI-1 function in cancer cells induces anti-apoptosis and cell proliferation process. Thus, the activities were attributed through inhibiting tumour suppressor protein ARF and blocking cyclin dependent kinase inhibitor Ink4a/p16

respectively. BMI1 therefore promotes cell proliferation and inhibit cell death activity. Pathway refers from [12, 13]. Note: ^{P} represents phosphorylation.

BMI1 in cancer stem cells downregulates the transcription activation of cyclin dependent kinase inhibitor INK4A (p16) and tumour suppressor ARF (p14) and constitutively activates self-renewal pathway (Figure 2). Overexpression of BMI1 abnormally regulates cell proliferation activity through phosphorylation of retinoblastoma protein (RB) and by blocking INK4A, which eventually activates a group of transcription factors (E2F) that result in initiation of cell cycle process from G1 to S phase [14]. In another mechanism, BMI1 also induces anti-apoptotic activity by blocking ARF expression, which inhibits MDM2 phosphorylation and therefore causes downregulation of p53 pro-apoptotic protein and thus results in survival of cancer cells [15]. There are also other possibilities that might cause an expansion of cancer stem cells refractory to anti-cancer therapy either with chemotherapy or TKIs [12].

1.2 Revised 2008 WHO guidelines for myeloid malignancy

The 2008 WHO classification system (revised version of the 2001 WHO classification) categorised myeloid malignancies depending on their pathological behaviour such as acute myeloid leukemia (AML), Myelodysplastic syndrome (MDS) and myeloproliferative neoplasm (MPN). In 2001, CML was categorised under myeloproliferative disorders, where now ‘disorder’ was replaced with ‘neoplasm’ in order to accurately reflect the neoplastic nature of the disease, refer to table 1 [16, 17].

Table 1: Classification of Myeloid Neoplasms According to the 2008 World Health Organization Classification Scheme
1. Myeloproliferative neoplasms (MPN)
1.1. Chronic myelogenous leukemia, *BCR-ABL1*–positive (CML)
1.2. Polycythemia Vera (PV)
1.3. Essential thrombocythemia (ET)
1.4. Primary myelofibrosis (PMF)
1.5. Chronic neutrophilic leukemia (CNL)
1.6. Chronic eosinophilic leukemia, not otherwise specified (CEL-NOS)
1.7. Mast cell disease [18]
1.8. MPN, unclassifiable
2. Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA, PDGFRB*, and *FGFR1*
3. MDS/MPN
3.1. Chronic myelomonocytic leukemia (CMML)
3.2. Juvenile myelomonocytic leukemia (JMML)
3.3. Atypical chronic myeloid leukemia, *BCR-ABL1*–negative (aCML)
3.4. MDS/MPN, unclassifiable
4. Myelodysplastic syndromes (MDS)
5. Acute myeloid leukemia (AML)

Another major change in this revised classification is a new class of myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA, PDGFRB* and *FGFR1,* which was previously subcategorized into chronic eosinophilic leukemia-not otherwise specified (CEL-NOS). The therapeutic regimens of class 2 patients categories into *PDGFR* mutation achieve good therapeutic responses on treatment with imatinib mesylate used as a *PDGFR* inhibitor. For the MDS/MPN class,

no changes have been introduced, therefore the document related to this family is similar to the 2001 WHO classification system [17]. My project was mainly focused on the oncogenic role of genetic variants identified in chronic myeloid leukemia (CML) patients at onset.

1.3 *Chronic Myeloid Leukemia (Ph+)*

Chronic myeloid leukemia is a hematological disorder (2008 WHO classified into class I MPN) characterized by inappropriate expansion of myeloid cells, which is caused by expression of the *BCR-ABL1* fusion gene [18, 19]. The fusion gene is derived from reciprocal translocation between 3' of *ABL1* on chromosome 9 to 5' of *BCR* from chromosome 22, termed as Philadelphia chromosome (Ph) [20]. The incidence of CML is very rare, with about 1 to 2 cases among 100,000 people annually. Gender-wise, men are more affected than women [21].

1.3.1 CML biology

In 1840s, the first case of leukemia was described by David Craigie from Scotland, with splenomegaly and elevated leucocytosis as key clinical features. In the following five years, more cases of CML were identified in France and Germany [22, 23]. The history of the findings in chronic myeloid leukemia since first discovery is summarized in table 2.

Table 2.	Timeline in chronic myeloid leukemia since discovery
1842	Report of probable case of leukemia by Donne in Paris
1845	Recognition of leukemia (probably CML) as a disease entity
1846	First diagnosis of leukemia in a live patient
1880s	Development of methods for staining blood cells
1951	Dameshek introduces the concept of myeloproliferative disorders
1960	Identification of the Philadelphia chromosome (22q-)
1973	Recognition of the reciprocal nature of the (9;22) translocation
1984	Description of the breakpoint cluster region (BCR) on chromosome 22
1985	Identification of the *BCR-ABL1* fusion gene and p210-Bcr-Abl
1989	Development of a reverse transcriptase PCR for measuring BCR-ABL transcripts
1990	Demonstration that the *BCR-ABL1* gene can induce a CML-like disease in mice
1996	Demonstration of selective blocking of Bcr-Abl1 kinase activity
1998	Blocking Bcr-Abl1 kinase activity reverses features of CML
2001	Recognition of non-random mutations in the Abl1 kinase domain

A century later, in 1960, Nowell and Hungerford were the first to find the presence of a minute chromosome as the basic etiology in chronic myeloid leukemic patients [24]. Since then, a similar observation was made by other researchers as well. The minute chromosome was later termed as Philadelphia (Ph) chromosome. Around 1970, a visualisation of minute chromosome was made possible with the application of fluorescence and Giemsa staining. On further characterisation, it was found that the Ph chromosome consists of a long arm of chromosome 22 that is fused with the short arm of chromosome 9 as shown in Figure 3 [25].

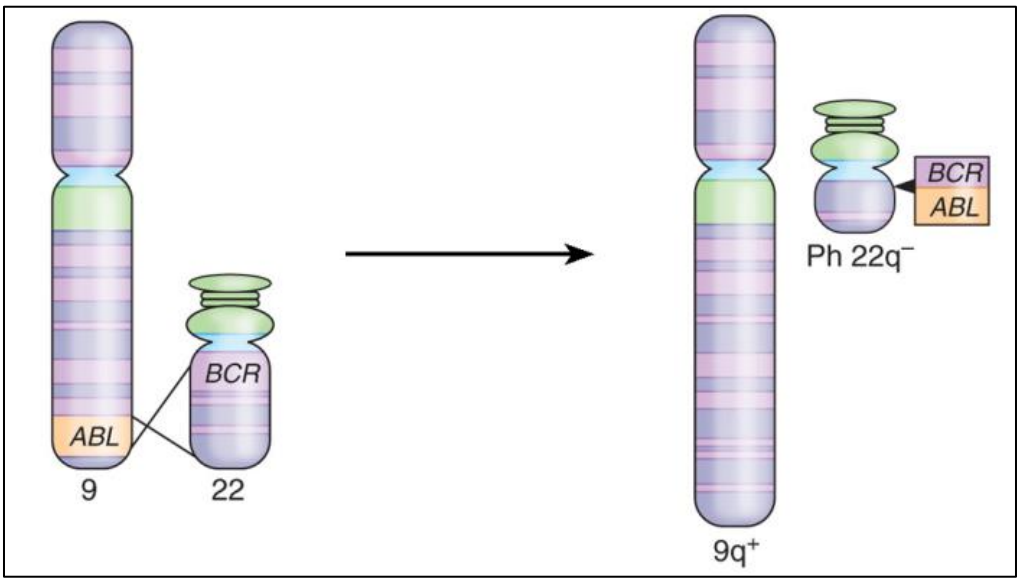

Figure 3: Illustrative picture of reciprocal rearrangement of Chromosome 9 and 22 to form a Philadelphia chromosome. On the left represent the normal chromosome, ABL and BCR situated on the long arm of both chromosome 9 and 22 respectively, and in right newly formed chromosome $9q^{+}$ and chromosome 22 derivative $22q^{-}$ that later termed as Ph^{+} [26].

The Ph chromosome was identified in more than 90% of CML cases and in 10-15% of children with B-ALL (Acute B Lymphoblastic Leukemia) [27]. The disease pathophysiology is best described according to their phases (Table 3). Initially, a chronic phase lasting on average 4 to 5 years [28] is characterized by the presence of both mature and immature cells along with confinement of differentiation in haematopoiesis [29]. This is followed by an accelerated phase in which the disease phenotype is rapidly moving in comparison with the chronic phase, characterized by the presence of at least 10% blastic population in bone marrow or in peripheral blood and reduction of HSC differentiation pattern [30, 31]. In the terminal blast crisis, the disease is most aggressive and holds features like complete differentiation block and large accumulation of genetic/chromosomal abnormalities such as Myc overexpression and/or trisomy of Chr8 [32], mutation in cancer causing genes, deletion or rearrangement of tumour suppressor genes like p16 [33, 34]. In 80% of CML cases,

a high incidence of genetic alterations is the main reason for blastic transformation along with *the BCR-ABL1* fusion gene [35].

Table 3: Phases of Chronic Myelogenous

CML phase	WHO definition
Chronic phase	Peripheral blood blasts fewer than 10% in the blood and bone marrow
Accelerated phase	Blasts 10-19% of white blood cells in peripheral and/or nucleated bone marrow cells ; persistent thrombocytopenia (< 100×10^9/L) unrelated to therapy or persistent thrombocytosis (> 1000×10^9/L) unresponsive to therapy; increasing white blood cells and spleen size unresponsive to therapy; cytogenetic evidence of clonal evolution
Blast crisis	Peripheral blood blasts ≥ 20% of peripheral blood white blood cells or nucleated bone marrow cells; extramedullary blast proliferation; and large foci or clusters of blasts on bone marrow biopsy

1.3.2 BCR-ABL1 Rearrangement

The actual reason for *BCR-ABL1* translocation is still unknown; a retrospective study on 443 CML patients showed that patients with prior exposure to ionizing radiation have lower tumour burden and prolonged survival as compared to *de novo* CML patients [36]. However, in the vast majority of CML cases we are not aware of any known predisposing factors [23]. The pathophysiology of CML is largely associated to the ABL1 tyrosine kinase activity. The *BCR-ABL1* fusion gene transcribes into a new fusion transcript that translates into a fusion protein with abnormal *ABL1* tyrosine kinase activity, under the control of the fused *BCR* endogenous promoter.

The normal *c-ABL1* (Abelson murine leukemia viral oncogene homolog 1) non-receptor tyrosine kinase is universally expressed and consists of several structural and functional domains and

generates a 145KD protein [37] (Figure 4). The amino terminal of c-Abl consists of three Src-homology (SH) domains, of which SH1 has tyrosine kinase activity and SH2 and SH3 domains are responsible for interaction with other proteins such as CrkL, Grb2 and Nck [38]. The Abelson protein structure also consists of 3 Proline-rich regions, nuclear localisation signals, DNA binding and actin binding domains at the carboxyl terminal portion [39-41]. Basically, normal ABL is involved in cell cycle regulation and in signal transduction between cellular environments [42].

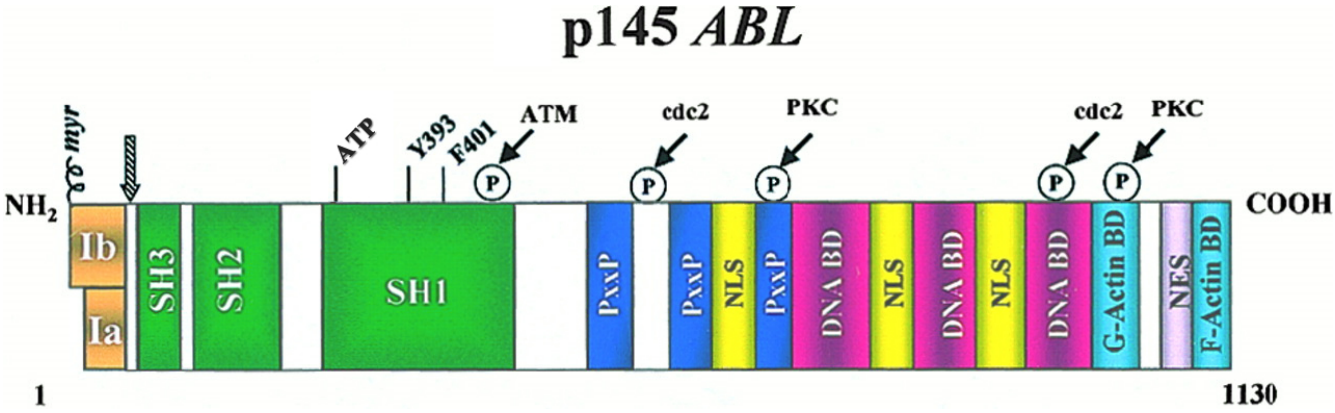

Figure 4: *ABL1* protein structure. Referred from [43].

The fusion partner in BCR-ABL1 is the breakpoint cluster region (*BCR)* gene, which is also ubiquitously expressed. The structural layout of *BCR* is shown in figure 5. The amino terminal region consists of a serine-threonine kinase domain (a member of the 14-3-3 family of proteins), an oligomerization domain and two cyclic adenosine monophosphate (cAMP) dependent kinase homologous domains. The middle portion contains Dbl-homology domain followed by a pleckstrin-homology (PH) motif; this unit works as a guanine nucleotide exchange factor for Rho family GTPases (Rho-GEF) [44-46]. The carboxyl terminal region consists of a Rac-GAP domain that regulates the activity through Rac protein to initiate polymerisation of actin and NADPH oxidase activity [47, 48]. *BCR* is also involved in the activation of Ras pathway via Grb2 binding, mediated by

autophosphorylation of a tyrosine residue at position 177. The physiological role of *BCR* is not well defined [49, 50].

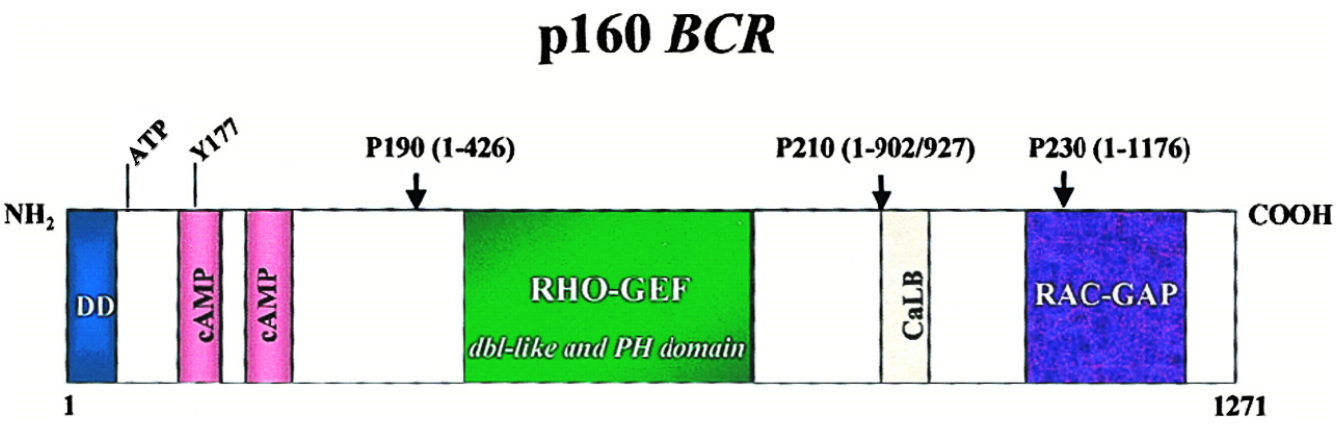

Figure 5: *BCR* protein structure. Refer from [43]

The Ph Chromosome carries the fusion of *ABL1* and *BCR* genes that are located respectively on 9q34 and 22q11. Breakpoint takes place on *ABL1* more frequently in a region between upstream of exon 1b and downstream of exon 1a, while in BCR (breakpoint cluster regions) it occurs at three different position [51]. The size of the fusion protein varies according to the different breakpoints on the BCR gene (Figure 6b). The breaks between exons 12-16 (major breakpoint cluster region, or M-*bcr*) generate a 210KD chimeric protein translated from the spliced fusion transcript of either b2a2 or b3a2 junction. The other 2 break points localize at exon 2 (minor breakpoint cluster region, m-*bcr*) and at exon 19 (µ-*bcr);* the resultant fusion protein from m-*bcr* is 190KD and µ-*bcr* is 230KD [43]. The p210 fusion protein is the major form observed in CML (>95% of cases). The 190KD fusion protein was predominantly detected in 2/3 of Ph+ B-ALL patients and 230KD fusion protein was associated with a rare Ph+ chronic neutrophilic leukemia [52].

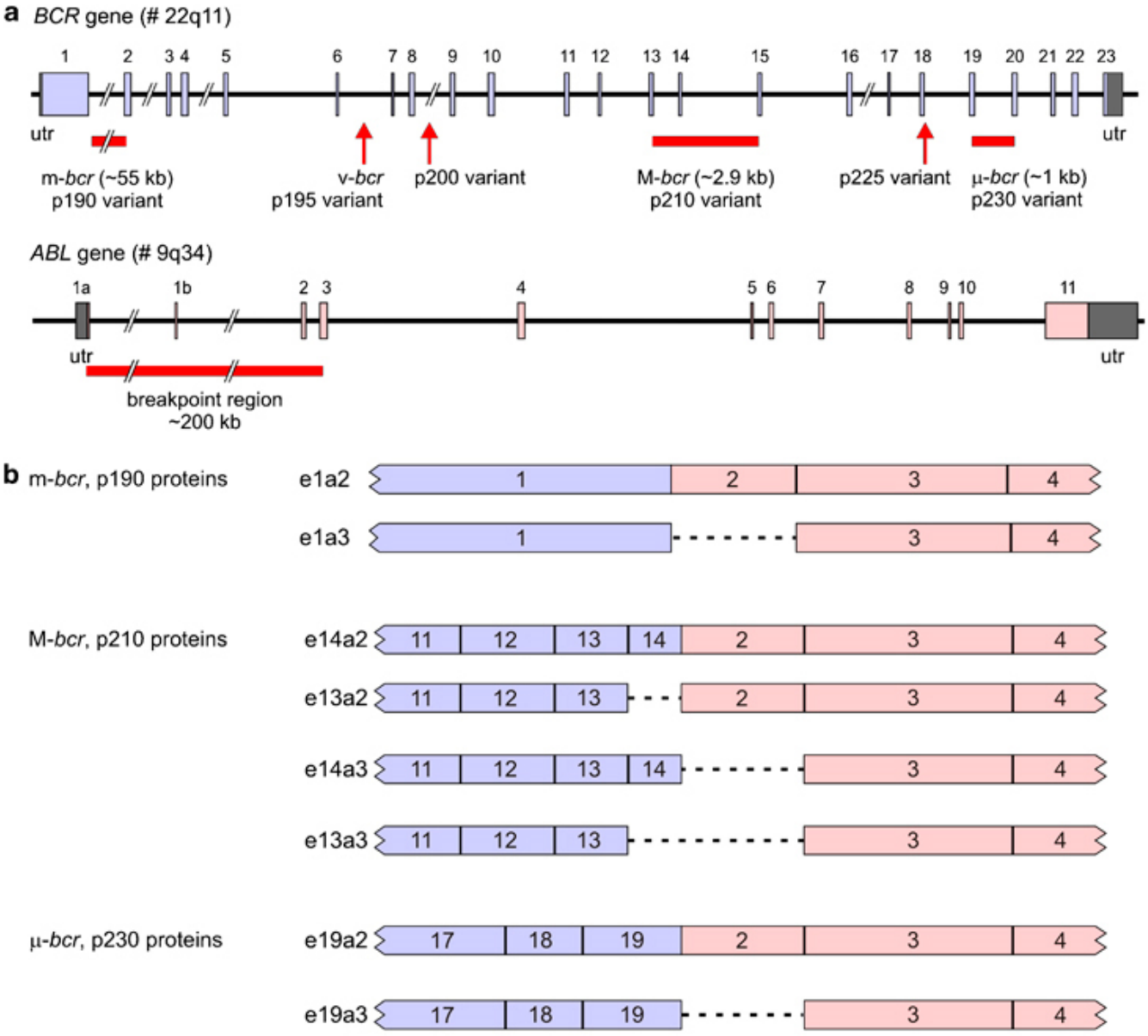

Figure 6: The genomic structure of *BCR* and *ABL* gene and different fusion protein derived from the various breakpoints on *BCR* gene. a) one large breakpoint on ABL gene occurred within ~200bp long region represent with red bar, whereas 3 breakpoint regions on BCR gene termed as m-*bcr*, M-*bcr*, μ-*bcr* (Indicated in red bar) that transcribe into a p190, p210, p230 fusion protein respectively. The red arrow indicates the rare breakpoint held in *BCR* gene that results into 3 different *BCR-ABL1* fusions and translates into a protein of p195, p200, p225 KD [53]. b) The 3 different fusion proteins derived

from the different breakpoints on BCR and alternative splicing from *ABL* gene. p190 and p230 has 2 different transcripts each and p210 has 4 different transcripts as illustrated. Figure referred from [54].

1.3.3 Function of BCR-ABL1 in Leukemia

There are various factors and signalling pathways, which are profoundly regulated by the *BCR-ABL1* oncogene, that result in the development of advanced leukemia (Figure 7). The BCR-ABL1 onco-protein deregulates the tyrosine kinase activity of *ABL* and induces pathogenesis of Ph+ human leukemia [55]. Several downstream signalling pathways were determined for disease progression through *BCR-ABL1* dependent mechanisms via altered cell adhesion properties, increased mitogenic activation and inhibition of apoptosis [56-58].

The *BCR-ABL1* tyrosine kinase (TK) remains constitutively active [59], while in normal *ABL1* the TK activity is well controlled and regulated under physiological pressure. The SH3 domain of c-*ABL1* plays a crucial role in the inhibitory process of TK; this inhibitory activity is initiated by interaction of SH3 with Abi-1 and 2 (Abl interactor proteins 1 and 2) [43].The N-terminal myristoyl group of c-Abl regulates auto-inhibition of the kinase through a conformational alteration that allows binding of SH2 and SH3 domains to the kinase domain and blocks the catalytic activity of Abl. The above mechanisms are disrupted in cells with *BCR-ABL1* fusion gene [60].

During *BCR-ABL1* fusion, the 5’ BCR sequences fused to the 3’ ABL, which destroys the SH3 domain and N-terminal myristoyl group and abrogates the auto-inhibited conformation of the kinase, thus making the TK domain constitutively active [43]. The CML mouse model had shown that *ABL1* kinase domain plays a crucial role in *BCR-ABL1* dependent leukemogenesis [61]. Gambacorti-Passerini *et. al*. has also demonstrated that inhibition of ABL1 kinase activity causes a marked

decrease in leukemic cells population *invitro* from a comparison between Ph+ and Ph- cell lines [62]. Besides the kinase domain, there are also other important motifs in *BCR-ABL1* that regulate binding to downstream effectors such as GRB2, PI3K, SRC, CRKL, CRK and CBL and activate downstream signalling pathways [29].

1.3.4 BCR-ABL1 mediated downstream signalling

There are numerous *BCR-ABL1* downstream signalling pathways, which can regulate cell growth and survival of leukemic cells [63]. In figure 7, the flowchart represents the signalling pathways and intermediate factors regulated by BCR-ABL1 gene, which are associated with oncogenic signal transduction.

1.3.4.1 Defective cell adhesion

Cell adhesion is the process where cells interact with other cells or with the extracellular matrix. CML progenitor stem cells lack the adhesion properties to marrow stromal elements and extracellular matrix; this may facilitate the cells passage to the systemic circulation [57, 64]. In normal individuals, hematopoietic progenitor cells adhere to the extracellular matrix which restricts cell proliferation. This attachment is mediated by cell-surface glycoprotein receptors composed of α and β subunits, specialised in ligand specificity and regulation of signal transduction respectively [65]. In normal progenitor cells, β1 integrin inhibits the signal that induces cell proliferation and this activity is damaged in CML cells. The presence of BCR-ABL1 protein in the cytoplasm disturbs the normal intracellular signal transduction conducted by Abl1 and causes leukemic transformation [66]. Similarly, CRKL (v-crk avian sarcoma virus CT10 oncogene homolog-like) is constitutively phosphorylated in *BCR-ABL1* transformed cells, but not in healthy controls [67]. Moreover, *BCR-ABL1* is involved in integrin mediated cell adhesion by forming a complex with other focal adhesion proteins like paxillin,

Fak adhesion kinase, p130Cas, Hef1, and in RAS-MAPK pathway activation via Grb2, to trigger further downstream oncogenic signalling activity [68-71]. Treatment with Interferon-α and tyrosine kinase inhibitors restored the defective cytoadhesion properties in CML cells and controlled cell invasion [72, 73].

