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CHARACTERIZATION OF SOME MOLECULAR MECHANISMS ASSOCIATED TO CML PROGRESSION

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ABSTRACT

Chronic Myeloid Leukemia (CML) is caused by the BCR/ABL fusion gene, the product of the reciprocal translocation between chromosomes 9 and 22, the so called Philadelphia (Ph) chromosome. If untreated CML progresses within 3 years from a mild and easy to control form, called chronic phase (CP), into an aggressive and deadly acute leukemia called blast crisis (BC). BC is marked, as any acute leukemia, by a differentiation block and the ensuing accumulation of blasts with frequent over-expression of BCR/ABL protein. Despite the aggressiveness of BC and the poor overall survival of BC patients, little is known about the molecular mechanisms responsible for the progression of the disease. To gain insight into the molecular lesions responsible for BC, I used a two prong approach.

First, I performed whole-exome-sequencing (WES) analysis of paired CP/BC CML samples from patients that underwent progression to BC. The access to matched CP/BC samples renders these data highly valuable. By comparing exome-sequences of 11 paired CP (used as control) and BC samples I found a total of 38 single nucleotide mutations occurring in BC and that were absent in the corresponding CP, showing a limited number of acquired mutations in BC patients (average=4 mutations/patient). By using this approach I found recurrent, somatic, single nucleotide changes in *RUNX1* and *UBE2A* in 2 out of 11 BC samples*. UBE2A* is described here as associated with CML progression for the first time. In addition, Copy Number Alteration (CNA) analysis of 9 matched BC/CP exomes revealed the presence of large chromosomal abnormalities acquired during BC transformation, among which the bulky alteration of chromosome 7 in 3/9 BC samples. Interestingly in patient #1/#9 and #4/#7 respectively, two major regulators of the cell cycle control (CDKN2A and p53) were also deleted as part of these CNA. In conclusion, despite some heterogeneity in the genetic alterations identified in BC samples, I was able to find 2 recurrently mutated genes associated with blastic transformation, *RUNX1* and *UBE2A*, with the last one never been

detected in CML samples. Ongoing analysis on additional BC/CP samples and *in vitro* experiments will help to clarify the role of UBE2A mutations in CML progression.

The second approach involves the study of the BCR promoter, the genomic region that after the oncogenic translocation regulates the expression of the BCR/ABL oncogene. Little is known about the regulation of BCR promoter. Dr. Manuela Marega, a previous PhD student in our laboratory, identified a deregulation of both BCR and BCR/ABL transcriptions during blast crisis transformation, but the molecular mechanisms associated with this phenomenon has not been completely elucidated until now. I thus performed *in-silico* analysis of 1444 bp of the BCR promoter (1444 bp from the ATG starting site) to identify putative binding sites (PBS) for transcription factors which can regulate BCR and BCR/ABL expression. By in-vitro Chromatin Immunoprecipitation (ChIP) experiments I confirmed that COUP-TF1, CTCF, MYC and MAX proteins bind at BCR promoter at specific sites. I focused on MYC and its cofactor MAX for further analysis. Quantitative PCR(qPCR) and Western Blot showed that, when overexpressed in K562 cells, MYC::MAX heterodimer significantly up-regulates the BCR and BCR/ABL expression in comparison with K562 empty cells. This induction seems to be stronger when both the MYC and MAX gene are over-expressed compared to single transfectants. In order to confirm the importance of MYC::MAX heterodimer in the regulation of BCR promoter, I created a HEK-293 shRNA stable cell line and used a BCR-luciferase reporter assay: I observed a significant 2 fold increase in luciferase activity in control HEK-293 shRNA cells in comparison to MYC silenced HEK-293 cells (shRNA). Interestingly, when 2 out of the 4 MYC::MAX binding sites previously identified were deleted (PBS3 and PBS4) a significant decrease of luciferase activity was observed, thus identifying these two regions as critical for BCR and BCR/ABL regulation. Furthermore, silencing of MYC in various BCR/ABL+ cell lines led to significant downregulation of BCR and BCR/ABL expression at both transcriptional and protein levels, which consequently led to decreased proliferation rate and induction of apoptosis. In conclusion, I have here clarified one of the molecular mechanisms regulating BCR/ABL expression. Since MYC is frequently over-expressed in

BC, this phenomenon could also play a critical role in BCR/ABL upregulation and blast aggressiveness acquired during CML evolution.

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1. INTRODUCTION

1.1 Normal Hematopoiesis

The generation of mature blood cells from a primitive pluripotent stem cell in an ordered sequence of maturation and proliferation is called hematopoiesis. Hematopoiesis is a well regulated process, established sequentially in different anatomic sites. The initial hematopoietic activity, known as primitive hematopoiesis, occurs in the blood island of the yolk sac (YS) where primitive erythrocytes are produced. The generation of the definitive/adult type hematopoietic stem cells (HSCs) occurs in the aorta-gonad.mesonephros (AGM) of embryo and thereafter it rapidly shifts to the fetal liver (FL) (1), where HSCs expands and generate large amount of various hematopoietic cells. Postnatally, hematopoiesis shifts to bone marrow (BM) and spleen. BM is the major site of hematopoiesis during adult life (1, 2).

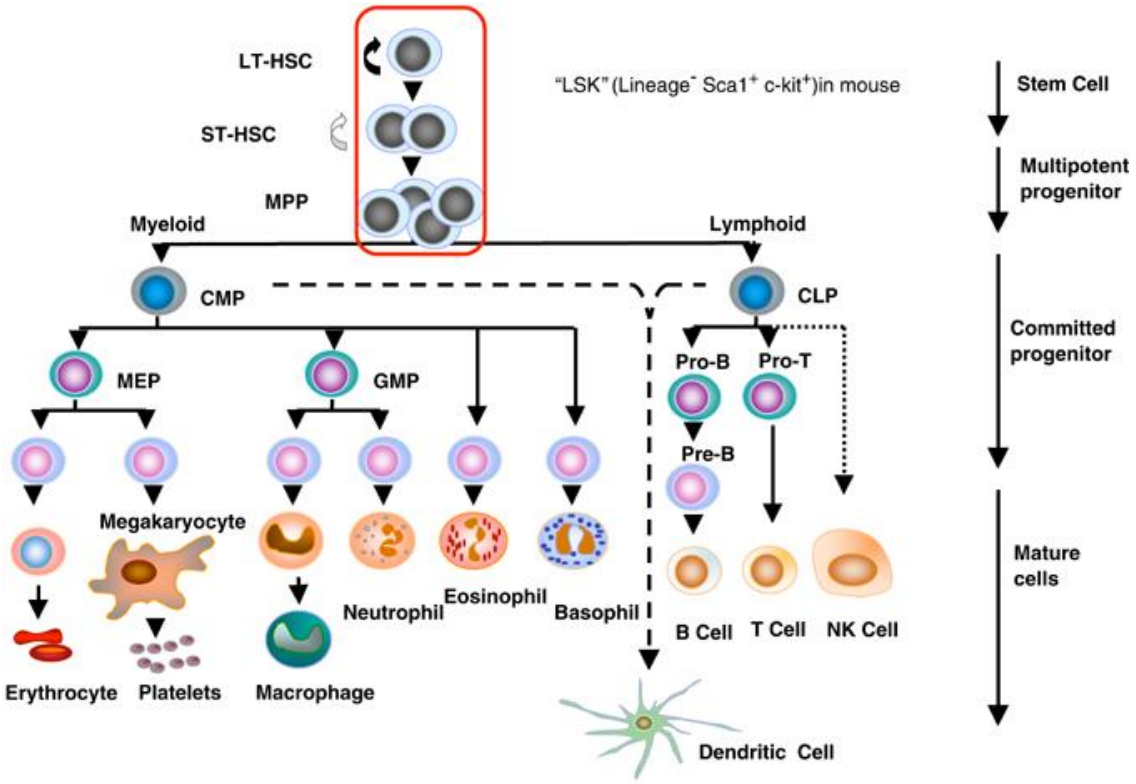

Figure 1: Schematic representation of Hematopoiesis: LT-HSC, long-term repopulating HSC; ST-HSC, short-term repopulating HSC; MPP, multipotent progenitor; CMP, common

myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte−macrophage progenitor. The encompass pluripotent population, LT-HSC, ST-HSC and MPP are Lin-, Sca-1+, c-kit+ as shown. Adapted from (2).