Chronic Myeloid Leukemia Signalling

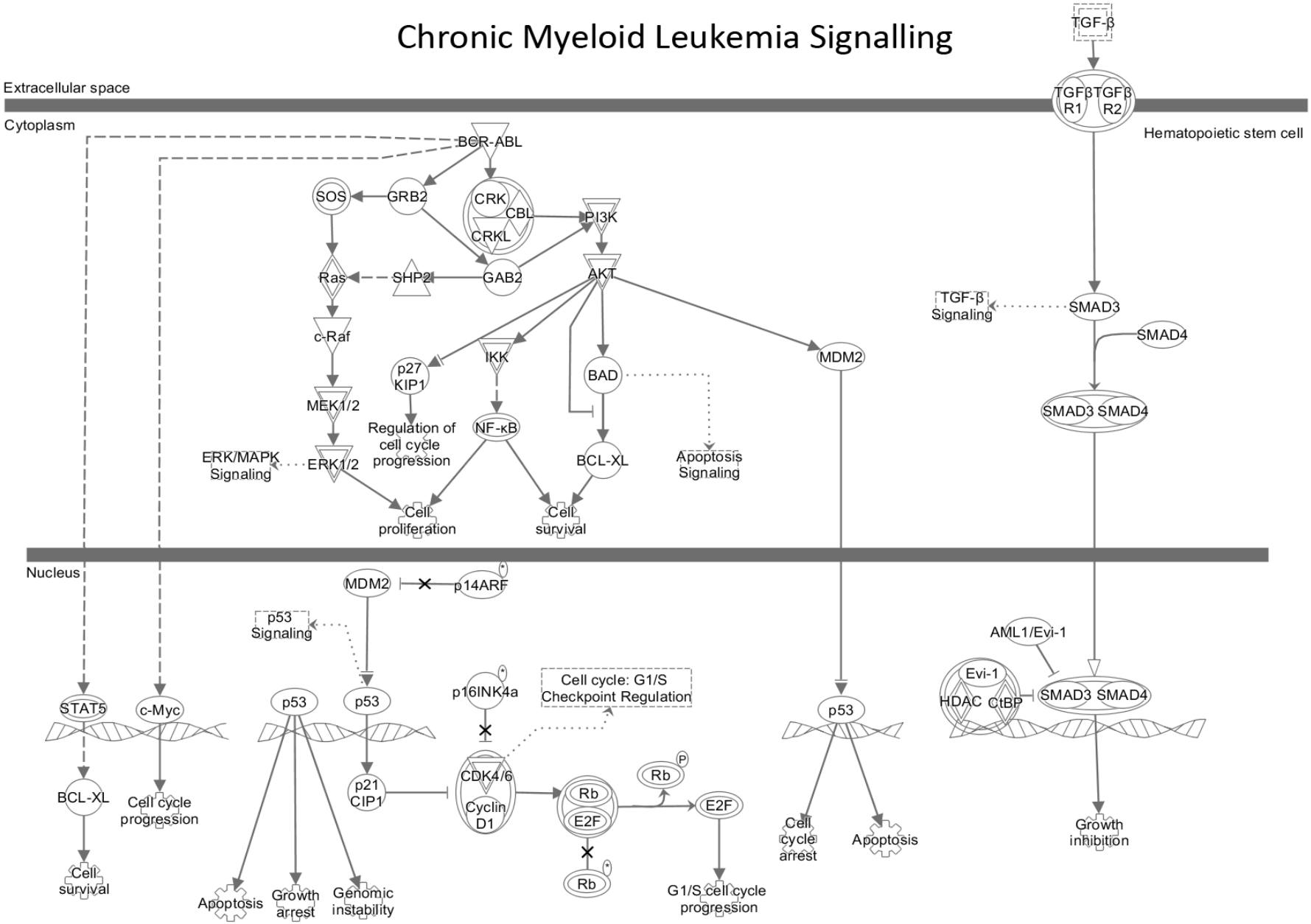

Figure 7: Signalling pathway operated by oncogene *BCR-ABL1* in CML cells. Activation of different downstream signalling pathways associated with cell proliferation and cell survivals through Ras/Raf/MEK/ERK and MAPK, PI3K/AKT, JAK/STAT, TGF-β signalling. Inhibition of apoptosis protein such as p53, BAD and tumour suppressor protein p16INK4a also a peculiar feature in CML cells and that increases cell survival and G1/S cell cycle transition. Diagrammatic representation of the *BCR-ABL1* signalling pathway was generated by IPA software.

1.3.4.2 Ras/Raf/MEK/ERK pathway

The Ras/Raf/MEK/ERK pathway is a major signal transduction pathway, which transmits the signals from multiple cell surface receptors to activate different transcription factors into the nucleus [74]. Approximately 30% of human cancer accumulates Ras mutations. These were frequently identified in hematological malignancies and result in Raf/Mek/Erk and PI3K/AKT pathways activation through two different Ras genes i.e. Ki-Ras and Ha-Ras respectively [75]. The Ras/Raf/MEK/ERK pathway is referred to as MAP (mitogen-activated protein) Kinase pathway, which is regulated by Ras stimulation. In another mechanism, autophosphorylation of BCR Tyr177 activates Ras via Grb2 binding [55, 76]. Moreover, Bcr-Abl1 initiates signal through two Ras activation adapter molecules such as Shc and Crkl that are considered as substrates of Bcr-Abl1. These substrates bind to SH2 (Shc) and SH3 (Crkl) domain of fusion protein and activate c-Raf, via Ras activation [67, 77, 78]. In murine CML model, substitution on *BCR-ABL1* at Tyr177Phe, abrogates Grb2 binding and thus weakens *BCR-ABL1* induced Ras activation [55, 79]. The immediate downstream effector of Ras in MAP kinase pathway is Raf that transduces the signalling cascade through the serine-threonine kinases Mek1/Mek2 and Erk, which then transfer signal into the nucleus and activate transcription factors responsible for cell proliferation. In CP-CML, stimulation of cytokine IL-3 is mandatory for the activation of Ras followed by Raf to Mek/Erk for downstream signalling, but in blastic crisis MAPK pathway remains constitutively active and is readily determined in CD34+ cells [80].

1.3.4.3 PI3K/AKT pathway

The *BCR-ABL1* tyrosine kinase forms a multi-protein complex with Cbl, Crk and Crkl to activate PI (Phosphoinositol)-3 kinase [81] pathway. The phosphorylated Tyr177 *BCR-ABL1* constitutively activates PI3K via recruiting GRB2-associated binding protein 2 (GAB2) [79]. PI3K activity is widely associated with cytoskeleton arrangement, cell division, apoptosis inhibition and glucose uptake [82-84]. Downstream of PI3K is a serine-threonine kinase, Akt, which has been implicated in anti-apoptotic signalling by phosphorylating a wide variety of targets [85, 86]. The pro-apoptotic Bad protein is phosphorylated at S136 by Akt that allows interaction with 14-3-3 protein and inhibits the complex formation with Bcl-2 and Bcl-X_L. In this way, Bcl-2 and Bcl-X_L remain bound to pro-apoptotic protein Bax and prevent the formation of Bax homodimers and thus inhibit apoptosis [87, 88]. In another mechanism, Akt activates the transcription of anti-apoptotic genes through phosphorylation of IKK and regulation of NF-κB by phosphorylating MDM2 and GSK-3, which promotes cell survival and cell cycle progression [89-91]. Thus, the *PI3K/AKT* pathway promotes leukemogenesis mediated by *BCR-ABL1* oncogene.

1.3.4.4 JAK/STAT pathway

The JAK or Janus family of tyrosine kinases is stimulated by activation of a cytokine receptor that results in STAT (Signal transducer and activator of transcription) factor activity. STAT family consists of seven proteins frequently overexpressed in many cancers. In particular, STAT3 protein is directly associated with oncogenicity while other STAT proteins interact with other oncoproteins to induce leukemogenesis [74]. STAT1 and STAT5 are constitutively active in *BCR-ABL1* positive cell and in primary CML cell lines [92, 93]. STAT5 appears to be regulated by SRC kinase family and triggers cell cycle transition from G1 to S phase by upregulating the cyclin D2 kinase [94]. In another mechanism, JAK/STAT appears to contribute to malignant transformation by inducing Bcl-X_L anti-apoptotic gene expression in *BCR-ABL1*+ K562 cell lines [95-97]. *Invivo*, the primary transplantation of fetal liver hematopoietic progenitor cells into STAT5 knockout recipient *BCR-*

ABL1+ mice failed to induced leukemia, which shows that STAT5 is required for leukemogenesis [98]. In chronic phase CML, the cells retain an almost normal differentiation pattern, and require external growth factors to induce anti-apoptotic and cell proliferation [99].

1.3.4.5 Myc Pathway:

c-Myc transcription factor is upregulated in many human malignancies and promotes cell proliferation, cell survival, differentiation block and anti-apoptosis activity [100]. c-Myc exerts its oncogenic activity by forming a heterodimer complex with MAX [101] and by inhibiting tumour suppressor protein PP2A through increasing CIP2A. [100]. Myc transcription factor activity depends on the SH2 domain of *BCR-ABL1* in Ph+ cells. Overexpression of Myc causes leukemic transformation, while dominant negative Myc blocks leukemic transformation in murine BM cells [102]. In blast crisis, Myc is overexpressed and plays a significant role in promoting leukemogenesis.

1.3.4.6 TGF-β signalling

The TGF-β1 (transforming growth factor beta1) signalling pathway consistently indulges in haematopoiesis by promoting cell growth, differentiation and apoptosis. Upon activation, dimerization of TGF-β type I and type II receptors activates SMAD3 (SMAD family member 3) which forms a heterologous complex with SMAD4. The SMAD3-SMAD4 complex then translocate into the nucleus and interacts with different transcription factors and regulates the expression of several target genes [103]. In CML, p27 (Cyclin-dependent kinase inhibitor 1B) activity is inhibited by the TGF-β1; this increases the cyclin dependent kinase cell cycle checkpoint transition, that initiates cell proliferation [104]. However, normal regulation of TGF- β1 signalling is restored in CML patient treated with tyrosine kinase inhibitor.

In conclusion, it is clear that *BCR-ABL1* kinase domain serves multi-integrated pathways in processing the cellular activity rather than a single unidirectional signalling. However, chronic

phase CML cells are indolent in nature and operate by communicating with various physiological pathways. Therefore, targeting the chimeric protein directly in early phase inhibits major downstream signalling pathways and restricts the process of leukemogenesis, but it may be re-activating in the presence of other genetic/cytogenetic abnormalities that could be a possible mechanism in CML progression.

1.3.5 Genetic/cytogenetic abnormalities in CML

Various factors apart from fusion gene *BCR-ABL1* exercise a significant role in worsening the disease. Several intrinsic and extrinsic factors have been identified as critical inputs in the advancement of disease apart from *BCR-ABL1* oncogene.

1) *TKI resistance mutation*

Introduction of Imatinib (IM) as Tyrosine kinase inhibitor (TKI) in treatment of CML as first line drug brought a significant achievement by improving the patient life expectancy through abolishing *BCR-ABL1* kinase activity. However, patients with point mutations in *BCR-ABL1* kinase domain display a suboptimal response to IM in 50-90% of cases, including 23% of IM naive patients; to counteract IM ineffectiveness, potent alternative TKIs were introduced [105, 106]. Patients in accelerated/advanced phase have higher chances to develop IM resistance compared to chronic phase patients [107]. The key mechanism for acquiring IM resistance is point mutations in the kinase domain that strictly interfere with Imatinib binding. The most frequently observed mutations were E255K or E225V, T315I and other substitutions at 250, 252, 253, 289, 317, 351, 396, and 486 [107]. Splicing mutations at ABL1 exon-intron junction were identified in three IM-resistant AP/BC patients, causing a frameshift (pC475YfsX11, pA424EfsX18 and pE275LfsX41, respectively) that resulted in loss of the C-terminal region of the BCR-ABL1 protein [108]. The presence of mutations in the KD alters IM activity, substrate utilization and transformation capability that

play a significant role in achieving the therapeutic responses in patients treated with TKIs [28].

2) *Clonal evolution*

One of the major causes in cancer development is a repetitive process of clonal expansion within a selective tissue environment. Tissue micro-environment is complex and linked to dynamic regulators, both systemic (Hormones, growth factors or cytokines) and local (cell type, adhesion defect) [109]. Cancer evolutionary process is increased by high reservoir of driver mutations that has been identified as a key feature in the biology of acute myeloid leukemia. Schmidt *et al* group has described 2 factors that drive clonal evolution in CML after destroying the majority of BCR-ABL1 positive cells by TKIs: a) persistence of *BCR-ABL1* independent clones, and b) consecutive acquisition of *BCR-ABL1* and alternative aberrations in CML patients [110].

Next generation sequencing on chronic phase CML samples reveals accumulation of genetic alteration in 33% of cases along with *BCR-ABL1* gene. Interestingly, the most commonly mutated genes found in CML to produce a significant clinical impact on patients overall survival are *ASXL1*, *EZH2*, *RUNX1*, *TP53*, *DNMT3A* and *TET2* [110, 111]. Follow-up analysis in similar CML patients revealed secondary events/mutations: a DNMT3A mutation that was present in pre-nilotinib treatment disappeared after treatment, but the others remain. This suggests that the DNMT3A mutation was a secondary event on a *BCR-ABL1* clone, but the order of acquisition may be the other-way around. So, the authors hypothesis was that prior to *BCR-ABL1* translocation, there could be a pre-existing stem cell clone with somatic mutations [110]. But it is difficult to say how the random mutations may contribute to disease progression, as most of the mutations in chronic phase CML are just passenger or age related.

In accelerated phase/blast crisis CML, the impact of clonal evolution is high and considered as an important feature in disease progression. The development of additional chromosomal and genetic abnormalities is highly associated with lower response rate and poor survival [112]. In advanced phase, most alterations were detected in BCR-ABL1 kinase domain, p53 tumour suppressor protein, and epigenetic modulation genes. Recently, by whole exome sequencing, a genetic alteration on SETBP1 was identified that relates to bad prognosis in different myeloid malignancies and found recurrently mutated in atypical chronic myeloid leukemia (aCML) patients [113] and also in one of our CML-BC patients. SETBP1 was somatically mutated in 25% of aCML patients, 17% of secondary acute myeloid leukemia and 15% of chronic myelomonocytic leukemia [114]. The same mutations were found germline in Schinzel-Giedion syndrome (SDS) patients [115]. Its biological role is poorly understood and requires in-depth research to further explore its mechanism in oncogenicity.

3) *BCR-ABL1 expansion*

The role of *BCR-ABL1* gene in the pathogenesis of CML is well known; its continued expression is required for the maintenance and survival of leukemic cells. BCR-ABL1 expression is higher in BC patients [116, 117].The relative elevation of BCR-ABL1 transcript has been detected in CD34+ progenitor cells in BC patients as compared to patients in CP CML [118]. *BCR-ABL1* overexpression activates the SRC family kinases [119, 120], which increases the cell survival by inhibiting the apoptosis signalling pathway [121] and increases clonogenicity on accumulating other oncogenic variants [122]. Interestingly, LYN kinase activation in patients expressing unmutated *BCR-ABL1* gene causes imatinib resistance [123]. It has been observed that resistance to imatinib gradually increases with *BCR-ABL1* expression and exhibit downstream signalling pathways in dose dependent manner [107].

4) *Differentiation block*

Cell differentiation pattern is tremendously altered in an advanced phase of leukemia, which is considered as a peculiar feature in diseases progression. The complete loss of differentiation is observed in BC CML by modulating the activity of several transcription factors involved in differentiation (figure 1) [124, 125]. Various differentiation regulating transcription factors such as *CEBP*α, *IKZF1*, *HOXA9* and *EVI1* are critically altered in haematopoiesis. The transcription factor CEBPα is a CCAAT/enhancer binding protein-α, which is an influential component for normal granulocyte differentiation. It is assumed that inhibitory action of hnRNP-E2 protein, which is stabilized by *BCR-ABL1*, down-regulates CEBPα expression in BC samples, which is normally expressed in normal bone marrow cells and even in CP CML samples [126]. Haploinsufficiency caused by a short deletion on IKZF1 gene, which encodes for Ikaros transcription factor, highly affected normal lymphoid development process [59]. In mice, decreased expression of Ikaros depletes the differentiation capability of B cells in BC CML and Ph+ ALL [127]. Another factor in disrupting the normal haematopoiesis is translocation of genes such as *NUP-98-HOXA9* and *AML1-EVL1* which produces a significant role in impairing the hematopoietic differentiation [128, 129]. Likewise, other transcription factors including *GATA3* and *RUNX1* altered the differentiation of progenitor cells in CML [130].

5) *Genetic instability*

Genetic instability results from abnormal mechanism that impair DNA repair operations. In CML cells, DNA damage is possibly stimulated by *BCR-ABL1* fusion gene, which deteriorates the normal mechanisms of DNA repair. Usually, the main cause for shattering the DNA structure is the reactive oxygen species, like superoxide radical anion, which cause double-strand breaks in S and G2/M cell-cycle phases. This DNA abrasion is repaired by HRR (homologous recombination repair) or NHEJ (non-homologous end

joining) in self-repair endogenous mechanisms, but this is impaired in CML. In CML cells, accumulation of mutations along with large deletion was detected in post HRR and NHEJ repair system, but not observed in counterpart *BCR-ABL1* negative cells [131]. c-MYC induces genetic instability by modulating the double stranded break repair mechanism through increased transcriptional activation of DNA ligase IIIα and PARP1 and induces myc associated error prone repair in TK activated cells. The mechanism of c-MYC in altering the NHEJ repair mechanism is by inhibiting microRNAs 150 and 22 which should block DNA ligase IIIα and PARP1 in leukemic cells [132]. Apart from HRR or NHEJ repair mechanism, the kinase activity of *BCR-ABL1* impairs the cell cycle damage sensor protein RAD3-related nuclear protein kinases [133] and ataxia telangiectasia mutated (ATM) protein, which is essential for the phosphorylation of cell cycle checkpoint 1 and inhibits the inappropriate DNA replication [134]. As discussed, the DNA damage is largely induced by ionizing radiation, ROS and genotoxic drugs in combination with faulty repair mechanism; this in-turn accumulates a high proportion of genetic alterations and cytogenetic/chromosomal abnormalities in CML cells [127, 135]. Telomerase shortening is also a cause of genomic instability in CML and associated with disease progression from chronic phase to advanced phase [136]. In CML cells, lower expression of TERT (Telomerase reverse transcriptase) enzyme increases the telomerase shortening process, which is inhibited by wild type ABL1 kinase [137]. But the exact mechanism involved in the deregulation of telomerase enzyme activity in cancer is still unclear. It has also been shown that a kinase inhibitor reduces the telomerase shortening activity in *BCR-ABL1* positive cells and delays the genomic instability process [136].

6) *Chromosomal aberration*

The cause of (9;22) translocation has still remained ambiguous: it could be a random event or resulting from a pre-existing condition associated with accumulation of genetic abnormalities in HSC. About 80% of CML patients develop additional non-random

cytogenetic abnormalities in Ph+ cells, which manifest a genetic instability that increases the chances of disease progression [138]. The most diverse cytogenetic abnormalities in advanced phase patients are trisomy 8, iso-chromosome 17, Ph chromosome duplication, loss of 17p and *BCR-ABL1* amplification. In few other CML cases chromosomal abnormalities such as trisomy 19, trisomy 21, trisomy 17 and deletion 7 has been observed (10% of cases) [139]. It has also been reported that chromosomal abnormality developed through the survival of LSC cells is highly upheld by the *BCR-ABL1* gene. In CP CML, genomic instability is most likely held in the LSC enriched CD34+CD38- population or in the LPC rich CD34+ pool. It has suggested that TKI resistance chromosomal abnormality progress CML-CP patient to either myeloid or lymphoid blastic phase, which is favoured by *BCR-ABL1* oncogene [135].

7) *Impairment of tumour suppressor genes:*

In malignancy, 25% to 30% of advanced phase patients were detected with impairment of tumour suppressor genes, which are either mutated or deleted. The p53 tumour suppressor gene is frequently impaired in CML and found mutated in a majority of blast crisis patients [140]. The p53 pathway plays a key role in inhibiting the *BCR-ABL1* kinase activity in patients with imatinib treatment. Inactivation of p53 by point mutation or deletion delays the responses to imatinib *invitro* and *invivo* [141]. Additionally, deletion and inactivation of p16 and p14/ARF gene expression alters the cyclin dependent kinase activity by upregulating the G1/S phase checkpoint transition. In some publications, an increase in the polyclonal cellular population of self-renewing pre-B cells was observed in p14 knockout mice [139, 142]. The (9,22) translocation gene inhibits the serine/threonine phosphatase 2A (*PP2A*), which acts as a tumour suppressor protein and activates SHP1-protein tyrosine phosphatase1 that catalyses the dephosphorylating and proteasome degradation of *BCR-ABL1*. It is also been shown that up-regulation/stabilisation of SET protein by *BCR-ABL1* kinase inhibits the tumour suppressor function of *PP2A* and result in cell survival [113, 143].

The other tumour suppressor gene that brings significant contribution to CML progression is *PTEN* (phosphatase and tensin homologue), which functions as a tumour suppressor gene by dephosphorylating the phosphatidylinositol (3,4,5)-triphosphate (PIP3) [144] and abrogates the activation of PI3K and the further downstream target Akt. In PTEN deficient CML mice the cell differentiation and proliferation were affected and resulted in loss of long-term HSC [145]. In another study, it was demonstrated that overexpression of *PTEN* prolongs survival of CML mice. [29].

The acquisition of genetic/cytogenetic abnormalities causes alterations in proliferation, cell survival and differentiation activity. The additional contribution of other abnormalities with *BCR-ABL1* in CML cells ignites the process of disease progression. Adjusting the treatment regimen in CML through proper examination of additional abnormality in patients at regular interval would be helpful in achieving higher therapeutic responses.

1.3.6 Strategic outline of CML treatment

The primary aim to treat CML cases is to recover their normal physiological functions with reduction in leukocyte counts and elimination of the Ph chromosome and BCR-ABL1 transcript. Different clinical parameters were evaluated in patients at diagnosis for better understanding the disease phenotype by calculating prognostic score, hematological profile, cytogenetic profile, fusion transcript measurement and kinase mutational status. Sokal and Hasford scores, two clinical-based patient risk scoring systems (Table 4) were implemented and their calculation is based on patient age, spleen size, WBC and platelet counts, percentage blasts cells, eosinophils and basophils. The factors were implemented in determining the patient risk as described in (table 4). In some studies, it was proved that patients with low Sokal scores achieved complete cytogenetic response in 50% and improved the overall survival by 10 years in >70% of patients in comparison to high Sokal score patients [133]. These scoring systems also help in predicting the possible outcome of the treatment with tyrosine kinase inhibitors [146, 147].

Table 4: Prognostic score calculation algorithm with different scale type		
	Sokal	Hasford
Score	= Exp. (0.0116 (age - 4.34))	= 0.6666 x age (0 when <50years, 1 otherwise)
	+0.0345 (spleen - 7.51)	+ 0.042 x spleen size (cm below costal margin)
	+0.188 ((platelets/700)2 - 0.563)	+0.0584 x %blasts
	+0.0887 (%blasts - 2.1)	+0.0413 x %eosinophils
		+0.2039 x basophils (0 when <3%, 1 otherwise)
		+1.0956 x platelet count (0 when <1500, 1 otherwise)
		x100
Risk category		
Low	< 0.8	≤ 780
Intermediate	0.8 - 1.2	> 780 and ≤ 1480
High	>1.2	> 1480

Post-treatment the patient responses are determined by evaluating the Hematological, cytogenetic and molecular response as classified according to European Leukemia Net [148] is summarised in table 5.

Table 5. Criteria for Treatment response [149]	
Type of Treatment Response	Criteria
Complete hematologic response	• Complete normalization of PB counts
	– WBC count <10^{10}/L
	– Platelet count <4.5x10^{11}/L
	• No immature cells in PB
	• No disease symptoms
Cytogenetic response	
Major	
Complete	0 Ph+ metaphase cells[a]
Partial	1-35% Ph+ metaphase cells[a]
Minor	>35% Ph+ metaphase cells[a]

Molecular Response	
Complete (CMR)	BCR-ABL1 transcripts undetectable by PCR
Major (MMR)	*BCR-ABL1*/*ABL1* ratio of ≤0.1% on the international scale[b]
PB, peripheral blood; WBC, white blood cell; Ph+, positive for the Philadelphia chromosome; PCR, polymerase chain reaction.	

a Out of ≥20 metaphase cells.

b Corresponds to a ≥3-log decrease in BCR-ABL1 transcript levels.

1.3.7 Treatment in CML

1) Conventional therapeutic compounds

Since the development of Imatinib as a tyrosine kinase inhibitor (TKI) as a first-line treatment for CML patient, a drastic improvement was observed in patients' life expectancy [150]. Prior to TKI treatment, there were other chemotherapeutic agents considered for the treatment such as Busulfan and hydroxyurea, in exceptional cases allogeneic stem cell transplantation was carried out when chemotherapy agents were insufficient to induce hematological response [151]. The first conventional therapy introduced in 1950 for the treatment of CML was busulfan. It is a cell-cycle non-specific alkylating anti-neoplastic compound, which prevents DNA from undergoing replication. Busulfan treatment in CML has never accomplished complete hematological response and fails to lower the risk of disease progression. It also produces severe side effects including myelosuppression, idiosyncratic pulmonary reaction, marrow fibrosis and endocardial fibrosis [152]. In 1972, a second compound was introduced in CML management: hydroxyurea, a cell-cycle specific inhibitor. It is better tolerated than busulfan and yields a complete hematological response in 40-60% patients, but never attained complete cytogenetic response when administered in chronic phase CML patient [152]. With conventional compounds, the median overall survival of the

patients was 3-5 years and the disease progressed to blastic phase and the patients died of serious disease conditions.

2) Allogenic stem cell transplantation (SCT)

Patients refractory or intolerant to chemotherapy, failing to achieve complete cytogenetic responses or at increased risk to disease progression are selected for SCT. Allogenic stem cell transplantation in CML produces long term survival in 50-70% of cases and achieves disease free survival in 30-70% of patients [153]. After 5 years since first transplantation, 15-30% of patients relapsed and progressed to advanced phase; in such cases a second transplantation was proposed. On second transplantation, the patient requires HLA-identical sibling and the result of disease free survival was found below 7-10% as compared to first transplantation [154]. Several factors were considered for successful transplantation. For example, transplantation in younger patients achieves highest disease free survival (70-80%) [155] as compared to older patients (30%) [156]. Hematopoietic stem cell transplantation in chronic phase patients produced a better prognosis than in advanced phase CML cases. The disease free survival in CP with SCT was 40-60% [157] and decreases as patient progresses to BC [158]. The frequency of post-transplantation relapses in chronic phase is very low (10-20%) and comparatively very high in blast crisis patients (70-80%) [159]. Also, pre-transplantation chemotherapy affects disease free survival rate, as patients at CP achieved higher disease free survival when pre-treated with hydroxyurea (61%) in comparison to Busulfan (45%) [157]. To reduce the toxicity and treatment related mortality rate, proper selection of chemotherapy should be made: for example, busulfan plus cyclophosphamide seems to be more effective to cyclophosphamide plus total body irradiation, but the latter produces more adverse effects [160]. The immune mediated treatment plays a significant role to achieve a cytogenetic response in CML. The relapsing rate was significantly higher in chronic phase patients who received T-cell depleted bone marrow in comparison to non-T cell depleted bone marrow [161]. Lately, the development of TKI changed the outcome of treatment in CML patients, whereas

HSCT is no longer the treatment of choice for early phase patient, except in cases of TKI failure where no available of alternative therapy.

3) Interferon Alpha

Until the introduction of TKI, interferon-α (IFNα) was predominantly used and preferred over other chemotherapeutic agents. IFNα successfully induced a complete hematological response in 70%, complete cytogenetic responses in 20% and partial cytogenetic responses in 40-60% of chronic phase CML patients [153, 162]. The mode of action of IFNα in CML is not entirely clear, but it appears that IFNα has an immune modulatory effect by increasing the expression of HLA class 1 antigens on cancer cells. However, toxicity with IFNα is a major concern and the presence of residual leukemia was found a major cause of disease relapses on accumulation of additional genetic abnormalities that render IFNα treatment ineffective [163]. Following the development of selective *BCR-ABL1* tyrosine kinase inhibitor Imatinib, IFNα was no longer a choice for treatment in CML. In early clinical trials, patients failing IFNα were treated with imatinib and achieved CCyR in 44% of Ph+ patients, with a 70% decrease in progressing rate to AP/BC at 6 years of follow-up and achieved a marked increase in patients overall survival with no serious side effects [164].

4) Tyrosine kinase inhibitors

No single *BCR-ABL1* selective standard therapy was accessible for chronic myeloid leukemia patients until the development of Imatinib (IM). Imatinib, or Imatinib mesylate (Salt form) or STI571 was developed by Novartis Pharmaceuticals, and commercially distributed as Glivec (European countries) or Gleevec (Unites states). Imatinib is a potent type-II inhibitor of *BCR-ABL1* tyrosine kinase activity and few other tyrosine kinases such as PDGFR (platelet derived growth factor receptor) and c-KIT. The initial trial of imatinib in chronic phase patients in IFNα failure showed an achievement of complete cytogenetic response in 41% and complete hematologic responses in 95% of patients. At 6 years of follow-up, progression-free survival and overall survival were found in 61% and 76%, respectively with imatinib [165, 166]. Following the success of initial trials, the

IRIS (international randomised study of Interferon and Imatinib) randomized trial was conducted on newly diagnosed patients with one group treated with Imatinib and other with Interferon-α; the crossover was allowed based on treatment failure or drug intolerance [167]. More than 5 fold increases in CCyR with imatinib versus Interferon-α were achieved in CP patients, at a well-tolerated oral dosage of 400mg daily. In 2002, based on the positive IRIS report, FDA approved imatinib as first-line treatment for patients with CML; continuous treatment induces durable responses and further decreases the relapse in CP-CML patients [149].

In multicentre clinical trials, CML related deaths were very low in a patient who achieves a complete cytogenetic response within 2 years of IM treatment. [168]. Recently, a study on 102 chronic phase CML patients treated with Imatinib observed that patients achieving CCyR and MMR at 12 months with IM treatment have an overall survival similar to that of the general population [169]. Despite achieving CCyR in chronic phase CML with standard dose of Imatinib and undetectable BCR-ABL1 by qPCR, there is a minute amount of residual disease persisting in CML [170]. So, high-dose imatinib trials were conducted and reported the efficacy and response rates in newly diagnosed CML patients (Table 6). The phase-II study with imatinib in patients at diagnosis showed a response rates at 12 months with distinct Sokal stages at different centres as described in table 6.

Table 6: High-dose imatinib multicentre study in phase-II trial

Study	Sokal risk	IM dose	Response at 12 months		Reference
			CCyR	MMR	
RIGHT	Low	800mg/d	85%	54%	[171]
GIMEMA	Intermediate	800mg/d	88%	56%	[172]
ELN	High	800mg/d	64%	Not achieved	[173]

The above study reported that CCyR and MMR responses with high-dose of imatinib were faster to achieve than standard dose (400mg daily). But it failed to provide higher freedom from

progression in high-dose imatinib versus standard dose, regardless of early molecular responses. The high dose of imatinib has only a limited role as first line therapy because of dose interruption, reduction and drug discontinuation observed in a substantial number of patients with serious toxicities of grade 3 or 4, which include neutropenia and thrombocytopenia [149, 174]. Despite the positive response to imatinib in CP-CML, there are cases with intolerance and resistance. To resolve this issue, alternative more potent therapy were implemented with the development of second generation TKIs as first line or second line treatment in accordance with ELN guidelines [175].

Imatinib resistance in CML:

Primary resistance (failure to achieve hematological remission within 3-6 months of treatment) and secondary resistance (due to kinase mutation or increased *BCR-ABL1* gene expression) are the two main categories described in patients with imatinib resistance.

Primary resistance is a rare event in CP-CML. Approximately 15-20% of IM treated patients does not achieve hematological responses at 6 months. Inadequate imatinib plasma level or intracellular IM concentration may be the cause for primary resistance. Firstly, it was observed that the majority of imatinib molecules in plasma are bound to α1-acid glycoprotein that significantly reduces the active drug concentration in leukemic cells. Therefore, free IM was found to be insufficient for effective inhibition of *BCR-ABL1* tyrosine kinase activity [176]. Secondly, overexpression of the multidrug resistance gene (MDR1) reduces the intracellular imatinib concentration and produces suboptimal response with similar dose of IM [177]. However, it was also found that low OCT1 transporter activity reduces the cellular uptake of imatinib and induces suboptimal responses. To overcome these OCT1 effects, high doses of imatinib would be considered [178]. Alternatively, low OCT1 expressing patients may be treated with either dasatinib or nilotinib because their therapeutic activity is independent of OCT1 mediated cellular uptake [179, 180].

Secondary resistance is predominantly *BCR-ABL1* dependent, through overexpression of the fusion gene and/or *BCR-ABL1* kinase domain mutations. However, the recent START-C study has showed that nearly half of imatinib resistant CP-CML patients did not carry any *BCR-ABL1* kinase mutation. This finding suggested that secondary resistance also depends on *BCR-ABL1* independent mechanisms, such as the presence of additional chromosomal abnormalities [181]. Among the mutations in BCR-ABL1 kinase domain, the most undruggable mutation is the T315I, which is still highly resistant to TKIs such as imatinib, bosutinib, nilotinib and dasatinib. Ponatinib is the only approved drug that shows activity against T315I mutation [182-184]. The potent second and third generation kinase inhibitors such as dasatinib, nilotinib, bosutinib and ponatinib were developed to increase the potency against imatinib resistance patient and reduce the risk of disease progression. New generation TKIs like dasatinib and nilotinib were already approved as first line treatment and in patients with imatinib resistance or intolerance [185, 186].

Second generation TKIs:

Dasatinib (Sprycel®) is a dual Src/ABL1 kinase inhibitor, which was found to be more potent than imatinib and has been approved for first-line CML treatment. It is structurally unrelated to imatinib and binds to both active and inactive conformations of *BCR-ABL1* [187]. Apart from blocking Src family kinases (FGR, FYN, HCK, LCK, LYN and YES), it also blocks KIT, PDGFR and ephrin receptor tyrosine kinases [148, 188]. Dasatinib inhibits 17/18 imatinib-resistant mutations, except T315I [183]. Positive data from clinical trials were observed in patients with imatinib resistance or intolerance at a recommended dose of 70mg twice daily. On the basis of the results, dasatinib was approved for treatment in all phases of CML [189]. Fluid retention is the main adverse event reported with dasatinib after long term use, similarly to imatinib. Hence, it has been suggested that the mechanism behind fluid retention caused by imatinib and dasatinib related to inhibition on anti-PDGFR activity [190].

Nilotinib (Tasigna®), developed by Novartis, is also approved for first–line CML therapy. The chemical structure is very similar to imatinib, as it was developed from the same

phenylaminopyrimidine derivative. Because of its improved binding to *BCR-ABL1,* Nilotinib exhibits 30-fold more potent ABL kinase inhibition compared to imatinib and shows similar inhibitory action on KIT and PDGFR receptor tyrosine kinases [191]. Most importantly, significant activity of nilotinib has been documented for the treatment of imatinib-resistant diseases, with enhanced cytogenetic responses in chronic phase CML. It is significantly active against 31/32 imatinib-resistant *BCR-ABL1* mutations [192]. Nilotinib safety profile is poor on long term use with increased incidence of metabolic syndrome in patients as compared to imatinib treatment patients [150].

Third generation TKIs:

Bosutinib (SKI-606; Wyeth) is another dual Src and ABL1 kinase inhibitor; inhibits phosphorylation of STAT5 and Src kinase family protein LYN/HCK and reduces proliferation of CML cells. However, unlike dasatinib, nilotinib and imatinib, bosutinib does not inhibit KIT or PDGFR and thus it does not cause serious side effects caused by additional blocking of these two targets. In a phase I/II clinical trial in imatinib resistant CML, patients receiving bosutinib achieved 43% CCyR with a good response against all *BCR-ABL1* mutants, except T315I [193]

Ponatinib (Iclusig®) was developed by ARIAD Pharmaceuticals and used against the non-treatable T315I mutant. Ponatinib acts as a potent pan-*BCR-ABL1* inhibitor but is also associated with several severe side effects, including blood clotting and narrowing of blood vessels [194]. Because of severe complications, this medication is not recommended for patients with *BCR-ABL1*+ disease until the failure of second generation TKIs. Several other potent compounds such as INNO-404 (NS-187), AZD0530 (AstraZeneca), DCC-2036, MK-0457 (Merck), PHA-739358 (Danusertib) were developed for treatment in CML and are active against most imatinib resistant mutants. All are under phase I/II clinical trials in patients with CML who have relapsed after imatinib therapy [191].

A recent study by Carlo Gambacorti-Passerini and Rocco Piazza demonstrated that management of treatment is very crucial in CML patients at diagnosis by considering the safety profile of TKI [195]. Different registration studies (DASISION and ENESTnd) on second generation

TKIs such as Dasatinib and Nilotinib versus Imatinib showed an increased rate of CCyR in chronic phase cases by 12 months. While Nilotinib showed a slight and temporary increase in progression free survival in ENESTnd study [196], dasatinib failed to show any survival differences in the DASISION trial [197]. After recognising the toxicity profile of 2^{nd} and 3^{rd} generation TKIs from the above studies, the patient safety should not be compromised and safest possible treatment should be considered for long term application (IM<DS<NI) along with highest efficacy. According to Gambacorti *et.al*, Imatinib is the best choice for first line treatment in CP-CML with a good safety profile on long term treatment [150].

Aim

In this project, my aim was to understand the genomic profile of chronic phase (CP) chronic myeloid leukaemia (CML) patients at diagnosis and to elucidate the oncogenic role of additional somatic events through next generation sequencing techniques. It is known that in CP CML patients the presence of oncogenic variants is minimal/absent compared to blast crisis patients. However, limited study was conducted and very little known about the presence of oncogenic variants in chronic phase CML patients. So, we implemented whole exome sequencing to study the presence of somatic variants and investigate whether these variants correlate with prognosis.

To perform this analysis, the genomic DNA from 23 CP-CML patients was isolated from peripheral or bone marrow samples collected at diagnosis and during disease remission. Thereafter, these samples were analysed for the identification of genetic events in CP patients through a Whole Exome Sequencing (WES) method. After WES analysis, a large number of somatic variants were identified as specific to CP samples by discarding the mutations present in the remission sample. Subsequently, we also sequenced 10 BC CML samples and identified SETBP1 mutation in one patient.

Overall, this project is based on two aspects, firstly identification of genetic alterations in 23 matched tumour/normal pairs by accessing high throughput sequencing technologies and secondly to determine their oncogenic role with respect to treatment response. In parallel, construction of a Setbp1 p.G870S targeting vector was carried out for in vivo modelling in order to understand the biological role of *Setbp1* mutation in relation to leukemia and SGS..

2 Material and Methods

2.1 Patient Samples

A series of 23 initially diagnosed CP-CML were studied. The myeloid and lymphoid cells from 23 CML patients were collected at diagnosis (Tumour) and at remission (Control) respectively. The study was approved by the Institutional Ethics code.

2.2 Fluorescence-activated cell sorting (FACS) analysis

The myeloid (tumour) cells were collected at diagnosis from 14 bone marrow with Ficoll-plaque PLUS (GE Healthcare, UK) gradient or from 9 peripheral blood with Buffy coat as previously described [113]. Lymphocytes (control) were collected from peripheral blood with Ficoll-plaque PLUS (GE Healthcare, UK) at remission in 11 patients. The lymphocyte of the remaining 12 patients were cultured with 2.5 µg/ml Phytohemagglutinin-M (PHA-M) (Roche Diagnostics GmbH, Germany [SR1]) and 200 UI/ml interleukin-2 (IL-2) (Aldesleukin, Novartis - Switzerland) from the sample collected after treatment The myeloid (positive for CD33, CD13 or CD117 staining) and lymphoid (positive for CD3, CD4, CD5, CD8 or CD19 staining) cell populations were evaluated by FACS and found to be over 70% and 80% respectively. After sorting, DNA was purified by using the PureLink^{TM} Genomic DNA Kit (Invitrogen, Life Technologies, NY) and the integrity of purified DNA was assessed by electrophoresis on 1% agarose gel.

2.3 Whole Exome enrichment and Sequencing

The exome libraries of tumour and non-tumour DNA from matched patient were prepared by Illumina TruSeq^{TM} DNA Sample Preparation Kit (FC-121-1001) and Illumina TruSeq^{TM} exome enrichment kit (FC-121-1008) referring to manufacturer protocol. Initially, 2µg of Genomic DNA (gDNA) were sheared to a size of 200-300bp using Bandelin Sonopuls Sonicator (30-65 Cycles, Processing time 10sec, Pulsation 20%, and Amplitude 10%). Fragmented DNA was concentrated by using AMPure XP beads (Invitrogen, Life Technologies, USA) and then DNA ends were

repaired and adenylation of 3' ends were performed according to standard Illumina TruSeq™ DNA Sample Preparation Kit protocol. The adapter-ligated DNA of 200-300bp fragments was isolated from 2% agarose gel with QIAGEN DNA Purification Kit. The extracted DNA was amplified for 10 Cycles and purified with AMpure XP beads. The amplified genomic libraries were pooled and hybridised with capture probes of exonic regions provided in the Illumina TruSeq™ exome enrichment kit. All the captured DNA libraries were subsequently sequenced on an Illumina Genome analyser IIx in paired-end mode to yield 76bp reads using Illumina TruSeq™ SBS Kit v5 (FC-104-5001). Following base calling and quality control metrics, the raw fastq reads were aligned to the reference human genome.

2.4 Base Calling and Sequence alignment

Base calling and quality control was executed on the Ilumina RTA sequence analysis pipeline (Illumina, v1.9.35 or newer). The binary bcl files were then converted into Qseq with the Off-Line Basecaller OLB v1.9.0. Further, Qseq files deindexed and reformed into Sanger-FastQ file format using in-house scripts. The FastQ sequences were aligned to the reference human genome database (GRCh37/hg19) with the Burrows–Wheeler-based BWA alignment tool [198]. Duplicated paired-end read were excluded from the analysis. Bam alignment files for tumour and control were cross-matched using dedicated in house software [113]. Variants present in less than 25% of the tumour reads were filtered-out. The mutation identified by WES were then re-confirmed by Sanger sequencing [199].

2.5 Validation of mutations by Sanger sequencing

Variant DNA sequences were downloaded from the UCSC genome browser and respective primers were designed with the Vector NTI Software (Invitrogen, v10. 3.0) as listed in supplementary table 2. DNA was amplified by polymerase chain reaction (PCR) (FastStart High Fidelity PCR system, Roche Applied Science, Germany). The PCR amplicons of tumour and

normal DNA were processed for Sanger sequencing at GATC Biotech AG (Cologne, Germany) and the presence of substitution was confirmed by using Chromas platform (Technelysium©).

Table 7: List of primers used for PCR and Sanger sequencing.

Mutation	Primer	
	Forward	**Reverse**
PATZ1	CATCCTAAAGGGCAGCAGGC	AGCCGATTTGTGGACAGTGC
KBTBD7	TGCTACTGCTGCTGCTGCTGT	GTGTGATGTGACCATCGAG
ASXL1	ACTGCCGCCTTATCCTCTAAATGG	CCACCAAGCCCTAATTCGTCAT
MAP3K4	TGGTACTTCAGTAGAAGGGC	ACTGGATGTCGAAGCTGTTATCT
HMCN1	CTGACTTGTCCTAGATCACCTAGC	GTGACAACCTACTCCAACTCACTC
SPHKAP	GCAGTAACTCAAATCCTCTCCC	GTCCTTGTTAGGGGATGACC
TMED4	GACTGTGCTACAAAGTTTCACAATC	CAGGGGCTCTACTTCCACATC
PTPRD	GGAATTATTTGCTTGTGAATGC	CCATCCTTTCTTTTCTTTATTCTC
DLG5	GATGGTATCACACTGCTGCC	GAGGCTGTGGGAATGTGATC
TEP1	GAATTGCTTGAACCCAGGAG	CCAGGTTCAGGTGTGGTCAG
C17orf53	CAGAGAAGAGTGACAGTGACAGAAG	AACAGGAGACCCAATGGAAG
C1orf62	AGTGTAGCCTGTGATGCTTCTC	AAAAGGGATGGTGAAAACAGC
SCN10A	AGAAGAGCATCCCAAAGACC	GAGATGGATTACTCAAGACTGTTG
POLN	CAGCATTTCTGTCTGGAGAGG	GTACTGTTCCCCATATCTCTGTG
AKAP4	CATCCCACTGGTACAGTCAAG	GGCAAAATGAAATCAGACCC
AC013553.1	ATGTGTGCTGATAGGGTGAGAG	GGCAAAACTGAAGATTGTGAAG
HSF2	CAAAACAGAAAAGAGCAACAAG	CAGGGTGTGTTTTTCTGTAAATAC
CBWD6	CAGAACAAGAAAGAACTCAGGG	GGTAATGTGAAATCTGAACTTGC
MYOF	TGATTAGCTCTTTCCTTCTGC	CAAGTCAGTGTCACCAAGAGG
PLXNB1	CAAGGACTGCTCACCTCTACTC	TCTCCACACCCATTTCTGAC
POU6F1	CCTCAGAAGGACAGGGTCAG	GAGGAATGTGGGTGACCAAC
TRAFD1	CTGAGCAGTTGTCTTCTATCTGG	GCCACCTTCACTCCTACCAC
TCEB3B	GATCATCTTTGCCTCTCTGCC	AGAAGCACTCTCTTCACCAAAG

IGSF21	GCTCTGGAAGAACTGGCATC	CATTTGTGCTCATCCTGACTC
LIMS2	CTGGAGTACCCATGAGGTTT	GACAGTACACATCCTGGGCT
TUBA1B	GCTGTGGAAAACCAAGAAGC	CAGATGCCAAGTGACAAGACC
RBM19	CCTGTCAACACCACTGTCAATC	CTGGGTCTTCTGACTCCTGATG
ASB2	CTGGACACAGCCTCAGCATC	CTGTGCCTCACCTTCCTTATC
OR10H2	CACTAGACACCCCCATGCTC	CATGACATAAAAAATGCTGGATC
ROR2	ATGAAGACCATTACCGCCACT	CTCAGGGAGCTGGTCTGCGT
ARNTL	AGCGTCTATTGGGCAAGAACC	GTGGACTGGTGGCTGTTCAT
MYO16	GTTATCAAAAGCCTTGCCAGG	GCCACATCTACCTTCAGTCCA
KARS	TGGGTTTGGCTGGTGGACTG	GCTGTGGGTGTGGAATTGAG
PCDHA5	CCTTTCATCCACATGATGTCG	CCTTCACTGCCACCTCCACA
SH3PXD2B	GACTTGTCTTGGGACTTGGCA	TGGAAGGGCAGTAAGGATGTC
STC2	CATCTCACCTGTCCGTTCCG	CCGAACCCAAGACAAGCACT
DUSP22	CCAAAGCAGGATTATTTGTACG	ACCAGTCAACCACAAGGAGCC
GPR30 (GPER1)	CCATCGGCTTTGTGGGCAACAT	GGTGGGCATGGCGGAAAGACT
BX255925.17-2	GGGCATGGGTGGGCAATGTGA	TCAGGTGGACAGCAGCTAGAC
MYST2	CATGAAGGAACAGCGTTCTGATCC	CCGAGGTGGAAAGCAGGGAATAAA
UNC13C	ATTCTCTCTGTCACTTTGC	GTCTTTGAGTTGAATATCCAG
BRSK1	GTTGGGATTACAGGCATGAGC	TCATCTTCTTAGGGACTCCT
CACNA1I	AGGAGAGGGTGTATCTGGCA	GGAATGAATGTGCTGTGG
AR	GTTGGTCTAGCACAAGAATCAG	TGGCTGGTCCATAGGAGCGT
NAG	AAGTTCCAAGACAGCTAGGATGCC	GCCTCTACCCATGAGATGCTAGTAG
HNRNPH3	GCTGTGGGACTTGACTGATGC	GCACGAAAAGGCAACCCTCTC
CADM1	GCTGAGATTTCAATCTTAGCGGG	GCAAATGCCAGGATTACCAAC
OR11G2	AGCAACTCCAGCACCTTCA	TGAGCCGTAGAACAGTGAAACC
TINAGL1	CAGATGTGAGAGTGAGGGAGATG	GGCTTTGGTAGTCTTGAGTGG
LEPR	CATGGTGCTTGGCACACAGC	CTGTTCTCCATCAGATTGACCCT
TNNI3K	GCACTGAGATAGTTAATAGTTT	ACTGTGTCCATTTGCCTGCCCT
FCAMR	ACCACATGGAAATACTCAGGGA	CAGAGGGTTCTGTCAAAGCACCT
CCDC80	TGCCTCTCTGATTCTCTCCCACAC	GTAATGTTCACAGCAGCCTTGC