HSCs have unique properties such as ability to choose between self-renewal (persist as a stem cell after cell division) or differentiation (initiate the path toward becoming a mature hematopoietic cell). In addition HSCs migrate in regulated fashion and are subject to regulation by apoptosis (programmed cell death). The equity between these activities decide the number of stem cells that are present in the body (3).

Based on the ability of self-renew, HSCs are categorized in three different populations: long-term self-renewing HSCs (LT-HSC), short-term self-renewing HSCs (ST-HSC) and multipotent progenitors without self-renewal potential (MPPs). The LT-HSC give rise to the ST-HSC and which further give rise to MPP. During HSCs maturation from the long term self-renewing pool to multipotent progenitors, they progressively lose their ability of self-renew, but these cells will become more mitotically active (4). All these cells constitute c-$Kit^{hi}Lineage^{-}Sca\text{-}1^{+}$ (KLS) bone marrow fraction (5, 6). MPP is the first differentiated precursor of HSC, which then differentiate into Common lymphoid (CLPs) and Common myeloid progenitors (CMPs) (Figure. 1).

CMPs consequently generate two more restricted progenitors: granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs). MEPs give rise to megakaryocytes/platelets and erythrocytes (7). GMPs further differentiate into neutrophils, eosinophils and possibly basophils/mast cells (8, 9) (Figure. 1). Lineage specific signals from osteoblasts or human bone forming cells support the growth of the specific hematopoietic lineage. Human bone-forming cells, or osteoblasts, produce important hematopoietic cytokines as IL-3 (multi-CSF), GM-CSF (granulocyte-monocyte colony-stimulating factor), M-CSF (monocyte-macrophage colony-stimulating factor) and G-CSF (granulocyte colony-stimulating factor) which can potently induces proliferation and differentiation of early myeloid

progenitors (9). Lineage-selective knockout establishes the critical role of transcription factor GATA-1 in erythroid and megakaryocytic lineage or platelet development (10, 11). PU.1 and /or CCAAT-enhancer-binding proteins (C/EBPα) are involved in regulating many macrophage and granulocyte restricted promoters (12). In addition these factors also cooperate in the regulation of the genes encoding the myeloid growth factor receptors M-CSFR, G-CSFR and GM-CSFR (12, 13).

CLPs give rise to uncommitted lymphoid progenitors pro-B and pro-T cells, that will differentiate further into mature B and T cells. In addition CLPs also produce NK lineage cells (7, 14). Interleukin 5 (IL-5) receptor signaling induces terminal B-cell differentiation. Interleukin 7(IL-7) receptors signaling are involved in the B-cell development (15). Complex transcriptional networks integrated around the bimodal 'switch' of the transcription factors GATA-1 and PU.1 represent a paradigm for myeloid-versus-erythroid lineage specification (16). In contrast, differentiation into lymphoid lineages follows more linear network “architecture”. Successive and unavoidable activation of the transcription factors E2A, Ebf1 and Pax5 (in distinct progenitor populations) are required for the establishment of lymphoid identity (17). Bcl-11A, Sox4 and TEAD1 are the lymphoid transcription factors that act upstream of known mater regulators of B cell commitment (17). Ikaros and PU.1 transcription factors help in development of all lymphoid specific progenitors (18, 19). The complex set of transcription factors involved in B cell development are PU.1, Ikaros, EBF, E2A, and Pax5. T cell development is arrested at the earliest distinct T cell progenitor stage in mice lacking either GATA3 or Notch1. Thus indicating the importance of GATA3 and Notch 1 in the T cell maturation (20, 21). In addition the transcription factors involved in the development of the natural killer cell lineage are Ikaros-, PU.1-, and Id2 (21).