GPR156	GCTTCTCTTGGACTGACCTCTG	GCTCACACTTTCCTGGCTCTG
CTNNA1	ACCTACCAAACCATACTGCCTTCT	GAACCAAACCCATATCCTCAGTA
JAKMIP2	CTCTCCAACTTGTCAGGTAATCC	GAGGCTCCTTGATCCACATAACTCA
SPRED3	CCTCAGGTTTGGCGAGCTGATTA	CTCTTCCAGGAGTTCCCCA
VN1R1	GATGAGGACAAAAGGGCTGCG	TGAGACCCACGGACTTGATTCT
DSCAM	AATCAGATGCCCAGGCGGATG	GCTGAAGGAGGATCTTGAGAAAG
AMACR	TCAGAGGGCATGGGAGAATGGGA	GTGGTACATGAGGATGATCGCT
CYLN2	GTGCGTGCTGGGATGGTGA	CAGGCTGGTTTCGGTCTCG
MYH7	TGAAGGAGGTGGGAGAGGAA	GTTGGGATCTCTGTTCTTGCT
BBS2	TGCCAAGGAACGAACTGAAG	TTGCCACATAAGGTTGCTCTAA
WDR63	CTCTCTCCTTCCAACCCTAC	CGTGTCTAACTGGCAGTGAGC
SHPRH	CAAGAACTCTCCACGAACAGC	GCAAGAAGAATCCTCAGCATC
QKI	GCTCGGGCTTTGAAGTTAAT	GTGCTTGGTGCTCTGTGTCA
IFNA8	TAGAGAGTACATAAAGGAAAGCAA	TCCAAAGCAGCAGATGAGTCC
MARK3	TGAGGCTTTGTCTCATTATG	GTGTTATGAAGACTCTGAGC
AC099524.5	GTTCAGAGAGGCGTGGGATTGT	CACAGTGGCAATGTACTACAGC
DDX4	TCTCCTCACCTTGTGATCCAC	CTTGTACTACCACTGAAGCCAGG
TINAG	CAGATGTGAGAGTGAGGGAGATG	GGCTTTGGTAGTCTTGAGTGG
FAM82A1	CACATAGTAGGTACTCAGTAA	TATCATCTTCAAAGCCAGTA
SORCS2	AGGAAGAATCTCCAGGGCAG	ATATCACCACCAGCTACCAG
SOX8	AGCAATGTGGACATCTCGGA	TCGCCATAGTCGCCCTGTGA
C2orf51	CTTGATGATGGAGTGACATGC	ACCCTCCACAACAGCACTTG
TRA2B	GGGCTAAGGAGTTACACCATTG	GAAATACTGGAGGAGAGCTTGTGG
SC4MOL	GTGAATGATCTGTGGCAGCAA	GATACTAGAAGCTCACCTTCC
GFRAL	ACGACCTGGGCATATCATTG	GGCTACCATATTTTGACATGCTGAT
SVEP1	GGGTAGCACCTAACCATATT	GTTTTGATGATGCACTGGCA
ARHGAP23	TGGTGGAGGCTGTCTGAAGC	CATAGTAGTGTCCTGGGAGATC
C19orf54	AGGTGGAGTTAGAGAGATGG	ACCAGATGAGGAAACTGAAGC
RLF	AATAGAAGCTGTGAATCAGAG	ATGAGGTATGTCAACTTCAGGT
MLXIPL	GACAGACAGACCCACAGAAAGAC	GTTCAAGGGACAGAGTGGCA

GTPBP1	GGAAGTCCCATCCTGCTCTG	GCATCTACCGCTTCAGAGTGC
FRMD4B	GGTCCTCAGACCAAGACAGTAG	CTCCTGGAAATGGAAATGCTGG
NLGN4X	GTTACTGCTGTGTGTGTAAGCGT	CTGGTGGCTCTCTTTACTGAC
OR5B17	CGTCTTTGTTCCTCAGGGTAT	CGCTACGCAGCAGTGTGTAAC
NCAM1	TGTCATTCCAGCAGCCATACT	GAGAGAGAATGGGACTGGTTA
ACSM4	CTCAAAGACGAACTCAGGGC	AGCCTTGGTTTCCTCTTCTG
FAM124A	GCTGAATCTCCTCCTAGCCTCG	ATGCCTCCACTCTGATGCCC
NALCN	TGATGGTGAGGGTGAGACAT	TGACTCAGTCTGTCCTCCTCT
CARD14	ACATGCTCACCCGCCCACAT	TCGTCCTTCAGCCTCAGCACC
SSTR4	TGTTCACCAGCGTCTTCTGTC	CGTGGTTGACGGTGGCATCA
SLC12A5	AGATGGGGTCACATTATGTTGCC	AGGATGGAGAGGATGACACAAC
FBN3	ACAGGGACAAGGTCAGAACTGG	GCCGCTGAGAAGGTGTTAGAG
SLC6A17	GCTGGTGTGTCCTGAAGCCT	ACTGAGTTATCTGAGCATGGA
PCDH1	AGGAAGTCCACGCTGAAGGG	GTGTGTGTGCCTGTGAGTTC
MYST3	GAGGCTGTGGTGCTGGTTGTGGTT	GAGTTACACCCAGGCTGACGAG
PATE2	TGTCCTCACTAACCTTTCTCTA	GAACCCTGACCTCCTGAAATC
RECQL	CAGGAGGCAAAGATGAACA	ATCTTCCAGGAATATGC
VAT1L	CTGTTCTCTCCTTGAGCCTCTG	TTGCTGTCCATGTGAACTCTACC
SCNM1	ATTAGGACAGCAGGAGTACC	GTGATAAGTCGTGTCTGAGT
ESRRG	TAACTATGCTCTTGTAAACTCTA	GCTGTTCGTCCTTCATCAAGA
PCM1	ACCTTCGTTGGGTGTCAGA	TGCTTGCTCTATAAGGCTTA
TMC1	CTCACTCCTAAGGGGTAT	AAGACATTCAGCCTGACC
OR10A5	CACAACCATCTCCTTCCTT	CAACAGTGTAGGATAATGATA
SERPINB2	CCAGAGACACTATCCCCCAA	TGGGCATAGACTCACAATTA

2.6 Clinical Scoring index

The Sokal score was evaluated at diagnosis [147]. In addition, the potential oncogenic scoring of all the somatic variants was determined by a) GeneRanker Score (http://cbio.mskcc.org/tcga-generanker/) [200] and b) Oncoscore an in-house software that automatically calculates the oncogenic score of individual genes by performing specific data-mining queries on PubMed (NCBI).

2.7 Statistical assay

Statistical tests were performed by using Graph Pad Prism. Correlations and linear relationship between categorised variables were analysed by using Pearson's correlation co-efficient [201] and linear regression [202] respectively. Comparison between 2 variables was performed with 95% confidence interval. The sampling distribution was reported as mean and standard mean error across groups analysed by the unpaired *t-test* [203] where statistical p value < 0.05 was considered significant.

2.8 BAC clone and Reagents

C57BL/6J strain genomic Setbp1 BAC (ID no. RPCIB731I22405Q) and cDNA *Setbp1* (ID no. IRAVp968G09121D) clones were obtained from the RPCI23 BAC library (Source Bioscience). Sodium dodecyl sulphate (SDS, GIBCOBRL, Life Technologies, cat no. 15525-025), Sodium hydroxide (NaoH, Sigma-Aldrich, CAS no. 1310-73-2), Ethylenediamine-tetraacetic acid (EDTA, Fluka, Biochemika, 03609), Sodium Chloride (NaCl, Sigma-Aldrich, CAS no. 7647-14-5), Tris (hydroxymethyl)-aminomethane (TRIS, Eurobio, Lot no. 088943), Formamide (Sigma-Aldrich, CAS no. 75-12-7), UltraPure™ Salmon Sperm DNA Solution (Invitrogen, Life Technologies, cat no. 15632-011), PIPES (P-1851, Sigma-Aldrich, CAS no. 5625-37-6) and One Shot® INV110 (ThermoFisher Scientific, cat no. C7171-03) were purchased. The southern blot film developer (ref

no. 190-0943) and fixer (ref no. 190-1875) solutions were brought from Carestream Health Inc, United States. All the restriction enzymes used for building targeting vector were ordered from New England Biolabs (NEB).

2.9 Pre-cloning and Site Directed Mutagenesis

Setbp1 cDNA exons 4-6 (Accession no. NM_053099) and *Setbp1* genomic regions from intron 3 (left homology arm) and from exon4/intron4 (right homology arm) were pre-cloned into pCR4TOPO® sub-cloning vector by using TOPO® TA Cloning® Kit (ThermoFisher Scientific, cat no. K4530-20) as described in company manual. The regions of cDNA (Exon4-6) and exon4/intron4 were amplified with primers designed with unique restriction sites (Table 8) and then cloned into the TOPO vector. Positive colonies were selected by using Ampicillin (A9393, Sigma-Aldrich, CAS no. 69-53-4) and Kanamycin (K4378, Sigma-Aldrich, CAS no. 25389-94-0) as bacterial antibiotic resistance marker. Additional PCR-amplified sequences of *Setbp1* splice acceptor site, BGH-PolyA sequence and SV40-Stop sequence were ligated with T4 DNA Ligase (NEB, cat no. M0202S) into the TOPO cloned cDNA fragment at KpnI, NheI and SacII restriction sites, respectively. The *Setbp1* p.G870S mutation was introduced into the TOPO cloned exon4 by site-directed mutagenesis using a PfuUltra High Fidelity enzyme (Agilent, Santa Clara, CA, USA). The primers used in developing the construct are listed in table 8. The presence of the p.G870S mutation was verified by Sanger sequencing.

2.10 In-Fusion® HD Cloning

pDELBOY-3X, originally built by D. J. Rossi, University of Helsinki [204] was used as a background vector for constructing the *Setbp1* p.G870S expressing targeting plasmid. For homologous recombination, two genomic Setbp1 sequences were selected as homology arms (Int3 on the left arm and Int4 on the right arm). The fragment containing the right homology region also includes a duplicate copy of mutant Exon4 p.G870S. The wild type *Setbp1* exon4-6 was

cloned within LoxP sites. The In-Fusion® HD Cloning kit (Catalogue# 638910, Clontech lab, TaKaRa) was employed for final fragments cloning into pDELBOY-3X. The In-Fusion cloning procedure was carried out using the pre-cloned sequences as templates for PCR amplification with infusion primers that share 15bp homology region with the ends of the linearized vector. The PCR was performed with Expand^{TM} Long template PCR system (Roche Diagnostics, Cat. no. 11681842001). In-fusion primers were designed as listed in table 8 with online In-fusion primer designing Tool. The cloning reaction was performed with a molarity ratio of vector:insert 1:2 as recommended, followed by transformation. The cloning reaction was transformed into Stellar^{TM} competent cells (Catalogue no. 636763*,* Clontech lab Inc.) and spreaded on ampicillin® agar plate and then incubated overnight at 37ºC. Finally, colonies were picked, grown overnight and DNA was extracted using a miniprep kit (Zymo Research, USA) and later analysed for the presence of insert by digestion with appropriate restriction enzymes. Positive clones were finally sequenced for verification of any unwanted sequence or artifacts.

TABLE 8: Primer used for constructing Setbp1 G870S targeting vector.

REGION	FORWARD (5'-3')	REVERSE (3'-5')
cDNA456-TC	GCATACGAGAGGCCCCAGAAACACTCA AC	ATCGATCCGCGGGCTAGCCTAGGGAAGGACATCACTCTCGCTGGC TC
Set-SpAc	GAT CGG TAC CAT CGA TGT ACA GTT CTT CTC AGA CTG CAA T	GGA TGG TAC CAC ACT TCC CAA GAG
POLYA-BGH	GAT CGC TAG CCT GTG CCT TCT AGT TGC CAG CCA	GAT CGC TAG CCT CAG AAG CCA TAG AGC CCA CC
STOP-SV40	GAT CCC GCG GTG CTG GAG TTC TTC GCC CAC	GAT CCC GCG GCG ACG GTA TAC AGA CAT GAT AAG A
MUTAGENSIS G870S	CTG TCC CGA TGC TAC TGT CGC TCG	CGA GCG ACA GTA GCA TCG GGA CAG
INT3-INFUSION	GCT CAA CCG CCT CGA GTG GGA TGT CAA TGG TCA CAA	CCG CCA CCG CCT CGA GTA GCT CTG GCT GTC CTG GAA C
EX4 -TC & INFUSION	ACG AAG TTA TGT CGA CGT ACA GTT CTT CTC AGA CTG CAA T	GGT AGA ATT CGT CGA CGT TTA AAC CTG GCA AGT CCA AGT CTA GAG
CT-CD1 INFUSION	GAG GGG GGG CCA TCG ATG TAC AGT TCT TCT CAG ACT GCA AT	GTA CCG GAT CCA TCG ATC ATA TGG CAC CGG GTA ATA CTG ATC AA
CT-CD2 INFUSION	TTA CCC GGT GCC ATA TAT CCA GTA CGA CCC ATT GCT CTA T	GAT CCA TCG ATC ATA CGA CGG TAT ACA GAC ATG ATA AGA
CT-INT4-1 INFUSION	GGACTTGCCAGGTTTAAACTTAATTAATTGTTTAAGT CGTGGCCTTTGC	AATTCGTCGACGTTTAAACATTTAAATCAGGACACAGGCAATGATAG GT
CT-INT4-2 INFUSION	CCTGTGTCCTGATTTAAATCATGCCCTCTCTAATTCT GCA	GACGTTTAAACATTTAAATTCCTTTGCTGGTCTTCTTGC
Rox-Hyg-Rox	CCAGGTTTAAACTTAATTAATAACTTTAAATAATGC CAATTATTTAAAGTTATGTGTCAGTTAGGGTGTGG AAAGTC	CGACTTAAACAATTAATTAATAACTTTAAATAATTGGCATTATTTAAA GTTAATAAAAATAGGCGTATCACGAGGC

3 Result

3.1 Patient clinical characteristics

The clinical features of 23 chronic myeloid leukemia patients are shown in table 9. The median age of male (n=15) and female (n=8) patients was 49.53 (±18.37) and 51.38 (±28.55) years, respectively. All the patients were diagnosed in chronic phase and initially prescribed with 400mg of Imatinib as first line treatment. The Sokal score identified in female patients was slightly higher than in male patients (1.30 ±0.65 versus 0.91 ±0.30, non-significant). The number of mutations obtained by whole exome sequencing is described in table 9.

Two different gene scoring systems were used to predict the oncogenic potential of each mutation (table 9). Firstly, GeneRanker score, which is based on information extracted from the literature and from other curated sources as described in [200]. The ranking of a gene is annotated as an overrepresentation index, where the index is proportional to the gene-disease association, with a score >1 predicted as oncogenic. Secondly, we used Oncoscore, an in-house developed cancer gene scoring system based on automatic literature data mining (unpublished). To determine the Oncoscore, the queries of genes were submitted and information was retrieved from PubMed based on the gene of interest being cited in cancer-related papers, with respect to total gene citations in scientific literature. The score of a queried gene is annotated in a range from 0 to 100, where higher scores associated to a stronger oncogenicity. The oncogenic cut-off score of a particular gene was determined based on intersection point between scores obtained from cancer and non-cancer causing gene. From this analysis, Oncoscore >20 was predicted as a potential oncogene (data not shown). However, the reported values (table 9) in GeneRanker and Oncoscore columns represent the sum of all individual scores of somatic variants identified by exome sequencing in each patient.

Table 9: Clinical features of 23 chronic phase CML patient at diagnosis.

No.	Patient	Age/Gender	Sokal Score	Phase at onset	Synonymous Mutation	Non-Synonymous Mutation	GeneRanker Score	Oncoscore	CCyR at 3 months with IM	Follow-up CCyR at 2 years
1	CMLPh+001	25/M	0.8	Chronic	1	1	2	67.88	Achieved	Achieved
2	CMLPh+002	23/M	0.8	Chronic	2	3	2.25	116.49	Failed	Achieved
3	CMLPh+003	68/M	1.6	Chronic	20	4	7	50.37	Failed	Achieved
4	CMLPh+004	55/F	2.6	Chronic	11	7	5	192.61	Failed	Failed
5	CMLPh+005	52/M	0.66	Chronic	2	5	0.5	96.13	Achieved	Achieved
6	CMLPh+006	45/M	0.91	Chronic	0	1	0	23.71	Achieved	Achieved
7	CMLPh+007	49/M	0.65	Chronic	2	4	0.5	24.14	Achieved	Achieved
8	CMLPh+008	22/F	1.52	Chronic	2	5	3.5	55.63	Failed	Achieved
9	CMLPh+010	82/F	0.91	Chronic	0	0	0	0.00	Failed	Achieved
10	CMLPh+011	81/M	0.91	Chronic	6	9	4	179.56	Achieved	Achieved
11	CMLPh+012	32/M	0.79	Chronic	0	1	2	56.61	Achieved	Achieved
12	CMLPh+013	21/F	1.79	Chronic	1	4	9.5	69.19	Failed	Achieved
13	CMLPh+014	24/F	0.57	Chronic	1	1	0	14.60	Achieved	Achieved
14	CMLPh+015	70/M	1.5	Chronic	1	11	10.75	219.68	Achieved	Achieved
15	CMLPh+016	35/M	0.9	Chronic	2	5	3	117.39	Achieved	Achieved
16	CMLPh+017	66/M	1.2	Chronic	3	6	2.5	103.60	Achieved	Achieved
17	CMLPh+018	72/M	0.83	Chronic	4	4	3	63.38	Achieved	Achieved
18	CMLPh+019	51/M	0.63	Chronic	3	3	1	26.12	Achieved	Achieved
19	CMLPh+020	41/M	0.6	Chronic	1	7	1	79.76	Failed	Achieved
20	CMLPh+021	33/M	0.94	Chronic	1	3	0.25	37.54	Failed	Achieved
21	CMLPh+022	80/F	1.11	Chronic	3	11	9.25	178.63	Achieved	Achieved
22	CMLPh+023	40/F	0.86	Chronic	3	6	5	138.21	Failed	Failed
23	CMLPh+024	87/F	1.04	Chronic	7	6	4	111.96	Failed	Achieved

3.2 Calling of Somatic variants by Whole Exome Sequencing

To identify the somatic variants from 23 chronic phase CML patients at diagnosis, we performed a whole exome sequencing analysis on matched tumour/control CML samples. Collectively, 6-8 GBases sequencing data were gathered with mean exon coverage of 84 fold per sample and having 90% of targeted bases with mean coverage over ≥ 20x. Globally, we identified a total of 141 (65%) non-synonymous and 76 (35%) synonymous mutations (Table 10). No mutation was detected in patient 10. Of these, 141 non-synonymous mutations identified by exome sequencing, were validated by standard Sanger sequencing with a success rate of 75% (107/141). The identified mutations were not previously known, except one. The only known somatic alteration found was ASXL1 p. Q829* in patient 2.

In order to predict the functional effect of the somatic variants identified by whole exome sequencing, Polymorphism Phenotyping v2 (PolyPhen-2) software was applied [205]. The PolyPhen-2 predicted 18% of mutations as possibly damaging with transitions present in over 80% of the variants (figure 8). The number of mutations present in chronic phase CML cases was found to be correlated with age (P=0.0258; Pearson (r) 0.4638) (Figure 9A), suggesting that a significant fraction of the mutations that are present in chronic phase may be passenger variants that physiologically accumulate in individual cells over time and therefore having a minimal or no oncogenic effect. However, according to the GeneRanker score, 17.76% and 14.95% of the somatic variants identified in CP-CML at onset have a predicted score of ≥1 and ≥2 respectively (figure 10) and therefore should be considered as bonafide oncogenic variants. To test if the number and oncogenic score of the somatic variants found in CML at diagnosis were different from those at blast crisis, we performed exome sequencing in a set of 10 blast crisis samples and we compared the average number of mutations (CP 4.65 vs BC 3.80) and the oncogenic score by GeneRanker (Table 11). The mean GeneRanker score of the mutated genes identified in 10 blast crisis (BC; 1.97 ±0.53 SEM) patients was significantly higher (p=0.0048) in comparison to 23 chronic phase (0.72 ±0.14 SEM) CML patients (Figure 9B).

To examine the presence of copy number abnormalities in CP patients, we analysed exome data by using our in-house developed tool CEQer [206]. We didn't observed any copy number abnormality and no fusion gene was detected besides *BCR-ABL1* by analysing RNA seq data with FusionAnalyser software [207]

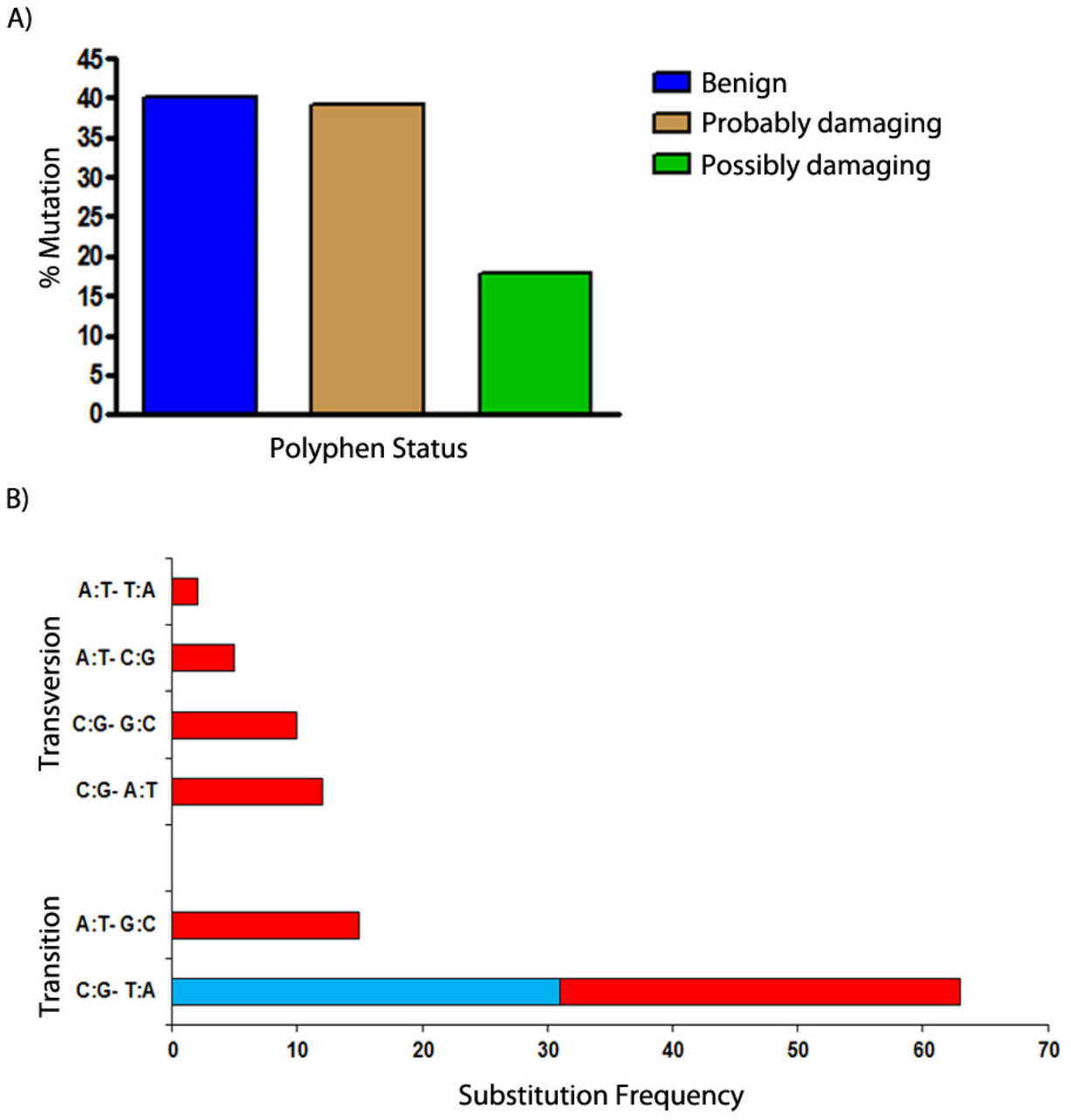

Figure 8: A) Structural and functional effect of somatic variation predicted by Polyphen-2 (Polymorphism Phenotyping). The potential influence of amino acid substitution on gene function

was categories into Benign, probably damaging and possibly damaging. B) The nucleotide substitutions into transition and transversion mutations obtained by WES from 23 CP CML samples at diagnosis. The red bar indicates the nucleotide substitution frequency between 6 different mutation types. The blue bar represents C→T substitutions representing in the first base of CpG dinucleotide.

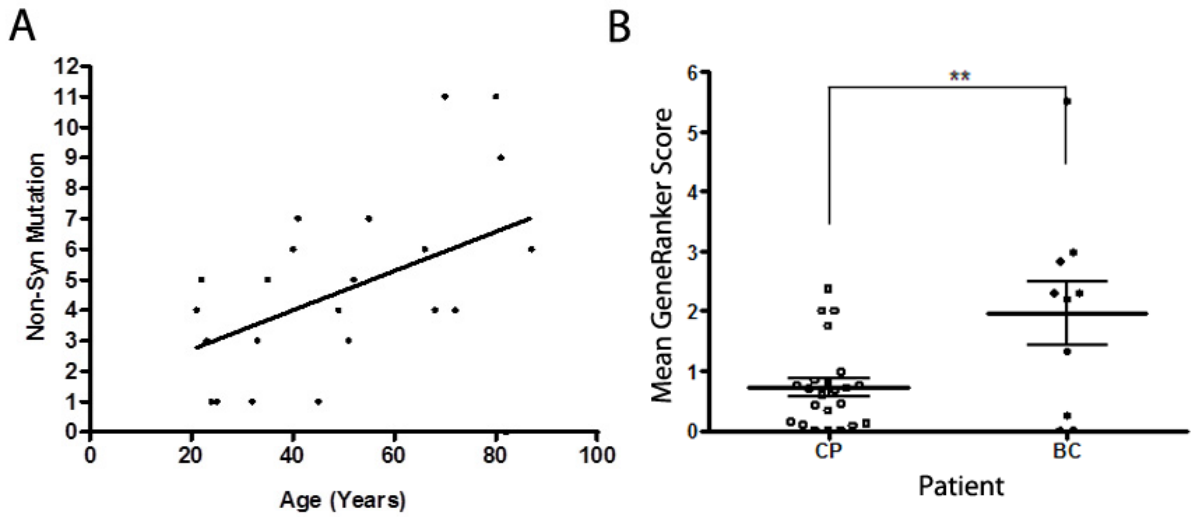

Figure 9: A) Pearson's correlation coefficient analysis between patient age and number of non-synonymous mutations from chronic phase CML patients. B) Comparison of gene ranker score in 23 chronic phase and nine blast crisis patients. Statistically $p<0.05$ was considered significant.

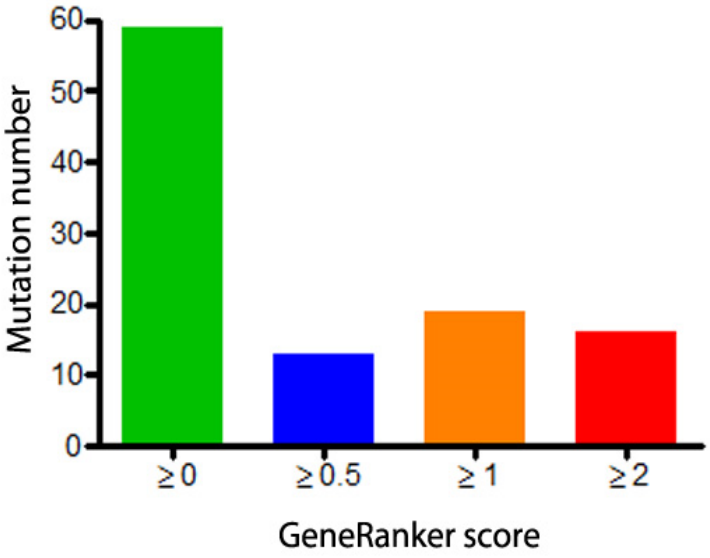

Figure 10: GeneRanker score of a particular gene was queried by using web based tools (URL: http://cbio.mskcc.org/tcga-generanker/). The mutations were distinguished into four ranges; the different bars represent indexes with ≥0 (Green), ≥0.5 (Blue), ≥1 (Orange) and ≥ (Red).

Table 10: Somatic variants from 23 chronic phase CML patients by whole exome sequencing at diagnosis.

Patient	Mutation	Gene Description	Amino acid	Co-ordinates	Base change	Gene Ranker	Oncoscore	Mutation ratio
CMLPh+001	PATZ1	POZ (BTB) and AT hook containing zinc finger 1	F539L	31724801	G/T	2	67.88	47.8
CMLPh+002	KBTBD7	Kelch repeat and BTB (POZ) domain containing 7	I196V	41767808	T/C	0	0.00	52.5
	ASXL1	Additional sex combs like 1 (Drosophila)	Q829*	31023000	C/T	0.25	77.56	36.0
	MAP3K4	Mitogen-activated protein kinase kinase kinase 4	R433*	161470601	C/T	2	38.93	54.2
CMLPh+003	HMCN1	Hemicentin 1	C1241Y	185964163	G/A	0	12.50	38.8
	SPHKAP	SPHK1 interactor, AKAP domain containing	N1027K	228882489	G/T	3	0.00	38.2
	TMED4	Transmembrane emp24 protein transport domain containing 4	G76D	44621356	C/T	0	0.00	45.3
	PTPRD	Protein tyrosine phosphatase, receptor type, D	L760F	8460424	G/A	4	37.87	39.3
CMLPh+004	DLG5	Discs, large homolog 5 (Drosophila)	V924I	79572041	C/T	0	63.23	55.6
	TEP1	Telomerase-associated protein 1	K1981R	20845585	T/C	1	59.45	53.1
	C17orf53	Chromosome 17 open reading frame 53	A247V	42225911	C/T	0	0.00	37.7
	C1orf62	AKNA domain containing 1	A281V	109394445	G/A	0	0.00	48.6
	SCN10A	Sodium channel, voltage-gated, type X, alpha subunit	I1482T	38743542	A/G	0	7.12	50.0
	POLN	Polymerase (DNA directed) nu	F475L	2082722	A/G	2	34.72	54.7
	AKAP4	A kinase (PRKA) anchor protein 4	A579V	49957628	G/A	2	28.09	47.8
CMLPh+005	AC013553.1 (SLC51B)	Organic solute transporter subunit beta	I50R	65343904	T/G	0	10.26	54.2
	HSF2	Heat shock transcription factor 2	Q375R	122744779	A/G	0	24.45	47.6
	CBWD6	COBW domain containing 6	C239Y	69238176	C/T	0	0.00	39.2
	MYOF	Myoferlin	Q177P	95169400	T/G	0.5	20.87	47.6
	PLXNB1	Plexin B1; Receptor for SEMA4D	R825H	48459884	C/T	0	40.54	53.6

CMLPh+006	POU6F1	POU class 6 homeobox 1	D140G	51585526	T/C	0	23.71	56.4
CMLPh+007	TRAFD1	TRAF-type zinc finger domain containing 1	V526M	112589901	G/A	0	0.00	45.2
	TCEB3B	Transcription elongation factor B polypeptide 3B (elongin A2)	A576T	44559910	C/T	0	0.00	45.8
	IGSF21	Immunoglobin superfamily, member 21	H279Q	18692013	C/G	0.5	0.00	47.7
	LIMS2	LIM and senescent cell antigen-like domains 2	F138C	128412016	A/C	0	24.14	52.2
CMLPh+008	TUBA1B	Tubulin, alpha 1b	I115V	49523057	T/C	0.5	0.00	52.2
	RBM19	RNA binding motif protein 19	H512R	114383724	T/C	0	0.00	48.1
	ASB2	Ankyrin repeat and SOCS box containing 2	V173M	94417564	C/T	0	27.36	45.8
	OR10H2	Olfactory receptor, family 10, subfamily H, member 2	V65I	15839046	G/A	0	0.00	51.3
	ROR2	Receptor tyrosine kinase-like orphan receptor 2	A643T	94486849	C/T	3	28.26	51.8
CMLPh+0010	No Mutation	-	-	-	-	-	-	-
CMLPh+0011	ARNTL	Aryl hydrocarbon receptor nuclear translocator-like	K259N	13391271	G/C	0.5	13.03	44.8
	MYO16	Myosin XVI	R747H	109617187	G/A	1,000	0.00	58.8
	KARS	Lysyl-tRNA synthetase	A54P	75675524	C/G	1,000	18.36	43.0
	PCDHA5	Protocadherin alpha 5	R12W	140201394	C/T	0	0.00	45.5
	SH3PXD2B	SH3 and PX domains 2B	A566T	171766413	C/T	0	12.02	43.6
	STC2	Stanniocalcin 2	R225C	172745086	G/A	0.5	50.48	45.0
	DUSP22	Dual specificity phosphatase 22	A56V	335142	C/T	1,000	49.32	32.7
	GPR30 (GPER1)	G protein-coupled receptor 30	D202N	1131968	G/A	0	36.35	31.8
	BX255925.17-2	Uncharacterized protein FLJ40246	W365*	140147839	G/A	0	0.00	71.9
CMLPh+0012	MYST2	K(lysine) acetyltransferase 7	R311G	47893243	C/G	2	56.61	46.6
CMLPh+0013	UNC13C	Unc-13 homolog C (C. elegans	R626Q	54786821	G/A	0.5	5.61	50.55
	BRSK1	BR serine/threonine kinase 1	R701Q	55820019	G/A	1.25	39.31	39.13

	CACNA1I	Calcium channel, voltage-dependent, T type, alpha 1I subunit	I226V	40030665	A/G	1.5	8.44	46.40
	AR	Androgen receptor	K659N	66931335	G/T	6.25	15.83	46.02
CMLPh+0014	NAG	N-acetylglucosaminidase	I1685V	15427282	T/C	0	14.60	42.6
CMLPh+0015	HNRNPH3	Heterogeneous nuclear ribonucleoprotein H3 (2H9)	Y156C	70098927	A/G	0.5	0.00	51.4
	CADM1	Cell adhesion molecule 1	A262V	115088648	G/A	1.25	66.97	51.2
	OR11G2	Olfactory receptor, family 11, subfamily G, member 2	R160P	20665973	G/C	0	0.00	51.1
	TINAGL1	Tubulointerstitial nephritis antigen-like 1	V443I	32052503	G/A	0	36.05	50.0
	LEPR	Leptin receptor	S882F	66088636	C/T	1	2.77	45.5
	TNNI3K	TNNI3 interacting kinase	M717L	74954900	A/C	2	2.08	53.4
	FCAMR	Fc receptor, IgA, IgM, high affinity	G374*	207133966	C/A	0	0.00	39.7
	CCDC80	Coiled-coil domain containing 80	S648I	112349052	C/A	1	47.41	52.5
	GPR156	G protein-coupled receptor 156	A705T	119886211	C/T	0	0.00	48.5
	CTNNA1	Catenin (cadherin-associated protein), alpha 1	N257S	138160400	A/G	4	64.39	54.4
	JAKMIP2	Janus kinase and microtubule interacting protein 2	D409H	147021327	C/G	1	0.00	43.4
CMLPh+0016	SPRED3	Sprouty-related, EVH1 domain containing 3	P159L	38885335	C/T	0.5	0.00	57.5
	VN1R1	Vomeronasal 1 receptor 1	S199N	57967259	C/T	1	4.69	47.6
	DSCAM	Down syndrome cell adhesion molecule	V1954M	41385098	C/T	0	8.60	55.9
	AMACR	Alpha-methylacyl-CoA racemase	E173K	34024078	C/T	1	83.04	48.2
	CYLN2	CAP-GLY Domain Containing Linker Protein 2	K39E	73731991	A/G	0.5	21.07	40.4
CMLPh+0017	MYH7	Myosin, heavy chain 7	A161T	23901869	C/T	0.5	3.99	50.00
	BBS2	Bardet-Biedl syndrome 2	G224D	56540078	C/T	0	1.72	51.00
	WDR63	WD repeat domain 63	V807I	85595682	G/A	0	0.00	40.96
	SHPRH	SNF2 histone linker PHD RING helicase	R936C	146256227	G/A	0	31.10	49.02

	QKI	QKI, KH domain containing	D74V	163876389	A/T	2	34.60	46.32
	IFNA8	Interferon, alpha 8	A2T	21409179	G/A	0	32.19	32.50
CMLPh+0018	MARK3	MAP/microtubule affinity-regulating kinase 3	R573*	103958244	C/T	1	22.35	56.16
	AC099524.5 (CMIP)	c-Maf-inducing protein isoform C-mip	NA	81739201	C/T	0	9.70	43.75
	DDX4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	R596H	55110902	G/A	2	14.36	55.56
	TINAG	Tubulointerstitial nephritis antigen	G54E	54173509	G/A	0	16.97	40.38
CMLPh+0019	FAM82A1	Family with sequence similarity 82	R428I	38208444	G/T	0.25	0.00	53.09
	SORCS2	Sortilin-related VPS10 domain containing receptor 2	E671Q	7725526	G/C	0.5	13.03	51.47
	SOX8	SRY (sex determining region Y)-box 8	A306V	1034962	C/T	0.25	13.09	52.17
CMLPh+0020	C2orf51	Testis expressed 37	P7S	88825181	C/T	0	0.00	60.71
	TRA2B	Transformer 2 beta homolog (Drosophila)	R65Q	185643391	C/T	0	24.94	64.52
	SC4MOL	Methylsterol monooxygenase 1	P111L	166259017	C/T	1	15.14	52.98
	GFRAL	GDNF family receptor alpha like	C210W	55216310	T/G	0	0.00	48.51
	SVEP1	Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	T523M	113261434	G/A	0	39.68	47.87
	ARHGAP23	Rho GTPase activating protein 23	I1002M	36646362	C/G	0	0.00	44.95
	C19orf54	Chromosome 19 open reading frame 54	R110*	41251099	G/A	0	0.00	44.87
CMLPh+0021	RLF	Rearranged L-myc fusion	E1748G	40705617	A/G	0.25	13.60	63.89
	MLXIPL	MLX interacting protein-like	R552L	73011211	C/A	0	23.94	46.15
	GTPBP1	GTP binding protein 1	A311S	39132191	G/A	0	0.00	45.45
CMLPh+0022	FRMD4B	FERM domain containing 4B	P129T	69351525	G/T	0	0.00	44.32
	NLGN4X	Neuroligin 4, X-linked	G503S	5821212	C/T	1	2.64	42.86
	OR5B17	Olfactory receptor, family 5, subfamily B, member 17	P235L	58125839	G/A	0.5	0.00	35.09
	NCAM1	Neural cell adhesion molecule 1	V100I	113075208	G/A	5.75	32.17	56.32

	ACSM4	Acyl-CoA synthetase medium-chain family member 4	V119M	7459282	G/A	0	0.00	48.28
	FAM124A	Family with sequence similarity 124A	R368Q	51854746	G/A	0	0.00	40.63
	NALCN	Sodium leak channel, non-selective	R297C	101944628	G/A	1	5.57	52.00
	CARD14	Caspase recruitment domain family, member 14	R152Q	78157817	G/A	0	29.82	59.46
	SSTR4	Somatostatin receptor 4	R251M	23016872	G/T	0	43.47	52.38
	SLC12A5	Solute carrier family 12 (potassium/chloride transporter), member 5	R231H	44669091	G/A	1	24.14	52.83
	FBN3	Fibrillin 3	R233C	8206866	G/A	0	40.80	57.14
CMLPh+0023	SLC6A17	Solute carrier family 6, member 17	W206S	110717446	G/C	0.50	0.00	40.18
	PCDH1	Protocadherin 1	S1147N	141233881	C/T	0.00	11.50	46.77
	KAT6A	K(lysine) acetyltransferase 6A	G1549S	41791093	C/T	3.50	75.32	42.69
	PATE2	Prostate and testis expressed 2	T47I	125647834	G/A	0.00	0.00	42.03
	RECQL	RecQ protein-like (DNA helicase Q1-like)	G375V	21628494	C/A	1.00	51.39	34.33
	VAT1L	Vesicle amine transport protein 1 homolog (T. californica)-like	R125S	103566702	C/A	0.00	0.00	36.36
CMLPh+0024	SCNM1	sodium channel modifier 1	Y41C	151139612	A/G	0.00	0.00	49.21
	ESRRG	Estrogen-related receptor gamma	T91N	216850549	G/T	1.00	30.69	45.00
	PCM1	Pericentriolar material 1	N1071K	17823974	T/A	3.00	34.66	46.67
	TMC1	Transmembrane channel-like 1	L216F	75369705	C/T	0.00	4.96	40.88
	OR10A5	Olfactory receptor, family 10, subfamily A, member 5	D181E	6867456	C/G	0.00	0.00	45.16
	SERPINB2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2	G179S	61565078	G/A	0.00	41.64	56.00

Table 11: Somatic variants detected in 10 blast crisis samples by WES

Patient	Gene name (aa substitution)	Ratio	GeneRanker
1	RTP2(A190V)	43%	0
	KCNH3(A314V)	55%	0.5
2	FAT4 (R1698W)	53%	1
	FUT3 (R354C)	38%	1
	RUNX1(K194N)	67%	6.5
3	SMARCA4(A945T)	55%	8
	UBE2A(D114V)	93%	1.5
	ABL1(F486S)	50%	7
4	PTPN11(G503V)	38%	5.5
	FAM123C(R709H)	41%	0.5
	LAMA2(P1025S)	44%	1.75
	GRIN3A(R1024*)	39%	1
5	SETBP1	32%	0
6	NRAS(Q61R)	32%	7.5
	DEFB119(R42H)	48%	0
	IKZF1(N159S)	42%	2
	C9orf98(R125H)	64%	0
	CASK(K250R)	47%	2
7	-	-	-
9	PPT1(V168A),	41%	1
	MDH1B(A272V/A272T)	47%/47%	0
	GPR98(R1745C)	47%	0.5
	CEL(E216Q)	44%	1
	LRP4(D449N)	55%	0
	CYP2B6(R145W)	40%	2.25
	BCR(F615W)	44%	4.5
10	ASXL1(G641_fs)	37%	0.25
	EPB41L3(P963L)	37%	1.3
	FGFR4(V262M)	39%	5.3
	RUNX1(D171G)	25%	6.5
	UBE2A(I33M)	39%	1.5
11	ABL1(E255V)	28%	7
	BARD1(G527_fs)	28%	5
	BSN(R3264H)	26%	0.5
	EFCAB4B(V643M)	31%	0
	KRT7(R339W)	41%	1.3
	MUDENG(D289N)	38%	0
	ROBO2(P1055_fs)	24%	3
	XPO1(E571K)	32%	1.5

3.3 Predicting potential driver gene by Oncoscore

Understanding the presence of driver events in early disease phase is crucial. The implementation of corrective measures is required in order to achieve highest therapeutic response with current treatments or by developing new therapies. In chronic phase, accumulation of genetic events is limited, except BCR-ABL1 fusion and therefore to detect initial driver event is always challenging. So, we developed a new driver gene predicting software termed Oncoscore. Besides Oncoscore, there are also other tools available, which predict the cancer-gene association such as GeneRanker score [200], Chasm score [208], S-score [209], but their application is limited only to genes associated with solid tumours. In addition, periodic up-date of database is required to include the new oncogene and updating the score of known genes. Therefore, by considering this, we introduced a new scoring system that detects the cancer-gene association from all cancer types including hematological malignancies and updates automatically in real-time thanks to PubMed curation of literature.

To test the authenticity of Oncoscore, we performed a statistical linear regression analysis between GeneRanker score and Oncoscore of somatic variants identified in 23 chronic phase CML patients by WES. The strength of association found between two variables was highly positive (r=0. 6615) as per Pearson's correlation co-efficient (Figure 11A). This correlation suggested that predicting score of a particular gene by Oncoscore is at least as reliable as by GeneRanker tool, with the advantage of real-time updating. According to Oncoscore, 43/107 (40%) somatic variants were predicted to have oncogenic score >20 and surprisingly none of the 43 variants except ASXL1 oncogene have been reported to be a real driver variant in hematological malignancies.

We hypothesize that the high scoring somatic variants could be therapeutically relevant. To study this, the relationship between disease prognostic score (Sokal score) and Oncoscore of somatic variants was determined. The positive correlation co-efficient (r 0.4328) between Sokal score and total Oncoscore predicted that somatic variants of oncogenic activity were observed in patient with intermediate and high Sokal score (figure 11B). Therefore, it was considered that the

variant weigh high Oncoscore have significant oncogenicity that could affects the treatment response, and could be associated with bad prognosis.

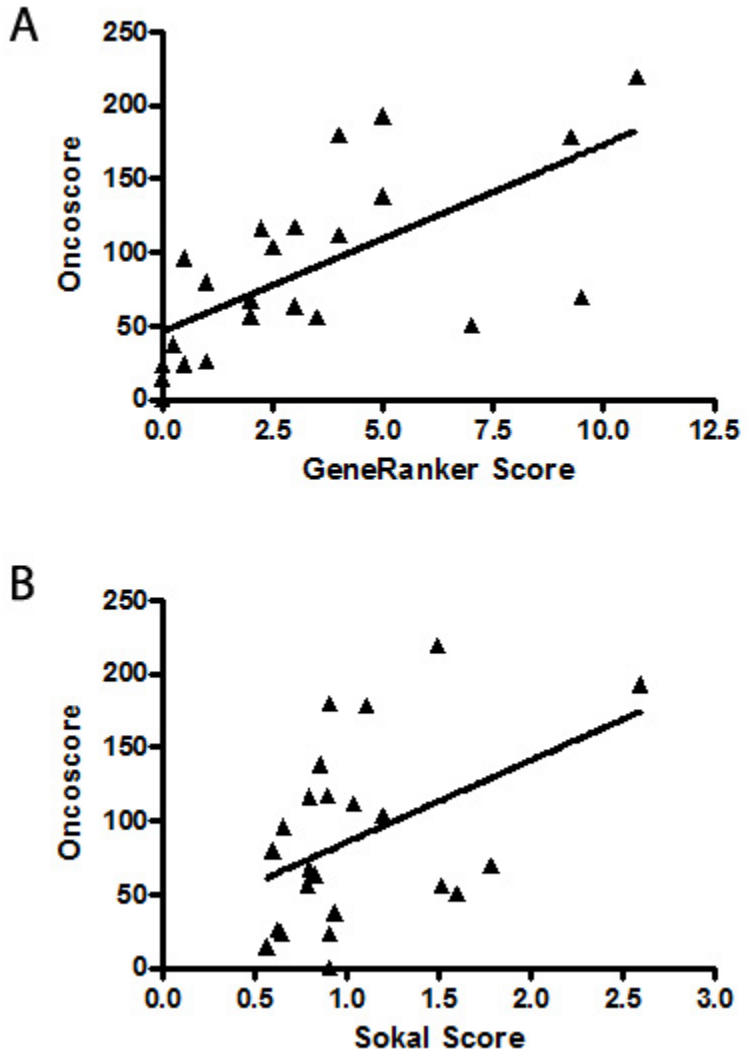

Figure 11: Linear regression model analysis between clinical scores from 23 CML patients. The correlation analysis between Oncoscore against A) GeneRanker and B) Sokal score on each CML cases.

3.4 Therapeutic responses to TKI treatment:

All the 23 CP-CML cases at diagnosis were prescribed with first line tyrosine kinase inhibitor imatinib (TKIs). The cytogenetic and BCR-ABL1 transcript profile was periodically examined in accordance with NCCN Clinical Practice Guidelines [149]. At 3 months following diagnosis, 13/23 (56%) patients achieved complete cytogenetic responses (CCyR). No significant differences was detected in average number of mutations (4.77 vs 4.50) and Oncoscore (90.11 ±18.69 vs. 85.18 ±17.62, SEM; p=0.9753) between patients with or without CCyR on Imatinib at 3 months (figure 12A and 12B).

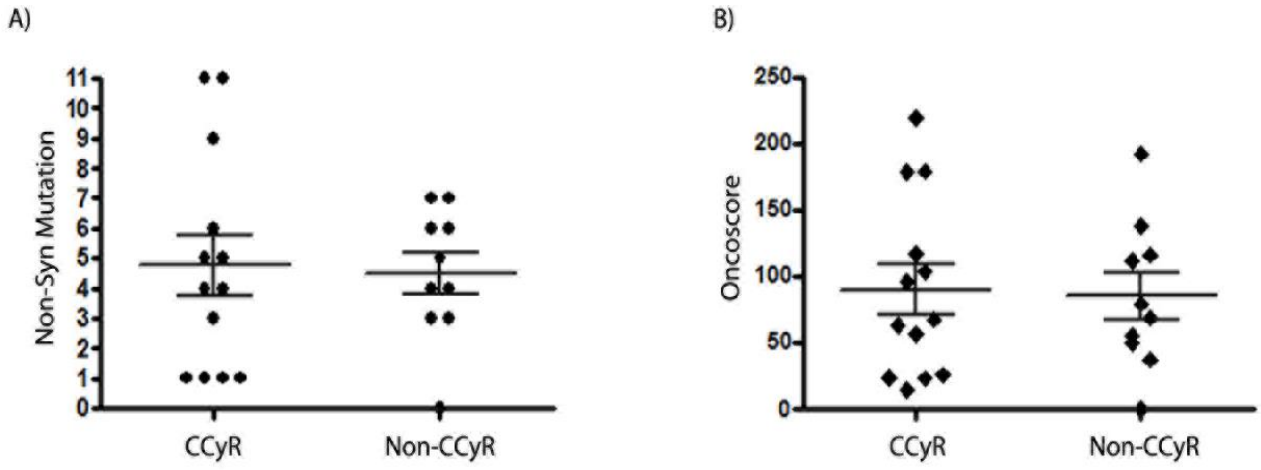

Figure 12: Cytogenetic response of 23 CML patients at 3 months. Analysis between A) non-synonymous mutation and B) Oncoscore in patients with CCyR and Non-CCyR at 3months. A significant test was calculated by using two-tailed unpaired *t-test* method.

On long-term follow-up, a total of 21/23 patients achieved complete cytogenetic responses, while 2/23 patients (pt4 and 23) never achieved CCyR with all generations TKIs and repetitively showed signs of relapses. The prognostic Sokal score in these two non-responding patients was found to be significantly higher (P 0.0307) than that of the 21 responding patients (figure 13A). Similarly, the total Oncoscore in non-responding patients (165.4 ±27.20 SEM) was 2-fold higher compared to responding patients (80.59 ±12.72 SEM) (figure 13B). Thus, the patients with bad prognosis carry somatic variants that might associate to CML progression.