Both CMPs and CLPs can give rise to functionally equivalent and phenotypically indistinguishable myeloid or lymphoid dendritic cells. In fact, dendritic cells can also be generated from the progenitors downstream of CMPs and CLPs such as GMPs and pro-T cells excluding pro-B cells (22, 23). Dendritic subtypes are decreased in number or completely absent, in a various transcription factor knockout mice (24). In particularly Mac-

1/CD11b$^+$ dendritic cells are absent in the mice lacking relB expression (25). In contrary, Id2-knockout mice lack CD8α+ dendritic cells as well as Langerhans cells, and CD11b+ dendritic cells are also reduced in these mice (26). Both types of dendritic cells are lacked in the mice carrying a dominant-negative form of Ikaros (27).

1.2 Chronic Myeloid Leukemia (CML)

The primary cause of chronic myeloid leukemia (CML) was identified in 1960 by Nowell and Hungerford (28). They identified the Philadelphia (Ph) chromosome and its association with the development of CML (28, 29) analyzing leukemia cells from patients. The discovery of Philadelphia (Ph) chromosome was the first demonstration of a chromosomal rearrangement linked to a specific cancer. The Ph chromosome accounts also for 10-15% of samples from patients with adult B-ALL (30).

Chronic myeloid leukemia is a rare disease, with a worldwide incidence of 10 to 15 cases per 10^6 inhabitants without any major geographical association and/or genetic predisposition to acquire this condition (31). The average age at diagnosis ranges from 60 to 65 years. It occurs less frequently in younger age and a tendency to increase exponentially with age has been observed. Its incidence rate is higher in male than in female and females seems to have survival advantage over males (32).

The majority of cases are diagnosed in chronic phases (85%) and about 50% of them are discovered by routine blood tests, since around 40% of the patients are asymptomatic (31). Clinical symptoms include fatigue, weight loss, abdominal fullness, bleeding, purpura, splenomegaly, leukocytosis, anemia, and thrombocytosis.

CML evolves through three main phases (Table 1). The initial stage of CML, which is a mild and easy control form, is called chronic phase (CP). Most of the patients in these phase have few or no symptoms. Blood cells hold their capacity to differentiate normally, until the disease progresses to the accelerated phase (AP) (33). At this stage the condition of patient become worse and symptoms begin to appear and the diseases progresses. In this phase the count

of white blood cells and immature cells increases. Symptoms frequently seen in accelerated phase are development of fever, night sweats, weight loss, and progressive splenomegaly (34). The final and aggressive phase of CML is Blast Crisis (BC) (Table 1). During blast crisis, more than 30% of the cells in peripheral blood and in the bone marrow are immature cells, called blasts. This phase morphologically resembles acute leukemia, with a complete differentiation block and blasts accumulation. Patients in blastic phase have approximately 70% of myeloid phenotype, 25% have a lymphoid phenotype and 5% have an undifferentiated phenotype. Historically, patients in the blastic phase have a median survival of 3 to 6 months. Patients in blast crisis most likely experience symptoms, including weight loss, fever, night sweats, and bone pain, anemia, infectious complications, and bleeding are common. Subcutaneous nodules or hemorrhagic tender skin lesions, lymphadenopathy, and signs of CNS leukemia may also occur (31, 34).

Table 1. The 3 phases of chronic myeloid leukemia (CML), as defined by the World Health Organization (WHO) (35).