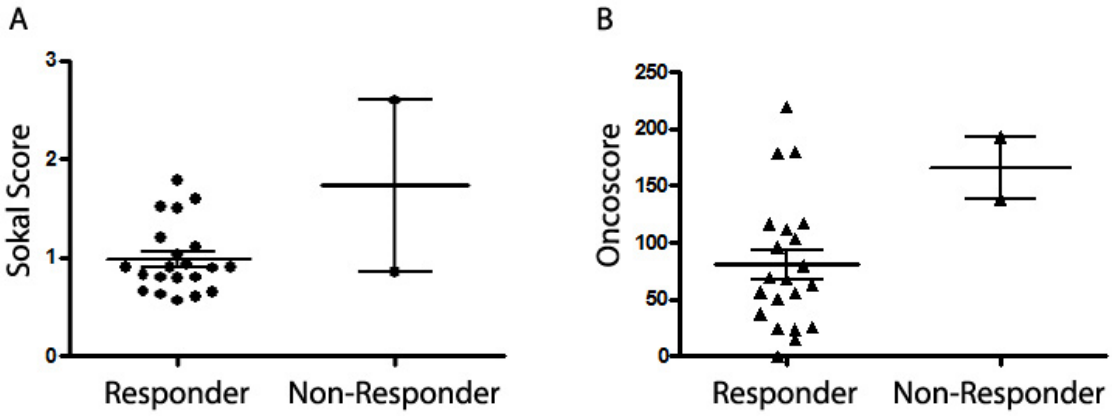

Figure 13: Column statistical analysis on patient cytogenetic status at long term follow-up. Distribution of A) Sokal score (left) and B) Oncoscore (Right) in responding and non-responding CML patients with different TKIs. A significant test was calculated by using unpaired *t-test* method considering $P<0.05$ as statistically significant, whereas deviation is referred as SEM.

Currently, 19 of the 21 responding patients are still in complete remission and none of them progressed to AP or BC during their follow-up, while two responders (pt. 10 and 11) have died of unrelated causes. Of the 2 non-responding patients, one maintains disease stability and the other one is in blastic phase and proposed for hematopoietic stem cell transplantation.

3.5 SETBP1 in BC-CML patient

After analysing the exome sequencing of 10 CML-BC patients (Table 11), we identified a somatic SETBP1 p.L842M mutation in patient 5 (Figure 14). The patient identified with SETBP1 mutation was associated with very poor prognosis. SETBP1 was also found recurrently mutated in 25% of aCML samples [113]. *Piazza, R. et.al.* has explained that mutation on SETBP1 SKI homology domain increases the half-life of the protein. From the collective analysis of other hematological malignancies, the mutations were predominantly accumulated in the SKI homology

domain within a 14AA residue stretch. The mutations detected in CML-BC and aCML patients were clustered in SKI homology, which is a prime region of β-TrCP1 (E3 ligase ubiquitinase enzyme) binding, required for proteosomal degradation [113]. So, we considered to construct a conditional knock-in Setbp1^{G870S} mouse model to unveil the tumorigenic properties of the gene, which is not completely illustrated.

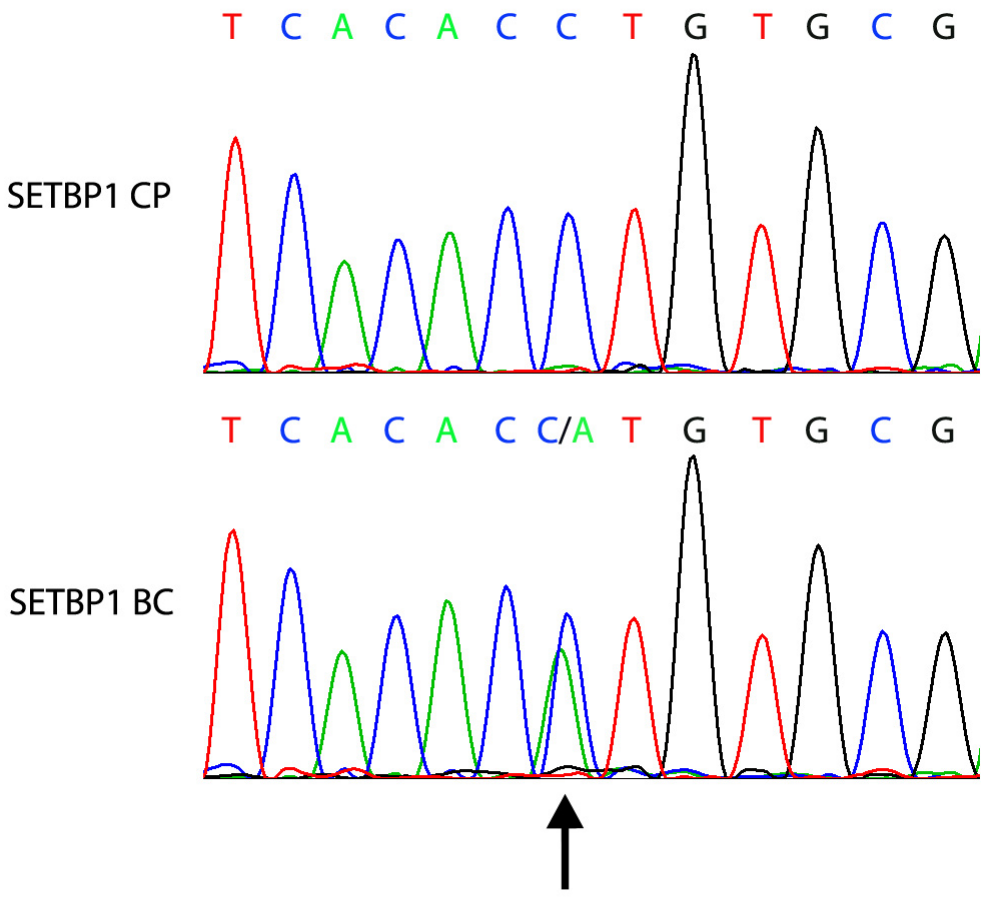

Figure 14: Chromatogram of *SETBP1* heterogeneous mutation identified in CML-BC patient 5. The confirmation of somatic mutation was performed by Sanger sequencing from a matched CP/BC samples. The arrow indicates the presence of double peak in BC as absent in CP samples.

3.6 Construction of conditional Knock-in Setbp1^{G870S} system

Genetically engineered knock-in mouse models are extremely useful tools for dissecting gene function [210]. Here, we describe a strategy for creating Setbp1^{G870S} mice in which a point mutation allele replaces the wild type allele in a conditional manner. The benefit of a conditional knock-in system is to induce disease phenotype through controlled Cre-loxP system. The following conditions must apply for a correct conditional knock-in model in vivo [210]:

a) Before induction of the knock-in mutation, the wild type allele must function normally under the control of endogenous promoter in terms of expression pattern.
b) Before induction, the mutated coding sequence must be transcriptionally inactive.
c) The transcriptional control of the mutated allele would be identical to that of the wild type allele.
d) After Cre recombination, the wild type coding sequence must be completely replaced by mutated sequence.

For the construction of *Setbp1*^{G870S} vector, the genomic Setbp1 fragments were initially pre-cloned into a pCR4-Topo shuttle vector and later cloned into pDelboy-3X backbone vector. The fragments were introduced one-by-one by seamless directional cloning of PCR-generated sequences using the In-Fusion cloning technology. The final construct, which would then be applied to modify the 3' end of the endogenous Setbp1 gene by homologous recombination (HR) technique as described in figure 15, consists of the following parts, from the left a) ~3.2kb homology *Setbp1* intronic sequence located downstream of exon3 required for HR, b) neomycin selection cassette that was flanked by frt recombination sites, c) WT exon4-6 Setbp1 cDNA sequence (including a splice acceptor site at its 5' end and a triple transcriptional stop at the 3' end) flanked by loxP recombination sites, followed by d) Exon4^{G870S} mutation and e) at rear, downstream of exon4 p.G870S mutation is a ~3kb intron4 sequence for HR.

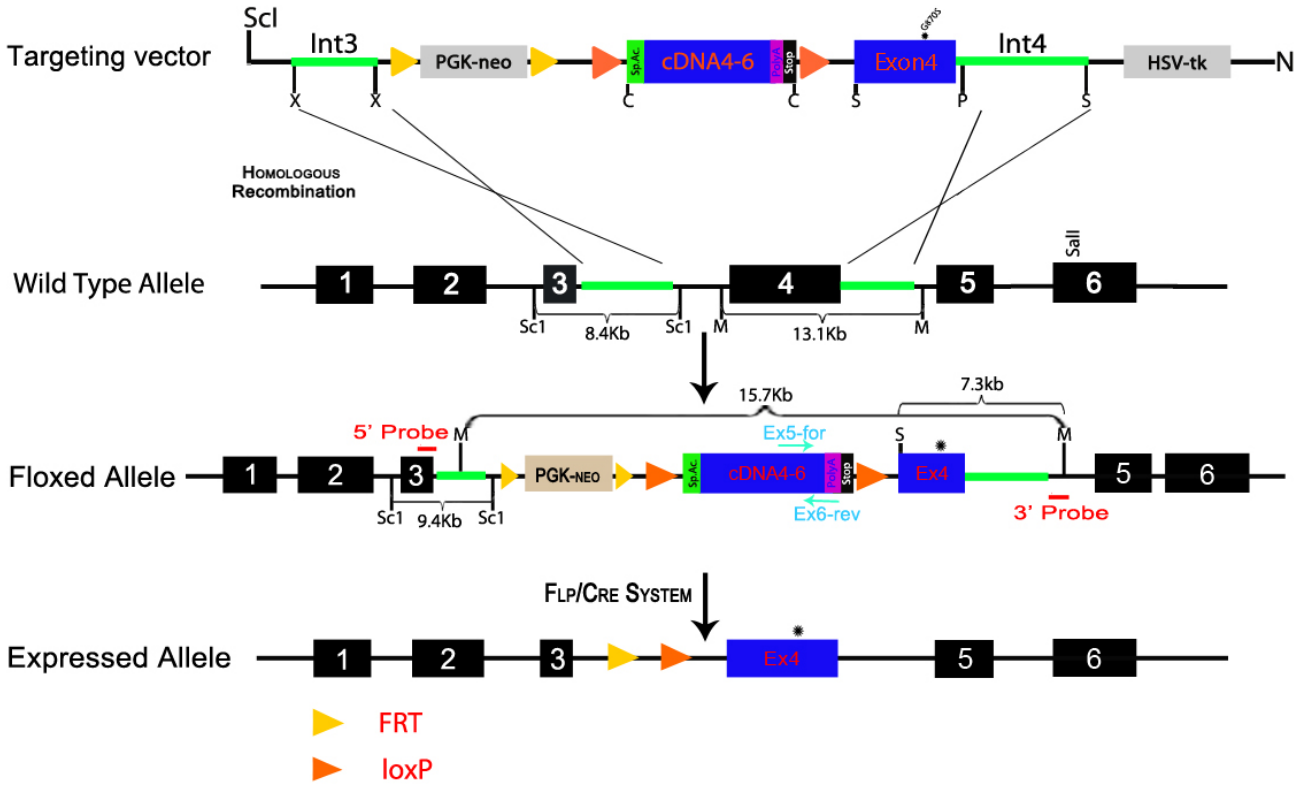

Figure 15: Conditional knock-in *Setbp1*^{G870S} targeting strategy and genotype evaluation. The labelled targeting vector is consisting of *Setbp1* sequences that were sub-cloned into the conditional replacement vector pDelBoy-3x. The intronic homology arms are indicated as green lines (Int3 and Int4) and the exon represent as blue boxes (cDNA4-6 and Exon4^{G870S}). LoxP and frt sites are displayed as orange and yellow filled triangles respectively. The neomycin cassette (positive selection of recombinant clones) and herpes simplex virus thymidine kinase (HSV-tk; negative selection of recombinant cells) shown as grey boxes in the vector are expressed under the control of mouse phosphoglycerate kinase 1 promoter. Neomycin cassette is flanked by flip recombinase (frt) sites which permit efficient excision of Neo^{R} selection marker. The fragment between loxP sites is a WT sequence of mouse Setbp1 is encompassing exons 4, 5 and 6, with a splice acceptor (SpAc) site at 5' end to facilitate correct splicing and a polyadenylation sequence (PolyA) followed by a triple transcriptional stop codon derived from SV40 promoter sequence at 3' end. Restriction sites are denoted as follows: X, XhoI; C, ClaI; S, SalI; P, PmeI; ScI, ScaI; M, MfeI; N, NotI. The corresponding Exon4 downstream of loxP sites carries the mutation at p.G870S introduced by site directed mutagenesis. The wild type allele shows complete genomic organisation of the wild type murine Setbp1 locus. Following homologous recombination in C57BL/6N strain embryonic stem cells, floxed allele positive cells were screened. The floxed allele panel represents a cyan coloured arrow for PCR screening at Ex5 and Ex6 junction which

indicates presence of targeted vector in the genome either correctly targeted or randomly integrated. The red lines indicate 5' and 3' external probes used for final screening by southern blot analysis for detection of correct recombination. Restriction sites relevant to southern blot analysis are shown, the probes hybridise the fragments referred by braces (number refers to size of fragments in wild type and floxed allele). In subsequent breeding, the neo cassette will be removed by Flp recombinase mediated deletion. The bottom panel shows the final organization of the conditionally rearranged locus, expressing the G870S mutation. The mutated allele will be obtained *in vivo* by mating the mouse bearing the floxed allele with a Cre-expressing mouse.

Hence, these genetic engineering techniques will allow the better understanding of Setbp1^{G870S} neoplastic role in mice. Also explore the secondary clonal events by high throughput sequencing methods and useful for the discovery of new therapeutic compound that will able to inhibit the overexpression effect of *Setbp1*.

4 Discussion

Chronic myeloid leukemia is diagnosed by the presence of *t*(9;22) chromosomal translocation, which forms *BCR-ABL1* fusion gene [24]. The disease is largely well managed by first line treatment Imatinib in chronic phase, but few cases develop resistance through *ABL1* kinase mutation and require second-generation TKIs to induce CCyR.

It is known that clonal evolution occurs in the advanced phases of chronic myeloid leukemia. However, little is known about the presence of clonal evolution in chronic phase that might associate with disease transformation or progression. To test whether other somatic variants may co-exist along with *BCR-ABL1* fusion gene in CML at onset, we performed a WES on matched samples of 23 CP patients. Analysis of the somatic variants identified by WES reveals that a large number of mutations accumulated in CP patients are passenger or age-related mutations, predicting that disease phenotype is more stable in CP as compared to situation in BC.

ASXL1 (additional sex combs 1) variants are known in myelo-dysplastic syndromes and myelo-proliferative disorders, which play a significant role in leukemogenesis and represent a factor for poor survival [211]. The biological function of *ASXL1* is not completely understood; it is believed to be involved in epigenetic regulation such as in histone modification. We detected evidence of *ASXL1* mutation in a single CML sample at diagnosis, undetectable in remission phase. Somatic variants, copy number abnormalities and the presence of additional fusion genes were also investigated from exome and RNA seq data: no further abnormalities were identified except somatic single nucleotide variants in 23 CP CML cases.

During our bioinformatics analysis, we implemented a very stringent filtering criterion and considered only variants present at a frequency ≥25%, which corresponds to the presence of a heterozygous mutation in 50% of cells. However, no recurrent genetic lesions were identified in CP CML samples; consider that the leukemogenesis was efficiently induced by existence of Ph+ chromosome. Simultaneously, we examined the somatic variants in matched 10 blast crisis samples by WES and found same number of mutation as compared to chronic phase patients. However, the mean GeneRanker score of variants in BC was found significant higher as compared

to variants in CP patients. Therefore, it seems that the mutations identified in BC were genuine driver variants and could be associated to CML progression.

Study by Schmidt et.al. has explained that mutations acquired during chronic phase could have neoplastic activity rather than just be a passenger mutations [110]. To understand this phenomenon they tested for BCR-ABL1 independent gene mutation in Ph-positive and Ph-negative clones by targeted deep sequencing of known oncogene. They identified 43% of genes (*DNMT3A*, *EZH2*, *RUNX1*, *TET2*, *TP53*, *U2AF1* and *ZRSR2*) mutations in Ph-negative clones were found similar in 33% of Ph-positive clones. So, their hypothesis that pre-BCR-ABL1 independent mutation was acquired in a clone providing a multistep pathogenesis of CML, one causing abnormal proliferation of a clone of pluripotent hematopoietic stem cells and the other inducing Ph1 in descendants of these progenitors [212, 213].

So, to understand the oncogenicity role of a somatic variant present in early phase of 23 Ph+ samples, we developed a new gene predicting software Oncoscore. The reason for developing a new scoring tool is to include the oncogene identified in hematological malignancies, which was unable to encompass by counterpart GeneRanker tool. Moreover, it also provides up to date analysis and retrieved information from all types of malignancies, which was not possible with other tools. The acquisition of oncogenic variants in high risk Sokal score patients in relationship to Oncoscore was found positive, which could be overtime either expanded or diminished during neoplastic transformation. However, one reason for obtaining positive trend because of patient age as one of the factor in calculating Sokal score, it is possible that majority of the somatic variants identified are just passenger or age related in chronic phase CML cases.

Around 40% (43/107) of the identified genes by Oncoscore were cancer oriented and some of the highly scored genes are *PATZ1*, *ASXL1*, *TEP1*, *MYST2*, *AMACR* and *KAT6A*. Apparently, none of the cancer oriented genes except ASXL1 were previously known to have pathological feature in leukemia. In this situation, we determined the oncogenic score of somatic variants in cohorts of treatment responsive (CCyR) and non-responsive (non-CCyR) patients by Oncoscore.

We observed that Oncoscore based oncogenes were enriched in individuals who failed to achieve complete cytogenetic responses on long term follow-up (Figure 13B), with 2 fold higher oncoscore values in 2 non-responsive patients than the one in 21 responsive patients. In fact the presence of polyclonal variants with low frequency at diagnosis have been correlated to sub-optimal or no response CP patients with TKIs as explained by [214]. Additionally, author also described that 50% of the chronic phase patients with pathogenic variants progressed to blast crisis within a median of 9 months from diagnosis. Thus, from the preliminary result, it is suggested that patients at high risk of CCyR failure accumulated cancer causing genes affect therapeutic responses. This model further requires an in-depth analysis on larger chronic phase CML population for confirmation.

Additionally, we exome sequenced 10 matched BC/CP samples and detected a *SETBP1* mutation in one of CML-BC patient, which was found mutated in various myeloid malignancies. However, the physiological role of *SETBP1* protein is not exactly known, apart from anti-PP2A activity. Piazza R. *et.al* has shown that the mutation inhibits SETBP1 protein degradation by restricting the binding of β-TrCP1 ubiquitination enzyme. This in turn increases SETBP1-Set protein interaction and inhibits the *PP2A* anti-tumour activity (Figure 16). It is found that patients carrying a *SETBP1* mutation experience worst prognosis with shorter survival in comparison to wild type *SETBP1* patients [113].

Formerly, germline mutations in SETBP1 were observed in Schinzel-Giedion syndrome (SGS) patients, a rare congenital disorder characterised by mental retardation, distinct facial features and multiple congenital malformations [215]. Apart from p.G870S, mutations at 868 and 871 were also associated with SGS. It is unclear whether these mutations result in a gain of function or have a dominant negative effect in SGS patients. Individuals with SGS have severe developmental dysregulation and neurological defects including severe feeding problems, epilepsy, dementia and visual or hearing impairment [216]. SGS also affects normal organ functioning in heart, kidney, and genitals. In the heart inadequate activity of heart valves is observed. In kidney, SGS patient suffer from hydronephrosis, in which urine deposits in the kidneys. The affected SGS individuals can also

have genitals abnormalities, condition like hypospadias in male and interlabial sulcus in female [217, 218]. The only known etiology of SGS is mutations on SETBP1 gene.

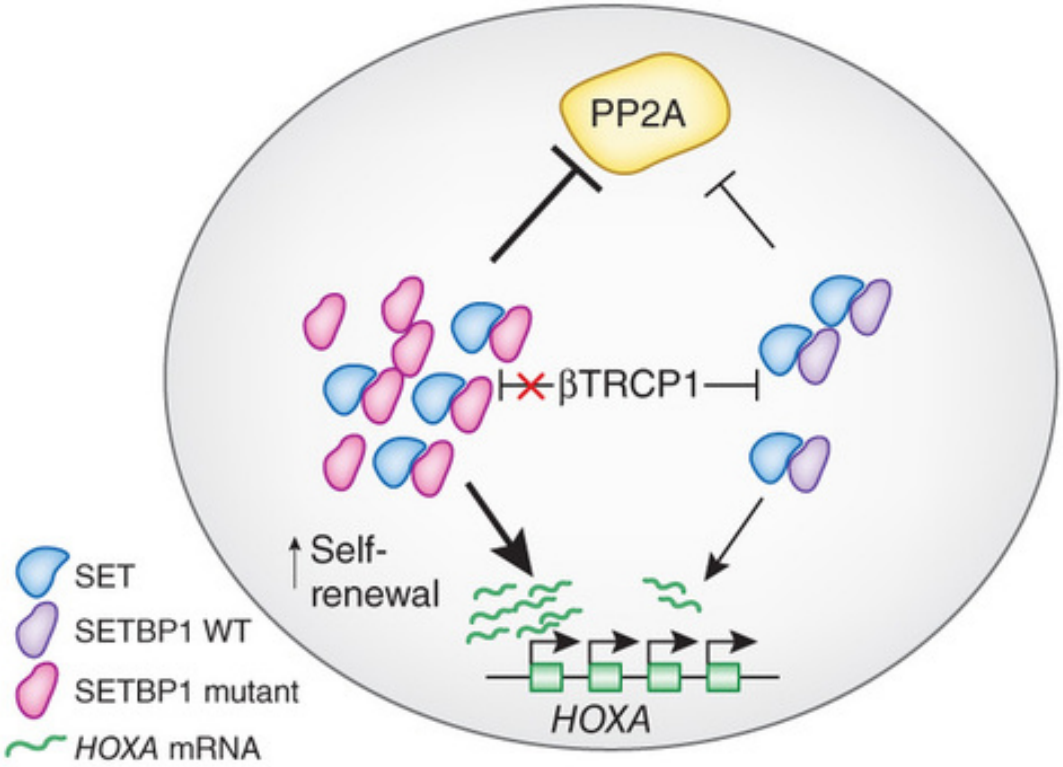

Figure 16: SETBP1 alterations in atypical myeloid leukemia inhibit the function of PP2A and subsequently impaired the binding of β-TRCP1 leads to halt in SETBP1 protein degradation and increases self-renewal characteristic of myeloid cells by transactivation of HOXA genes. Figure adapted from [219].

A better research model is required for understanding the unknown consequences of mutated *SETBP1*. Recent work has suggested that overexpressing Set protein induces leukemia like feature in mice and predicts adverse outcomes in patient with acute myeloid leukemia [220, 221]. Thus, to examine the role of *Setbp1* mutation in the leukemogenic process, we designed a conditional *Setbp1*^{G870S} knock-in system.

The tailoring of mouse genome at specific locus allows us to elucidate the physiological role of a transgene through homologous recombination based gene targeting system [222]. In this thesis, we have described the strategy in creating the conditional point mutant knock-in mice. By developing the conditional *Setbp1*^{G870S} mice, we will overcome all the limitation of definite *Setbp1*^{G870S} knock-ins. We focused to introduce a point mutation on exon4 of the *Setbp1* gene. Considering lethality of the *Setbp1*^{G870S} germline mutation, our strategy was to create a floxed allele that would function identical to the normal endogenous wild type ahead of Cre-recombination. Following Cre-recombination, the wild type *Setbp1* exon4-6 from floxed allele would be irreversibly deleted and the p.G870S exon4 would express somatically and translate into Setbp1^{G870S} protein.

The Cre/LoxP mediated recombination provides an extremely useful technique in introducing expression of the mutated transgene in a restrictive manner. There are two possibilities to induce transgene expression by Cre-mediated excision. In the first scenario, the conditional transgene is somatically activated by Cre recombinase, under the control of either a lineage/cell type specific promoter or a ubiquitous one. In the second strategy, the conditional gene is constitutively expressed during embryonic stage. Again, this can be driven by a ubiquitous or tissue-specific promoter [222, 223]. The generation of perfect conditional knock-in model, Cre/loxP is an ideal system.

Following the generation of *Setbp1*^{G870S} chimeric mouse by transplantation of knock-in ES cells into blastocyst of pseudo-pregnant mice aseptically, the 1st generation floxed/founder mice would be produced after cross breeding between chimeric mouse and wild type C57/BL6 strain mice. The *Setbp1*$^{wt/floxed}$ mice will express wild type *Setbp1* protein under the control of the endogenous promoter. After cross breeding of *Setbp1*$^{wt/floxed}$ with a panel of Cre-expressing transgenic lines, several cells/tissue specific expressing Setbp1^{G870S} phenotype will be generated. At least 7 Cre-expressing lines will be crossed with *Setbp1*$^{wt/floxed}$ mice to induce *Setbp1*^{G870S} expression

conditionally in a tissue specific manner or globally in all cells with their respective promoters. The various Cre-expressing lines that will be employed in this project are listed in table 12.

Before the *in-vivo* excision of a loxP site by Cre recombinase, the targeted allele containing selectable marker neo^R within the frt (figure 15) should be removed [224] because the presence of a selectable marker in the genome possibly influences gene expression and create a phenotype, that may confound the results [225]. Therefore, 129SvJ FLPer strain expressing mice would crossbreed to cleave the frt sites from the mouse genome [226].

Table 12: Different Cre-expressing C57/BL6 mice under various promoter activities.

Cre lines	Description	Cre-expression	Cell/tissue type	Examine *Setbp1*G870S disease phenotype	Reference
C57/BL6-*EIIa*-Cre	The expression of Cre recombinase under the control of the adenovirus *EIIa* promoter.	Constitutive-Germline	Ubiquitous	Schinzel-Giedion syndrome	[227]
C57/BL6-*Runx1*-MER-Cre-MER	Cre expression under the control of mice *Runx1* promoter.	Tamoxifen-inducible	Hematopoietic cells	Leukemia	[228]
C57/BL6-Cre-*ERT2*	Cre-mediated recombination will result in deletion of loxP flanked sequences in widespread cells/tissues.	Tamoxifen-inducible	Ubiquitous	*Setbp1*G870S effects in all tissues	[229]
C57/BL6-*Mx1*-Cre	The Cre recombinase is under the control of the *Mx1* promoter	Interferon-inducible	Hematopoietic cells	Leukemia	[230]
C57/BL6-*Col1a1*-Cre	Mouse *Col1a1* promoter directing expression of the Cre-recombinase	Tamoxifen-inducible / early embryo	osteoblast	Bone physiology and homeostasis.	[231]
C57/BL6-*Col2a1*-Cre	This strain expresses Cre recombinase under the control of a *Col2a1* promoter.	Constitutive-prenatal development	chondrocyte	Cartilage differentiation pattern	[232]
C57/BL6-*Vav1*-Cre	Cre transgenic mice can be used to delete gene sequences flanked by loxP sites under control of murine *Vav1* gene regulatory elements.	Constitutive-early embryo	Hematopoietic cells (and their progenitors) and limited to other cells	Development pattern in Hematopoietic system	[233]

Following Cre-mediated recombination, the wild type *Setbp1* exon4-6 coding sequence would be cleaved and simultaneously the targeted knockin mutated allele will be expressed. The further task would be to evaluate the molecular and physiological role of germline and somatically *Setbp1*^{G870S} expression in mice. Few mouse models are proposed to study *Setbp1*^{G870S} effects in hematopoietic cells and non-hematopoietic cell/tissue.

As the mutation was identified in patients with aCML, a rare hematological disorder, the Setbp1^{G870S} effect will be induced in hematopoietic cells by using a specific promoter controlling Cre expression. Heterozygous *Setbp1*^{G870S} expression would be induced somatically under the transcriptional control of *Mx1* or *Runx*1, which are driven in Cre expressing mice by interferon-alpha/beta [234] and tamoxifen [235] respectively. This *Setbp1*^{G870S} model will help in studying its oncogenic effect and accumulation of secondary events in MDS/MPN malignancies. Similarly, the effect of *Setbp1*^{G870S} in prenatal hematopoietic development will also be studied in C57/BL6 mice by executing Cre-expressing under the control of murine *Vav1* transcription factor (Table 12).

Simultaneously, we will investigate the effect of Setbp1^{G870S} in other tissues/cells as well. The germline ubiquitous or tissue specific *Setbp1*^{G870S} expression during embryonic development would be induced with *Ella* and *Col2a1*-driven Cre activation. This germline *Setbp1*^{G870S} expression strategy will help in understanding the development of Schinzel-Giedion syndrome (ubiquitous *Ella* promoter) and cartilage forming pattern (*Col2a1* promoter). Similarly, *Col1a1* -driven Cre-expression will help in studying the effects of *Setbp1* overexpression in bone deformities (Table 12). Finally, tamoxifen controlled Cre-*Ert2* will allow somatic induction of *Setbp1*^{G870S} expression in all tissues.

In some studies, Knock-in mutants expressed prenatally in mice do not always recapitulate the human disease [236]. Nevertheless, it would be interesting to study the tissue/cell specific germline expression of *Setbp1*^{G870S} also in the development process of osteoblast and chondrocyte. The

monitoring and screening of different phenotypes in mice expressing *Setbp1* G870S mutation will be considered at certain time interval to understand the above hypothesis.

The *Setbp1*^{G870S} oncogenic properties will be further evaluated by studying the proteins that are potentially involved in *Setbp1*^{G870S} triggered oncogenic signalling pathways. The functional genomic methods would be applied to explore the complete genetic and transcriptional profile of mice expressing mutated and wild type Setbp1. These studies will also help in identifying the clonal evolution of the disease along with additional chromosomal abnormalities accumulated during leukemic development. In the extreme contest these study will help in selecting existing or developing new therapeutic strategy that would possibly to overcome the effect of a mutated Setbp1 gene.

In conclusion, we identified *BCR-ABL1* independent gene mutations in chronic phase CML patients at diagnosis revealed by whole exome sequencing; no recurrent somatic alterations were found. Patients with resistance to treatment were found to accumulate more cancer gene mutations, which could be relevant to treatment response and progression of the disease. In chronic phase CML, *BCR-ABL1* oncogene is self-sufficient to induce leukemogenesis without any aid of somatic variants.

Simultaneously, a complete construction of Setbp1 knock-in targeting vector was generated and its biological function would be studied in wild type and mutated Setbp1 mouse, with the idea to develop a new targeted therapy for mutated Setbp1 patients.

5. Bibliography

1. Lichtman, M.A., *Battling the hematological malignancies: the 200 years' war.* Oncologist, 2008. **13**(2): p. 126-38.
2. Passegue, E., et al., *Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics?* Proc Natl Acad Sci U S A, 2003. **100 Suppl 1**: p. 11842-9.
3. Wang, J.C.Y. and J.E. Dick, *Cancer stem cells: lessons from leukemia.* Trends in Cell Biology, 2005. **15**(9): p. 494-501.
4. Bonnet, D. and J.E. Dick, *Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell.* Nat Med, 1997. **3**(7): p. 730-7.
5. Al-Hajj, M., et al., *Prospective identification of tumorigenic breast cancer cells.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 3983-8.
6. Frishman-Levy, L., et al., *Central nervous system acute lymphoblastic leukemia: role of natural killer cells.* Blood, 2015. **125**(22): p. 3420-31.
7. Look, A.T., *Oncogenic transcription factors in the human acute leukemias.* Science, 1997. **278**(5340): p. 1059-64.
8. Askmyr, M., et al., *Modeling chronic myeloid leukemia in immunodeficient mice reveals expansion of aberrant mast cells and accumulation of pre-B cells.* Blood Cancer Journal, 2014. **4**: p. e269.
9. Massard, C., E. Deutsch, and J.C. Soria, *Tumour stem cell-targeted treatment: elimination or differentiation.* Ann Oncol, 2006. **17**(11): p. 1620-4.
10. Lessard, J. and G. Sauvageau, *Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells.* Nature, 2003. **423**(6937): p. 255-260.
11. Taipale, J. and P.A. Beachy, *The Hedgehog and Wnt signalling pathways in cancer.* Nature, 2001. **411**(6835): p. 349-354.

12. Pardal, R., M.F. Clarke, and S.J. Morrison, *Applying the principles of stem-cell biology to cancer.* Nat Rev Cancer, 2003. **3**(12): p. 895-902.

13. Manoranjan, B., et al., *Medulloblastoma stem cells: where development and cancer cross pathways.* Pediatr Res, 2012. **71**(4-2): p. 516-522.

14. Jacobs, J.J., et al., *The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus.* Nature, 1999. **397**(6715): p. 164-8.

15. Lowe, S.W. and C.J. Sherr, *Tumor suppression by Ink4a-Arf: progress and puzzles.* Curr Opin Genet Dev, 2003. **13**(1): p. 77-83.

16. Vardiman, J.W., et al., *The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes*. Vol. 114. 2009. 937-951.

17. Tefferi, A., J. Thiele, and J.W. Vardiman, *The 2008 World Health Organization classification system for myeloproliferative neoplasms.* Cancer, 2009. **115**(17): p. 3842-3847.

18. Lucas, C.M., et al., *Low leukotriene B4 receptor 1 (LTB4R1) leads to ALOX5 down-regulation at diagnosis of chronic myeloid leukemia.* Haematologica, 2014.

19. Illert, A.L., et al., *Inhibition of Aurora Kinase B Is Important for Biologic Activity of the Dual Inhibitors of BCR-ABL and Aurora Kinases R763/AS703569 and PHA-739358 in BCR-ABL Transformed Cells.* PLoS One, 2014. **9**(11): p. e112318.

20. Rowley, J.D., *Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining.* Nature, 1973. **243**(5405): p. 290-3.

21. Berger, U., et al., *Gender aspects in chronic myeloid leukemia: long-term results from randomized studies.* Leukemia, 2005. **19**(6): p. 984-989.

22. Bennett, J.H., *Case of Hypertrophy of the Spleen and Liver: In which Death Took Place from Suppuration of the Blood*1845: Stark and Comp.

23. Goldman, J.M., *Chronic Myeloid Leukemia: A Historical Perspective.* Seminars in Hematology, 2010. **47**(4): p. 302-311.

24. Nowell, P. and D. Hungerford, *A minute chromosome in human chronic granulocytic leukemia.* Landmarks in Medical Genetics: Classic Papers with Commentaries, 2004. **132**(51): p. 103.

25. Rowley, J.D., *A New Consistent Chromosomal Abnormality in Chronic Myelogenous Leukaemia identified by Quinacrine Fluorescence and Giemsa Staining.* Nature, 1973. **243**(5405): p. 290-293.

26. Lydon, N., *Attacking cancer at its foundation.* Nat Med, 2009. **15**(10): p. 1153-1157.

27. Westbrook, C.A., et al., *Clinical significance of the BCR-ABL fusion gene in adult acute lymphoblastic leukemia: a Cancer and Leukemia Group B Study (8762).* Blood, 1992. **80**(12): p. 2983-90.

28. Griswold, I.J., et al., *Kinase domain mutants of Bcr-Abl exhibit altered transformation potency, kinase activity, and substrate utilization, irrespective of sensitivity to imatinib.* Mol Cell Biol, 2006. **26**(16): p. 6082-93.

29. Chen, Y., et al., *Molecular and cellular bases of chronic myeloid leukemia.* Protein & Cell, 2010. **1**(2): p. 124-132.

30. Wong, S. and O.N. Witte, *The BCR-ABL story: bench to bedside and back.* Annu Rev Immunol, 2004. **22**: p. 247-306.

31. Sokal, J.E., et al., *Staging and prognosis in chronic myelogenous leukemia.* Semin Hematol, 1988. **25**(1): p. 49-61.

32. Jennings, B.A. and K.I. Mills, *c-myc locus amplification and the acquisition of trisomy 8 in the evolution of chronic myeloid leukaemia.* Leuk Res, 1998. **22**(10): p. 899-903.

33. Sill, H., J.M. Goldman, and N.C. Cross, *Homozygous deletions of the p16 tumor-suppressor gene are associated with lymphoid transformation of chronic myeloid leukemia.* Blood, 1995. **85**(8): p. 2013-6.

34. Ahuja, H.G., et al., *Abnormalities of the retinoblastoma gene in the pathogenesis of acute leukemia.* Blood, 1991. **78**(12): p. 3259-68.

35. Mitelman, F., *The cytogenetic scenario of chronic myeloid leukemia.* Leuk Lymphoma, 1993. **11 Suppl 1**: p. 11-5.

36. Corso, A., et al., *Chronic myelogenous leukemia and exposure to ionizing radiation--a retrospective study of 443 patients.* Ann Hematol, 1995. **70**(2): p. 79-82.

37. Laneuville, P., *Abl tyrosine protein kinase.* Seminars in Immunology, 1995. **7**(4): p. 255-266.

38. Cohen, G.B., R. Ren, and D. Baltimore, *Modular binding domains in signal transduction proteins.* Cell, 1995. **80**(2): p. 237-248.

39. Kipreos, E.T. and J.Y.J. Wang, *Cell Cycleâ€"Regulated Binding of c-Abl Tyrosine Kinase to DNA.* Science, 1992. **256**(5055): p. 382-385.

40. McWhirter, J.R. and J.Y. Wang, *An actin-binding function contributes to transformation by the Bcr-Abl oncoprotein of Philadelphia chromosome-positive human leukemias.* EMBO J, 1993. **12**(4): p. 1533-46.

41. Van Etten, R.A., P. Jackson, and D. Baltimore, *The mouse type IV c-abl gene product is a nuclear protein, and activation of transforming ability is associated with cytoplasmic localization.* Cell, 1989. **58**(4): p. 669-678.

42. Lewis, J.M. and M.A. Schwartz, *Integrins regulate the association and phosphorylation of paxillin by c-Abl.* J Biol Chem, 1998. **273**(23): p. 14225-30.

43. Deininger, M.W.N., J.M. Goldman, and J.V. Melo, *The molecular biology of chronic myeloid leukemia*. Vol. 96. 2000. 3343-3356.

44. Denhardt, D.T., *Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling.* Biochem J, 1996. **318 (Pt 3)**: p. 729-47.

45. McWhirter, J.R., D.L. Galasso, and J.Y. Wang, *A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoproteins.* Molecular and Cellular Biology, 1993. **13**(12): p. 7587-7595.

46. Montaner, S., et al., *Multiple signalling pathways lead to the activation of the nuclear factor kappaB by the Rho family of GTPases.* J Biol Chem, 1998. **273**(21): p. 12779-85.

47. Diekmann, D., et al., *Bcr encodes a GTPase-activating protein for p21rac.* Nature, 1991. **351**(6325): p. 400-402.

48. Diekmann, D., et al., *Rac GTPase interacts with GAPs and target proteins through multiple effector sites.* EMBO J, 1995. **14**(21): p. 5297-305.

49. Wu, Y., J. Liu, and R.B. Arlinghaus, *Requirement of two specific tyrosine residues for the catalytic activity of Bcr serine/threonine kinase.* Oncogene, 1998. **16**(1): p. 141-6.

50. Ma, G., et al., *Bcr phosphorylated on tyrosine 177 binds Grb2.* Oncogene, 1997. **14**(19): p. 2367-72.

51. Melo, J.V., *The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype [editorial; comment]*. Vol. 88. 1996. 2375-2384.

52. Pane, F., et al., *Neutrophilic-chronic myeloid leukemia: a distinct disease with a specific molecular marker (BCR/ABL with C3/A2 junction) [see comments]*. Vol. 88. 1996. 2410-2414.

53. Hochhaus, A., et al., *A novel BCR-ABL fusion gene (e6a2) in a patient with Philadelphia chromosome-negative chronic myelogenous leukemia.* Blood, 1996. **88**(6): p. 2236-40.

54. Weerkamp, F., et al., *Flow cytometric immunobead assay for the detection of BCR-ABL fusion proteins in leukemia patients.* Leukemia, 2009. **23**(6): p. 1106-1117.

55. Pendergast, A.M., et al., *BCR-ABL-induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein.* Cell, 1993. **75**(1): p. 175-85.

56. Puil, L., et al., *Bcr-Abl oncoproteins bind directly to activators of the Ras signalling pathway.* EMBO J, 1994. **13**(4): p. 764-73.

57. Gordon, M.Y., et al., *Altered adhesive interactions with marrow stroma of haematopoietic progenitor cells in chronic myeloid leukaemia.* Nature, 1987. **328**(6128): p. 342-344.

58. Bedi, A., et al., *Inhibition of apoptosis by BCR-ABL in chronic myeloid leukemia*. Vol. 83. 1994. 2038-2044.

59. Mullighan, C.G., et al., *BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros.* Nature, 2008. **453**(7191): p. 110-4.

60. Thomas, S.M. and J.S. Brugge, *Cellular functions regulated by Src family kinases.* Annu Rev Cell Dev Biol, 1997. **13**: p. 513-609.

61. Zhang, X. and R. Ren, *Bcr-Abl efficiently induces a myeloproliferative disease and production of excess interleukin-3 and granulocyte-macrophage colony-stimulating factor in mice: a novel model for chronic myelogenous leukemia.* Blood, 1998. **92**(10): p. 3829-40.

62. Gambacorti-Passerini, C., et al., *Inhibition of the ABL kinase activity blocks the proliferation of BCR/ABL+ leukemic cells and induces apoptosis.* Blood Cells Mol Dis, 1997. **23**(3): p. 380-94.

63. Wang, J.Y.J., *Abl tyrosine kinase in signal transduction and cell-cycle regulation.* Current Opinion in Genetics & Development, 1993. **3**(1): p. 35-43.

64. Verfaillie, C.M., et al., *Integrin-Mediated Regulation of Hematopoiesis: Do BCR/ABL-Induced Defects in Integrin Function Underlie the Abnormal Circulation and Proliferation of CML Progenitors?* Acta Haematologica, 1997. **97**(1-2): p. 40-52.

65. Faderl, S., et al., *The Biology of Chronic Myeloid Leukemia.* New England Journal of Medicine, 1999. **341**(3): p. 164-172.

66. Lewis, J.M., et al., *Integrin regulation of c-Abl tyrosine kinase activity and cytoplasmic-nuclear transport.* Proc Natl Acad Sci U S A, 1996. **93**(26): p. 15174-9.

67. Oda, T., et al., *Crkl is the major tyrosine-phosphorylated protein in neutrophils from patients with chronic myelogenous leukemia.* J Biol Chem, 1994. **269**(37): p. 22925-8.

68. Schlaepfer, D.D., et al., *INTEGRIN-MEDIATED SIGNAL-TRANSDUCTION LINKED TO RAS PATHWAY BY GRB2 BINDING TO FOCAL ADHESION KINASE.* Nature, 1994. **372**(6508): p. 786-791.

69. Salgia, R., et al., *p130CAS forms a signaling complex with the adapter protein CRKL in hematopoietic cells transformed by the BCR/ABL oncogene.* J Biol Chem, 1996. **271**(41): p. 25198-203.

70. Sattler, M., et al., *The proto-oncogene product p120CBL and the adaptor proteins CRKL and c-CRK link c-ABL, p190BCR/ABL and p210BCR/ABL to the phosphatidylinositol-3' kinase pathway.* Oncogene, 1996. **12**(4): p. 839-46.

71. Sattler, M., et al., *Differential signaling after beta1 integrin ligation is mediated through binding of CRKL to p120(CBL) and p110(HEF1).* J Biol Chem, 1997. **272**(22): p. 14320-6.

72. Verfaillie, C.M., *Biology of chronic myelogenous leukemia.* Hematol Oncol Clin North Am, 1998. **12**(1): p. 1-29.

73. Bhatia, R., et al., *Interferon-alpha restores normal adhesion of chronic myelogenous leukemia hematopoietic progenitors to bone marrow stroma by correcting impaired beta 1 integrin receptor function.* J Clin Invest, 1994. **94**(1): p. 384-91.

74. Steelman, L.S., et al., *JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis.* Leukemia, 2004. **18**(2): p. 189-218.

75. Yan, J., et al., *Ras isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase.* J Biol Chem, 1998. **273**(37): p. 24052-6.

76. Zhang, X., et al., *The NH(2)-terminal coiled-coil domain and tyrosine 177 play important roles in induction of a myeloproliferative disease in mice by Bcr-Abl.* Mol Cell Biol, 2001. **21**(3): p. 840-53.

77. Pelicci, G., et al., *Constitutive phosphorylation of Shc proteins in human tumors.* Oncogene, 1995. **11**(5): p. 899-907.

78. Senechal, K., J. Halpern, and C.L. Sawyers, *The CRKL adaptor protein transforms fibroblasts and functions in transformation by the BCR-ABL oncogene.* J Biol Chem, 1996. **271**(38): p. 23255-61.

79. Sattler, M., et al., *Critical role for Gab2 in transformation by BCR/ABL.* Cancer Cell, 2002. **1**(5): p. 479-92.

80. Notari, M., et al., *A MAPK/HNRPK pathway controls BCR/ABL oncogenic potential by regulating MYC mRNA translation*. Vol. 107. 2006. 2507-2516.

81. Skorski, T., et al., *Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway.* EMBO J, 1997. **16**(20): p. 6151-61.

82. Roche, S., M. Koegl, and S.A. Courtneidge, *The phosphatidylinositol 3-kinase alpha is required for DNA synthesis induced by some, but not all, growth factors.* Proc Natl Acad Sci U S A, 1994. **91**(19): p. 9185-9.

83. Wennstrom, S., et al., *Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling.* Curr Biol, 1994. **4**(5): p. 385-93.

84. Yao, R. and G.M. Cooper, *Growth factor-dependent survival of rodent fibroblasts requires phosphatidylinositol 3-kinase but is independent of pp70S6K activity.* Oncogene, 1996. **13**(2): p. 343-51.

85. Skorski, T., et al., *Transformation of hematopoietic cells by BCR/ABL requires activation of a Plâ€□3k/Aktâ€□dependent pathway*. Vol. 16. 1997. 6151-6161.

86. Franke, T.F., D.R. Kaplan, and L.C. Cantley, *PI3K: downstream AKTion blocks apoptosis.* Cell, 1997. **88**(4): p. 435-7.

87. Datta, S.R., et al., *Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery.* Cell, 1997. **91**(2): p. 231-41.

88. Andreeff, M., et al., *Expression of Bcl-2-related genes in normal and AML progenitors: changes induced by chemotherapy and retinoic acid.* Leukemia, 1999. **13**(11): p. 1881-92.

89. Fukumoto, S., et al., *Akt participation in the Wnt signaling pathway through Dishevelled.* J Biol Chem, 2001. **276**(20): p. 17479-83.

90. Ozes, O.N., et al., *NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase.* Nature, 1999. **401**(6748): p. 82-5.

91. Zhou, B.P., et al., *HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation.* Nat Cell Biol, 2001. **3**(11): p. 973-82.

92. Ilaria, R.L. and R.A. Van Etten, *P210 and P190BCR/ABL induce the tyrosine phosphorylation and DNA binding activity of multiple specific STAT family members.* Journal of Biological Chemistry, 1996. **271**(49): p. 31704-31710.

93. Chai, S.K., G.L. Nichols, and P. Rothman, *Constitutive activation of JAKs and STATs in BCR-Abl-expressing cell lines and peripheral blood cells derived from leukemic patients.* J Immunol, 1997. **159**(10): p. 4720-8.

94. Nosaka, T., et al., *STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells*. Vol. 18. 1999. 4754-4765.

95. Sillaber, C., et al., *STAT5 activation contributes to growth and viability in Bcr/Abl-transformed cells*. Vol. 95. 2000. 2118-2125.

96. Horita, M., et al., *Blockade of the Bcr-Abl kinase activity induces apoptosis of chronic myelogenous leukemia cells by suppressing signal transducer and activator of transcription 5-dependent expression of Bcl-xL.* J Exp Med, 2000. **191**(6): p. 977-84.

97. de Groot, R.P., et al., *STAT5 Activation by BCR-Abl Contributes to Transformation of K562 Leukemia Cells*. Vol. 94. 1999. 1108-1112.

98. Hoelbl, A., et al., *Clarifying the role of Stat5 in lymphoid development and Abelson-induced transformation*. Vol. 107. 2006. 4898-4906.

99. Amos, T.A., et al., *Apoptosis in chronic myeloid leukaemia: normal responses by progenitor cells to growth factor deprivation, X-irradiation and glucocorticoids.* Br J Haematol, 1995. **91**(2): p. 387-93.

100. Lucas, C.M., et al., *c-Myc inhibition decreases CIP2A and reduces BCR-ABL1 tyrosine kinase activity in chronic myeloid leukemia.* Haematologica, 2015. **100**(5): p. e179-82.

101. Sharma, N., et al., *BCR/ABL1 and BCR are under the transcriptional control of the MYC oncogene.* Mol Cancer, 2015. **14**(1): p. 132.

102. Sawyers, C.L., W. Callahan, and O.N. Witte, *Dominant negative MYC blocks transformation by ABL oncogenes.* Cell, 1992. **70**(6): p. 901-10.

103. Su, E., X. Han, and G. Jiang, *The transforming growth factor beta 1/SMAD signaling pathway involved in human chronic myeloid leukemia.* Tumori, 2010. **96**(5): p. 659-66.

104. Warmuth, M., S. Danhauser-Riedl, and M. Hallek, *Molecular pathogenesis of chronic myeloid leukemia: implications for new therapeutic strategies.* Ann Hematol, 1999. **78**(2): p. 49-64.

105. Gambacorti-Passerini, C., R. Piazza, and M. D'Incalci, *Bcr-Abl mutations, resistance to imatinib, and imatinib plasma levels*. Vol. 102. 2003. 1933-1935.

106. Willis, S.G., et al., *High-sensitivity detection of BCR-ABL kinase domain mutations in imatinib-naive patients: correlation with clonal cytogenetic evolution but not response to therapy.* Blood, 2005. **106**(6): p. 2128-37.

107. Gambacorti-Passerini, C.B., et al., *Molecular mechanisms of resistance to imatinib in Philadelphia-chromosome-positive leukaemias.* Lancet Oncol, 2003. **4**(2): p. 75-85.

108. Ma, W., et al., *Three novel alternative splicing mutations in BCR-ABL1 detected in CML patients with resistance to kinase inhibitors.* Int J Lab Hematol, 2011. **33**(3): p. 326-31.

109. Bierie, B. and H.L. Moses, *Tumour microenvironment: TGF[beta]: the molecular Jekyll and Hyde of cancer.* Nat Rev Cancer, 2006. **6**(7): p. 506-520.

110. Schmidt, M., et al., *Molecular-defined clonal evolution in patients with chronic myeloid leukemia independent of the BCR-ABL status.* Leukemia, 2014. **28**(12): p. 2292-2299.

111. Bejar, R., et al., *Clinical effect of point mutations in myelodysplastic syndromes.* N Engl J Med, 2011. **364**(26): p. 2496-506.