CML Phase	WHO definition
Chronic phase	Blasts < 10% Basophils <20% No thrombocytemia Hypercellular bone marrow (myeloid: erythroid ratio from 10:1 to 30:1)
Accelerated Phase	Blast cells in blood or bone marrow 10-19% Basophils in blood ≥ 20% Persistent thrombocytopenia (< 100 x 10^9 /L) unrelated to therapy cytogenetic evidence of clonal evolution Splenomegaly Unresponsive to therapy
Blast Phase	≥ 20% bone marrow or peripheral blasts Extramedullary haematopoiesis with immature blasts large foci or clusters of blasts on bone marrow biopsy

1.3 The Philadelphia (Ph) chromosome

CML is characterized by the presence of the Philadelphia Chromosome (Ph), derived from the reciprocal translocation between chromosomes 9 and 22. It is the hallmark of CML and is diagnosed in up to 95% of patients (34, 36). During the translocation, the 3' segment of the Abelson (*ABL*) oncogene on chromosome 9q34 merges with the 5' part of the Break Point Cluster (*BCR*) gene located at 22q11,producing the BCR/ABL hybrid gene t(9;22)-(q34:q11) (34, 36). After translocation the BCR/ABL gene is transcribed in the chimeric BCR/ABL messenger RNA (mRNA) that encodes for the chimeric BCR/ABL oncoprotein, promoting growth advantage of leukemic cells.(34, 37).

1.4 The *ABL* Gene

The cellular c-*ABL* is the human homologue of viral ABL (v-*ABL*) oncogene (38). *ABL* gene is located on chromosome 9q34 and is expressed in all normal human cells. This gene has two alternative first exons, Ia and Ib: these regions are differentially transcribed from two different promoters Pa (proximal) and Pb (distal) (39, 40). Exon Ib is situated at the 5' end of the gene and is 150 to 200 kb upstream from exon 1a. The promoter Pa and a Pb are separated by 175 kb and they initiates the synthesis of two mRNA species of 6 and 7 kb, respectively. In approximately 90% of translocations the proximal promoter, Pa, is nested within the BCR/ABL transcriptional unit (41). In normal cells, the activity of the Pa promoter appears to be unaffected by the Pb promoter and both *ABL* promoters are active in gene expression (42). In most of CML cases, chromosomal breakpoints in the *ABL* gene occurs predominantly between exons Ib and Ia and thus the Ph chromosome contains the entire coding sequence of c-*ABL* (exons 2 to 11) and an intact exon Ia and its promoter(Pa) (41).

ABL can shuttle between the nucleus and the cytoplasm. In the nucleus it can bind to DNA, while in cytoplasm it binds to actin cytoskeleton (43). *ABL is a* non-receptor tyrosine kinase that is involved in cell differentiation (44), cell division (45) and stress response (46). Both positive and negative regulatory effects of *ABL* have been reported, based on the cell-cycle

phase studied (43, 47) *ABL* interacts with other molecules involved in the process of DNA repair, such as the ATM gene product (48). Nuclear *ABL* has been implicated in genotoxicity (49).

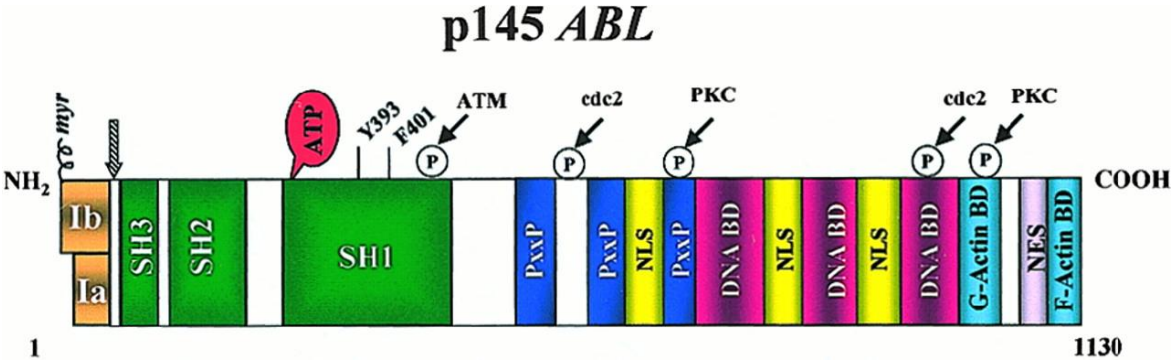

Figure 2: Structure of the ABL protein. Adapted from (36).

The two ABL isoforms originated by the alternative splicing of the first exon are known as Type Ia and Type Ib. Type Ia isoform is slightly shorter than type Ib, which contains a myristoylation (myr) site for attachment to the plasma membrane. Note the 3 SRC-homology (SH) domains situated toward the NH_2 terminus (Figure. 2): the SH1 domain bears the tyrosine kinase function, while the SH2 and SH3 domains permit for interaction with other proteins. In the center of the molecule there are Proline-rich sequences (PXXP), which in turn interacts with SH3 domains of other proteins, such as Crk (50). SH3 domain has a negative regulatory effect on the tyrosine kinase function. Deletion of SH3 or mutation in SH3 domain facilitates tyrosine kinase activity of ABL protein (40). The Proline rich region contains 1 of 3 nuclear localization signals (NLS). Y393 is the major site of auto phosphorylation within the kinase domain, and phenylalanine 401 (F401) is highly conserved in PTKs containing SH3 domains. The carboxy terminus contains DNA as well as G and F

actin–binding domains. Phosphorylation sites by ATM, cdc2, and PKC are shown (Figure. 2). The arrowhead indicate the region of the breakpoint of BCR/ABL fusion protein (36).

In normal *ABL*, phosphorylation is tightly regulated, presumably by motifs in the N-terminal region (47). During *BCR/ABL* translocation, motifs in the N-terminal are lost, resulting in high constitutive kinase enzymatic activity, a key factor in the oncogenic potential of the fusion protein (51, 52). Mutational studies were advantageous to determine the *ABL* domains which control *ABL* activation: N-terminal "cap" is required to achieve and maintain inhibition of *ABL* kinase activity, and its loss turns c-ABL into an oncogenic protein and led to deregulation of BCR/ABL (52). In addition myristoylated residue in the N terminus of the c-Abl 1b isoform binds within a hydrophobic pocket at the base of the c-Abl kinase domain and induces conformational changes that allow the SH2 and SH3 domains to dock onto it, thus preventing activation of the kinase by phosphotyrosine ligands (53, 54).

1.5 The BCR Gene

BCR gene is located on the long arm of chromosome 22 (22q11). It encodes for a protein with a molecular weight of 160 kDa (36, 55). During myeloid maturation BCR protein level are decreased (56). Similar to *ABL* protein, the normal *BCR* protein is ubiquitously expressed both in the cytoplasmic and nuclear compartments (56-58). In both interphase and metaphase *BCR* is associated with condensed DNA (59).

The *BCR* gene has many different functional motifs. In eukaryotes *BCR* gene is implicated in the two major signaling pathways, phosphorylation and guanosine triphosphate (GTP) binding (60, 61). The first exon of *BCR* is included in all known *BCR/ABL* fusion proteins. *BCR* has serine and threonine kinase enzymatic activity in its first exon (62). It can phosphorylate itself as well as key substrates and, hence, propagates cellular signals. Several Src homology-2 (SH2)–binding domains are also in the first exon of *BCR*. SH2 domains are highly conserved, noncatalytic regions of 100 amino acids that bind SH2-

binding sites consisting of 3 to 5 amino acids, including a phosphotyrosine. This interaction is critical in the assembly of signal transduction complexes (63).