112. O'Dwyer, M.E., et al., *The impact of clonal evolution on response to imatinib mesylate (STI571) in accelerated phase CML.* Blood, 2002. **100**(5): p. 1628-33.

113. Piazza, R., et al., *Recurrent SETBP1 mutations in atypical chronic myeloid leukemia.* Nat Genet, 2013. **45**(1): p. 18-24.

114. Damm, F., et al., *SETBP1 mutations in 658 patients with myelodysplastic syndromes, chronic myelomonocytic leukemia and secondary acute myeloid leukemias.* Leukemia, 2013. **27**(6): p. 1401-3.

115. Hoischen, A., et al., *De novo mutations of SETBP1 cause Schinzel-Giedion syndrome.* Nat Genet, 2010. **42**(6): p. 483-485.

116. Gaiger, A., et al., *Increase of bcr-abl chimeric mRNA expression in tumor cells of patients with chronic myeloid leukemia precedes disease progression*. Vol. 86. 1995. 2371-2378.

117. Guo, J.Q., J.Y. Wang, and R.B. Arlinghaus, *Detection of BCR-ABL proteins in blood cells of benign phase chronic myelogenous leukemia patients.* Cancer Res, 1991. **51**(11): p. 3048-51.

118. Jamieson, C.H., et al., *Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML.* N Engl J Med, 2004. **351**(7): p. 657-67.

119. Ban, K., et al., *BCR-ABL1 mediates up-regulation of Fyn in chronic myelogenous leukemia*. Vol. 111. 2008. 2904-2908.

120. Dai, Y., et al., *A Bcr/Abl-independent, Lyn-dependent form of imatinib mesylate (STI-571) resistance is associated with altered expression of Bcl-2.* J Biol Chem, 2004. **279**(33): p. 34227-39.

121. Cambier, N., et al., *BCR-ABL activates pathways mediating cytokine independence and protection against apoptosis in murine hematopoietic cells in a dose-dependent manner.* Oncogene, 1998. **16**(3): p. 335-48.

122. Barnes, D.J., et al., *Dose-dependent effects of Bcr-Abl in cell line models of different stages of chronic myeloid leukemia.* Oncogene, 2005. **24**(42): p. 6432-40.

123. Wu, J., et al., *Association between imatinib-resistant BCR-ABL mutation-negative leukemia and persistent activation of LYN kinase.* J Natl Cancer Inst, 2008. **100**(13): p. 926-39.

124. Perrotti, D., et al., *TLS/FUS, a proâ€• oncogene involved in multiple chromosomal translocations, is a novel regulator of BCR/ABLâ€• mediated leukemogenesis*. Vol. 17. 1998. 4442-4455.

125. Carlesso, N., D.A. Frank, and J.D. Griffin, *Tyrosyl phosphorylation and DNA binding activity of signal transducers and activators of transcription (STAT) proteins in hematopoietic cell lines transformed by Bcr/Abl.* J Exp Med, 1996. **183**(3): p. 811-20.

126. Perrotti, D., et al., *BCR-ABL suppresses C/EBPalpha expression through inhibitory action of hnRNP E2.* Nat Genet, 2002. **30**(1): p. 48-58.

127. QuintÃ¡s-Cardama, A. and J. Cortes, *Molecular biology of bcr-abl1â€“positive chronic myeloid leukemia*. Vol. 113. 2009. 1619-1630.

128. Dash, A.B., et al., *A murine model of CML blast crisis induced by cooperation between BCR/ABL and NUP98/HOXA9.* Proceedings of the National Academy of Sciences, 2002. **99**(11): p. 7622-7627.

129. Nucifora, G., et al., *Involvement of the AML1 gene in the t(3;21) in therapy-related leukemia and in chronic myeloid leukemia in blast crisis*. Vol. 81. 1993. 2728-2734.

130. Zhang, S.J., et al., *Gain-of-function mutation of GATA-2 in acute myeloid transformation of chronic myeloid leukemia.* Proc Natl Acad Sci U S A, 2008. **105**(6): p. 2076-81.

131. Nowicki, M.O., et al., *BCR/ABL oncogenic kinase promotes unfaithful repair of the reactive oxygen speciesâ€“dependent DNA double-strand breaks*. Vol. 104. 2004. 3746-3753.

132. Muvarak, N., et al., *c-MYC Generates Repair Errors via Increased Transcription of Alternative-NHEJ Factors, LIG3 and PARP1, in Tyrosine Kinase-Activated Leukemias.* Mol Cancer Res, 2015. **13**(4): p. 699-712.

133. Bonifazi, F., et al., *Chronic myeloid leukemia and interferon-Î±: a study of complete cytogenetic responders*. Vol. 98. 2001. 3074-3081.

134. Melo, J.V. and D.J. Barnes, *Chronic myeloid leukaemia as a model of disease evolution in human cancer.* Nat Rev Cancer, 2007. **7**(6): p. 441-453.

135. Skorski, T., *Genetic Mechanisms of Chronic Myeloid Leukemia Blastic Transformation.* Current Hematologic Malignancy Reports, 2012. **7**(2): p. 87-93.

136. Melo, J.V. and D.J. Barnes, *Chronic myeloid leukaemia as a model of disease evolution in human cancer.* Nat Rev Cancer, 2007. **7**(6): p. 441-53.

137. Kharbanda, S., et al., *Regulation of the hTERT telomerase catalytic subunit by the c-Abl tyrosine kinase.* Curr Biol, 2000. **10**(10): p. 568-75.

138. Cortes, J. and M.E. O'Dwyer, *Clonal evolution in chronic myelogenous leukemia.* Hematol Oncol Clin North Am, 2004. **18**(3): p. 671-84, x.

139. Calabretta, B. and D. Perrotti, *The biology of CML blast crisis.* Blood, 2004. **103**(11): p. 4010-22.

140. Quintas-Cardama, A. and J. Cortes, *Molecular biology of bcr-abl1-positive chronic myeloid leukemia.* Blood, 2009. **113**(8): p. 1619-30.

141. Wendel, H.-G., et al., *Loss of p53 impedes the antileukemic response to BCR-ABL inhibition.* Proceedings of the National Academy of Sciences, 2006. **103**(19): p. 7444-7449.

142. Williams, R.T., W. den Besten, and C.J. Sherr, *Cytokine-dependent imatinib resistance in mouse BCR-ABL+, Arf-null lymphoblastic leukemia.* Genes Dev, 2007. **21**(18): p. 2283-7.

143. Neviani, P., et al., *The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein.* Cancer Cell, 2005. **8**(5): p. 355-68.

144. Maehama, T. and J.E. Dixon, *The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate.* J Biol Chem, 1998. **273**(22): p. 13375-8.

145. Bruns, I., et al., *The hematopoietic stem cell in chronic phase CML is characterized by a transcriptional profile resembling normal myeloid progenitor cells and reflecting loss of quiescence.* Leukemia, 2009. **23**(5): p. 892-9.

146. Hasford, J., et al., *A new prognostic score for survival of patients with chronic myeloid leukemia treated with interferon alfa. Writing Committee for the Collaborative CML Prognostic Factors Project Group.* J Natl Cancer Inst, 1998. **90**(11): p. 850-8.

147. Sokal, J.E., et al., *Prognostic discrimination in "good-risk" chronic granulocytic leukemia.* Blood, 1984. **63**(4): p. 789-99.

148. Melnick, J.S., et al., *An efficient rapid system for profiling the cellular activities of molecular libraries.* Proc Natl Acad Sci U S A, 2006. **103**(9): p. 3153-8.

149. O'Brien, S., et al., *Chronic myelogenous leukemia.* J Natl Compr Canc Netw, 2012. **10**(1): p. 64-110.

150. Gambacorti-Passerini, C. and R. Piazza, *Imatinib-A new tyrosine kinase inhibitor for first-line treatment of chronic myeloid leukemia in 2015.* JAMA Oncology, 2015. **1**(2): p. 143-144.

151. Sawyers, C.L., *Chronic Myeloid Leukemia.* New England Journal of Medicine, 1999. **340**(17): p. 1330-1340.

152. Kantarjian, H.M., et al., *Clinical course and therapy of chronic myelogenous leukemia with interferon-alpha and chemotherapy.* Hematol Oncol Clin North Am, 1998. **12**(1): p. 31-80.

153. Faderl, S., et al., *Chronic Myelogenous Leukemia: Biology and Therapy.* Annals of Internal Medicine, 1999. **131**(3): p. 207-219.

154. Mrsic, M., et al., *Second HLA-identical sibling transplants for leukemia recurrence.* Bone Marrow Transplant, 1992. **9**(4): p. 269-75.

155. Gratwohl, A., et al., *Bone marrow transplantation for chronic myeloid leukemia: long-term results. Chronic Leukemia Working Party of the European Group for Bone Marrow Transplantation.* Bone Marrow Transplant, 1993. **12**(5): p. 509-16.

156. Kantarjian, H.M., et al., *Chronic myelogenous leukemia: a concise update.* Blood, 1993. **82**(3): p. 691-703.

157. Goldman, J.M., et al., *Choice of pretransplant treatment and timing of transplants for chronic myelogenous leukemia in chronic phase.* Blood, 1993. **82**(7): p. 2235-8.

158. Clift, R.A. and R. Storb, *Marrow transplantation for CML: the Seattle experience.* Bone Marrow Transplant, 1996. **17 Suppl 3**: p. S1-3.

159. Martinez, C., et al., *Relapse of chronic myeloid leukemia after allogeneic stem cell transplantation: outcome and prognostic factors: the Chronic Myeloid Leukemia Subcommittee of the GETH (Grupo Espanol de Trasplante Hemopoyetico).* Bone Marrow Transplant, 2005. **36**(4): p. 301-6.

160. Clift, R.A., et al., *Marrow transplantation for chronic myeloid leukemia: a randomized study comparing cyclophosphamide and total body irradiation with busulfan and cyclophosphamide.* Blood, 1994. **84**(6): p. 2036-43.

161. Goldman, J.M., et al., *Bone marrow transplantation for chronic myelogenous leukemia in chronic phase. Increased risk for relapse associated with T-cell depletion.* Ann Intern Med, 1988. **108**(6): p. 806-14.

162. Kantarjian, H.M., et al., *Prolonged survival in chronic myelogenous leukemia after cytogenetic response to interferon-alpha therapy. The Leukemia Service.* Ann Intern Med, 1995. **122**(4): p. 254-61.

163. Baccarani, M., et al., *Interferon-alfa for chronic myeloid leukemia.* Semin Hematol, 2003. **40**(1): p. 22-33.

164. Hochhaus, A., et al., *Favorable long-term follow-up results over 6 years for response, survival, and safety with imatinib mesylate therapy in chronic-phase chronic myeloid leukemia after failure of interferon-alpha treatment.* Blood, 2008. **111**(3): p. 1039-43.

165. Kantarjian, H., et al., *Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia.* N Engl J Med, 2002. **346**(9): p. 645-52.

166. Hochhaus, A., et al., *Favorable long-term follow-up results over 6 years for response, survival, and safety with imatinib mesylate therapy in chronic-phase chronic myeloid leukemia after failure of interferon-Î± treatment*. Vol. 111. 2008. 1039-1043.

167. O'Brien, S.G., et al., *Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia.* N Engl J Med, 2003. **348**(11): p. 994-1004.

168. Gambacorti-Passerini, C., et al., *Multicenter independent assessment of outcomes in chronic myeloid leukemia patients treated with imatinib.* J Natl Cancer Inst, 2011. **103**(7): p. 553-61.

169. Viganò, I., et al., *First-line treatment of 102 chronic myeloid leukemia patients with imatinib: A long-term single institution analysis.* American Journal of Hematology, 2014. **89**(10): p. E184-E187.

170. Franceschino, A., et al., *Imatinib failed to eradicate chronic myeloid leukemia in a patient with minimal residual disease.* Haematologica, 2006. **91**(6 Suppl): p. ECR14.

171. Cortes, J.E., et al., *High-dose imatinib in newly diagnosed chronic-phase chronic myeloid leukemia: high rates of rapid cytogenetic and molecular responses.* J Clin Oncol, 2009. **27**(28): p. 4754-9.

172. Castagnetti, F., et al., *Results of high-dose imatinib mesylate in intermediate Sokal risk chronic myeloid leukemia patients in early chronic phase: a phase 2 trial of the GIMEMA CML Working Party*. Vol. 113. 2009. 3428-3434.

173. Baccarani, M., et al., *Comparison of imatinib 400 mg and 800 mg daily in the front-line treatment of high-risk, Philadelphia-positive chronic myeloid leukemia: a European LeukemiaNet Study*. Vol. 113. 2009. 4497-4504.

174. Schiffer, C.A., *BCR-ABL tyrosine kinase inhibitors for chronic myelogenous leukemia.* N Engl J Med, 2007. **357**(3): p. 258-65.

175. Baccarani, M., et al., *Response definitions and European Leukemianet Management recommendations.* Best Pract Res Clin Haematol, 2009. **22**(3): p. 331-41.

176. Gambacorti-Passerini, C., et al., *Alpha1 acid glycoprotein binds to imatinib (STI571) and substantially alters its pharmacokinetics in chronic myeloid leukemia patients.* Clin Cancer Res, 2003. **9**(2): p. 625-32.

177. Thomas, J., et al., *Active transport of imatinib into and out of cells: implications for drug resistance*. Vol. 104. 2004. 3739-3745.

178. White, D.L., et al., *Most CML patients who have a suboptimal response to imatinib have low OCT-1 activity: higher doses of imatinib may overcome the negative impact of low OCT-1 activity*. Vol. 110. 2007. 4064-4072.

179. Giannoudis, A., et al., *Effective dasatinib uptake may occur without human organic cation transporter 1 (hOCT1): implications for the treatment of imatinib-resistant chronic myeloid leukemia*. Vol. 112. 2008. 3348-3354.

180. White, D.L., et al., *OCT-1- mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib*. Vol. 108. 2006. 697-704.

181. Mauro, M.J., et al. *Dasatinib 2-year efficacy in patients with chronic-phase chronic myelogenous leukemia (CML-CP) with resistance or intolerance to imatinib (START-C)*. in *ASCO Annual Meeting Proceedings*. 2008.

182. Ferri, C.A., et al., *Clinical activity of ponatinib in one patient with chronic myeloid leukemia in chronic phase with e19a2 transcript and T315I mutation.* Eur J Haematol, 2015. **94**(3): p. 270-2.

183. Redaelli, S., et al., *Activity of bosutinib, dasatinib, and nilotinib against 18 imatinib-resistant BCR/ABL mutants.* J Clin Oncol, 2009. **27**(3): p. 469-71.

184. Redaelli, S., et al., *Three novel patient-derived BCR/ABL mutants show different sensitivity to second and third generation tyrosine kinase inhibitors.* Am J Hematol, 2012. **87**(11): p. E125-8.

185. Kantarjian, H., et al., *Dasatinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia.* N Engl J Med, 2010. **362**(24): p. 2260-70.

186. Saglio, G., et al., *Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia.* N Engl J Med, 2010. **362**(24): p. 2251-9.

187. Tokarski, J.S., et al., *The structure of Dasatinib (BMS-354825) bound to activated ABL kinase domain elucidates its inhibitory activity against imatinib-resistant ABL mutants.* Cancer Res, 2006. **66**(11): p. 5790-7.

188. Das, J., et al., *2-aminothiazole as a novel kinase inhibitor template. Structure-activity relationship studies toward the discovery of N-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)-1- piperazinyl)]-2-methyl-4-pyrimidinyl]amino)]-1,3-thiazole-5-carboxamide (dasatinib, BMS-354825) as a potent pan-Src kinase inhibitor.* J Med Chem, 2006. **49**(23): p. 6819-32.

189. Talpaz, M., et al., *Dasatinib in Imatinib-Resistant Philadelphia Chromosomeâ€“Positive Leukemias.* New England Journal of Medicine, 2006. **354**(24): p. 2531-2541.

190. Jayson, G.C., et al., *Blockade of platelet-derived growth factor receptor-beta by CDP860, a humanized, PEGylated di-Fab', leads to fluid accumulation and is associated with increased tumor vascularized volume.* J Clin Oncol, 2005. **23**(5): p. 973-81.

191. Weisberg, E., et al., *Second generation inhibitors of BCR-ABL for the treatment of imatinib-resistant chronic myeloid leukaemia.* Nat Rev Cancer, 2007. **7**(5): p. 345-56.

192. Weisberg, E., et al., *AMN107 (nilotinib): a novel and selective inhibitor of BCR-ABL.* Br J Cancer, 2006. **94**(12): p. 1765-9.

193. Khoury, H.J., et al., *Bosutinib is active in chronic phase chronic myeloid leukemia after imatinib and dasatinib and/or nilotinib therapy failure.* Blood, 2012. **119**(15): p. 3403-3412.

194. Huang, W.S., et al., *Discovery of 3-[2-(imidazo[1,2-b]pyridazin-3-yl)ethynyl]-4-methyl-N-{4-[(4-methylpiperazin-1-y l)methyl]-3-(trifluoromethyl)phenyl}benzamide (AP24534), a potent, orally active pan-inhibitor of breakpoint cluster region-abelson (BCR-ABL) kinase including the T315I gatekeeper mutant.* J Med Chem, 2010. **53**(12): p. 4701-19.

195. Gambacorti-Passerini, C. and R. Piazza, *How I treat newly diagnosed chronic myeloid leukemia in 2015.* Am J Hematol, 2015. **90**(2): p. 156-61.

196. Larson, R.A., et al., *Efficacy and safety of nilotinib (NIL) vs imatinib (IM) in patients (pts) with newly diagnosed chronic myeloid leukemia in chronic phase (CML-CP): long-term follow-up (f/u) of ENESTnd.* Blood, 2014. **124**(21): p. 4541-4541.

197. Radich, J.P., et al., *A randomized trial of dasatinib 100 mg versus imatinib 400 mg in newly diagnosed chronic-phase chronic myeloid leukemia.* Blood, 2012. **120**(19): p. 3898-905.

198. Li, H. and R. Durbin, *Fast and accurate long-read alignment with Burrows-Wheeler transform.* Bioinformatics, 2010. **26**(5): p. 589-95.

199. Sanger, F., S. Nicklen, and A.R. Coulson, *DNA sequencing with chain-terminating inhibitors.* Proceedings of the National Academy of Sciences of the United States of America, 1977. **74**(12): p. 5463-5467.

200. Gonzalez, G., et al., *GeneRanker: An Online System for Predicting Gene-Disease Associations for Translational Research.* Summit on Translat Bioinforma, 2008. **2008**: p. 26-30.

201. Anthony J. Onwuegbuzie, L.D., Nancy L. Leech, *Pearson Product-Moment Correlation Coefficient. Encyclopedia of Measurement and Statistics. SAGE Publications, Inc*2007, Thousand Oaks, CA: SAGE Publications, Inc. 751-756.

202. Su, X., X. Yan, and C.-L. Tsai, *Linear regression.* Wiley Interdisciplinary Reviews: Computational Statistics, 2012. **4**(3): p. 275-294.

203. Armitage, P., G. Berry, and J.N. Matthews, *Statistical methods in medical research*2008: John Wiley & Sons.

204. Rossi, D.J., et al., *Inability to enter S phase and defective RNA polymerase II CTD phosphorylation in mice lacking Mat1.* EMBO J, 2001. **20**(11): p. 2844-56.

205. Adzhubei, I.A., et al., *A method and server for predicting damaging missense mutations.* Nat Methods, 2010. **7**(4): p. 248-9.

206. Piazza, R., et al., *CEQer: a graphical tool for copy number and allelic imbalance detection from whole-exome sequencing data.* PLoS One, 2013. **8**(10): p. e74825.

207. Piazza, R., et al., *FusionAnalyser: a new graphical, event-driven tool for fusion rearrangements discovery.* Nucleic Acids Res, 2012. **40**(16): p. e123.

208. Carter, H., et al., *Cancer-specific high-throughput annotation of somatic mutations: computational prediction of driver missense mutations.* Cancer Res, 2009. **69**(16): p. 6660-7.

209. de Souza, J.E., et al., *S-score: a scoring system for the identification and prioritization of predicted cancer genes.* PLoS One, 2014. **9**(4): p. e94147.

210. Skvorak, K., B. Vissel, and G.E. Homanics, *Production of conditional point mutant knockin mice.* genesis, 2006. **44**(7): p. 345-353.

211. Carbuccia, N., et al., *Mutations of ASXL1 gene in myeloproliferative neoplasms.* Leukemia, 2009. **23**(11): p. 2183-6.

212. Fialkow, P.J., et al., *Evidence for a multistep pathogenesis of chronic myelogenous leukemia.* Blood, 1981. **58**(1): p. 158-63.

213. Raskind, W.H., et al., *Further evidence for the existence of a clonal Ph-negative stage in some cases of Ph-positive chronic myelocytic leukemia.* Leukemia, 1993. **7**(8): p. 1163-7.

214. Susan Branford, P.P.W., Wendy Tara Parker, David T Yeung, Justine E Marum, Doris Stangl, Zoe Donaldson, Alexandra Yeoman, Nathalie Nataren, Ieuan Walker, Leanne Purins, Bradley Chereda, Christopher N Hahn, Hamish S Scott, Andreas Schreiber, and Timothy P. Hughes, *High Incidence of Mutated Cancer-Associated Genes at Diagnosis in CML Patients with Early Transformation to Blast Crisis*, in *American Society of Hematology*, S. Branford, Editor 2015: Orlando, FL, USA.

215. Hoischen, A., et al., *De novo mutations of SETBP1 cause Schinzel-Giedion syndrome.* Nat Genet, 2010. **42**(6): p. 483-5.

216. Shah, A.M., et al., *Schinzel-Giedion syndrome: Evidence for a neurodegenerative process.* American Journal of Medical Genetics, 1999. **82**(4): p. 344-347.

217. Özkinay, F.F., et al., *Agenesis of the corpus callosum in Schinzel-Giedion syndrome associated with 47, XXY karyotype.* Clinical Genetics, 1996. **50**(3): p. 145-148.

218. Joss, S. and J.C. Dean, *A Schinzel-Giedion-like syndrome--a milder version or a separate condition?* Clin Dysmorphol, 2002. **11**(4): p. 271-5.

219. Trimarchi, T., P. Ntziachristos, and I. Aifantis, *A new player SETs in myeloid malignancy.* Nat Genet, 2013. **45**(8): p. 846-847.

220. Cristobal, I., et al., *SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia.* Blood, 2010. **115**(3): p. 615-25.

221. Vishwakarma, B.A., et al., *Runx1 repression by histone deacetylation is critical for Setbp1-induced mouse myeloid leukemia development.* Leukemia, 2015.

222. Nagy, A., *Cre recombinase: the universal reagent for genome tailoring.* genesis, 2000. **26**(2): p. 99-109.

223. Wolfe, A., et al., *Temporal and spatial regulation of CRE recombinase expression in gonadotrophin-releasing hormone neurones in the mouse.* J Neuroendocrinol, 2008. **20**(7): p. 909-16.

224. Gu, H., Y.R. Zou, and K. Rajewsky, *Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting.* Cell, 1993. **73**(6): p. 1155-64.

225. Fiering, S., et al., *Targeted deletion of 5'HS2 of the murine beta-globin LCR reveals that it is not essential for proper regulation of the beta-globin locus.* Genes Dev, 1995. **9**(18): p. 2203-13.

226. Jansen, B. and T. Lucas, *A method for diagnosing a person having multiple sclerosis*, 2004, Google Patents.

227. Lakso, M., et al., *Efficient in vivo manipulation of mouse genomic sequences at the zygote stage.* Proc Natl Acad Sci U S A, 1996. **93**(12): p. 5860-5.

228. Sohal, D.S., et al., *Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein.* Circ Res, 2001. **89**(1): p. 20-5.

229. Ruzankina, Y., et al., *Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss.* Cell Stem Cell, 2007. **1**(1): p. 113-26.

230. Kuhn, R., et al., *Inducible gene targeting in mice.* Science, 1995. **269**(5229): p. 1427-9.

231. Kim, J.E., K. Nakashima, and B. de Crombrugghe, *Transgenic mice expressing a ligand-inducible cre recombinase in osteoblasts and odontoblasts: a new tool to examine physiology and disease of postnatal bone and tooth.* Am J Pathol, 2004. **165**(6): p. 1875-82.

232. Ovchinnikov, D.A., et al., *Col2a1-directed expression of Cre recombinase in differentiating chondrocytes in transgenic mice.* genesis, 2000. **26**(2): p. 145-146.

233. de Boer, J., et al., *Transgenic mice with hematopoietic and lymphoid specific expression of Cre.* Eur J Immunol, 2003. **33**(2): p. 314-25.

234. Thelemann, C., et al., *Interferon-gamma induces expression of MHC class II on intestinal epithelial cells and protects mice from colitis.* PLoS One, 2014. **9**(1): p. e86844.

235. Feil, S., N. Valtcheva, and R. Feil, *Inducible Cre mice.* Methods Mol Biol, 2009. **530**: p. 343-63.

236. Nishii, K., Y. Shibata, and Y. Kobayashi, *Connexin mutant embryonic stem cells and human diseases.* World J Stem Cells, 2014. **6**(5): p. 571-8.