BCR also interacts with G proteins (64). These proteins play an important role in intracellular signaling, cytoskeletal organization, cell growth, and normal development. G proteins cycle between an inactive guanosine diphosphate (GDP)–bound state and an active guanosine triphosphate (GTP)–bound state. Equilibrium between this process is regulated by guanosine triphosphatase (GTPase)-activating proteins (which turn off G proteins) and guanine nucleotide exchange factors (which turn on G proteins). *BCR* has a dichotomous role in the G protein-associated signaling pathways, because it has both GTPase-activating protein and guanine nucleotide exchange factor function. *BCR* (and p210 *BCR/ABL*) binds with the xeroderma pigmentosum group B protein (XPB). Xeroderma pigmentosum is an autosomal recessive genetic disorder whose sign is increased sensitivity to sunlight linked with an error in the DNA damage response process. Hence *BCR* may also be engaged in DNA repair (61). *BCR* can form complexes with *BCR/ABL*, by binding to SH2 domains of *ABL* (65). The result of synergy between *BCR* and *BCR/ABL* may be a functional feedback mechanism (66).

Functional sites in *BCR* protein can be delineated (Figure. 3). They include a serine and threonine kinase domain in N-terminal exon 1. The only substrates of this kinase identified so far are Bap-1, a member of the 14-3-3 family of proteins, and possibly Bcr itself (67). dbl-like and pleckstrin-homology (PH) domains are present in the center of the molecule, and can stimulate the exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP) on Rho guanidine exchange factors, that in turn may activates transcription factors such as NF-κB.27 (68).

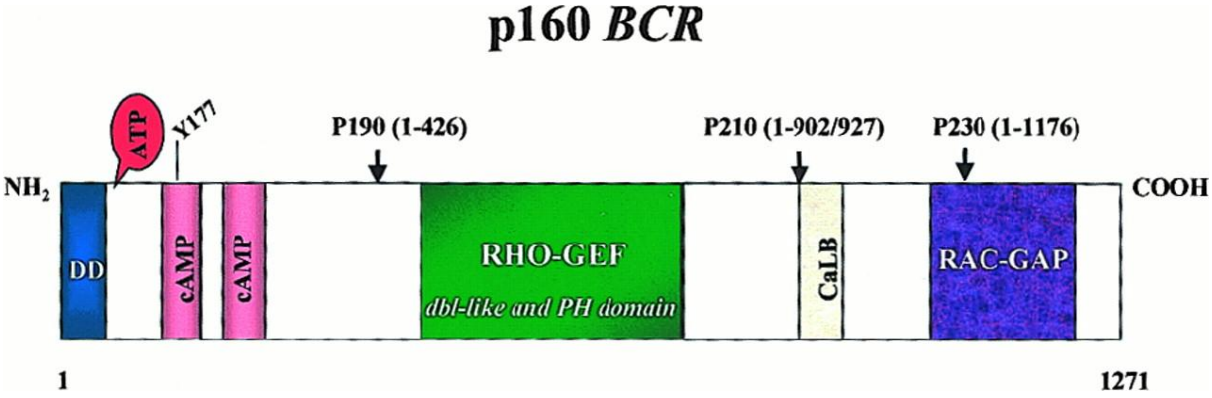

Figure 3: Structure of the BCR protein. Adapted from (36)**.**

BCR can be phosphorylated on several tyrosine residues, especially tyrosine 177, which binds Grb-2, an essential adapter molecule involved in the activation of the RAS pathway (69). The fact that *BCR* knockout (*BCR*−/−) mice are viable and the fact that an increased oxidative burst in neutrophils is thus far the only recognized defect probably reflects the redundancy of signaling pathways (70). The role of *BCR* in the pathogenesis of Ph +ve leukemias, is not clearly recognizable because the incidence and biology of $P190^{BCR/ABL}$-induced leukemia are the same in *BCR*−/− mice as they are in wild-type mice.(71, 72).

1.6 Molecular Biology of the BCR/ABL translocation

The breakpoint in the *ABL* gene at 9q34 can occur mainly over a large area at its 5' end: it may occur at the upstream of the first alternative exon Ib, downstream of the second alternative exon Ia, or, more regularly, between the two exons (73). Disregarding the exact position of the breakpoint, splicing of the primary hybrid transcript yields a fusion mRNA molecule in which *BCR* sequences are fused to *ABL* exon a2.

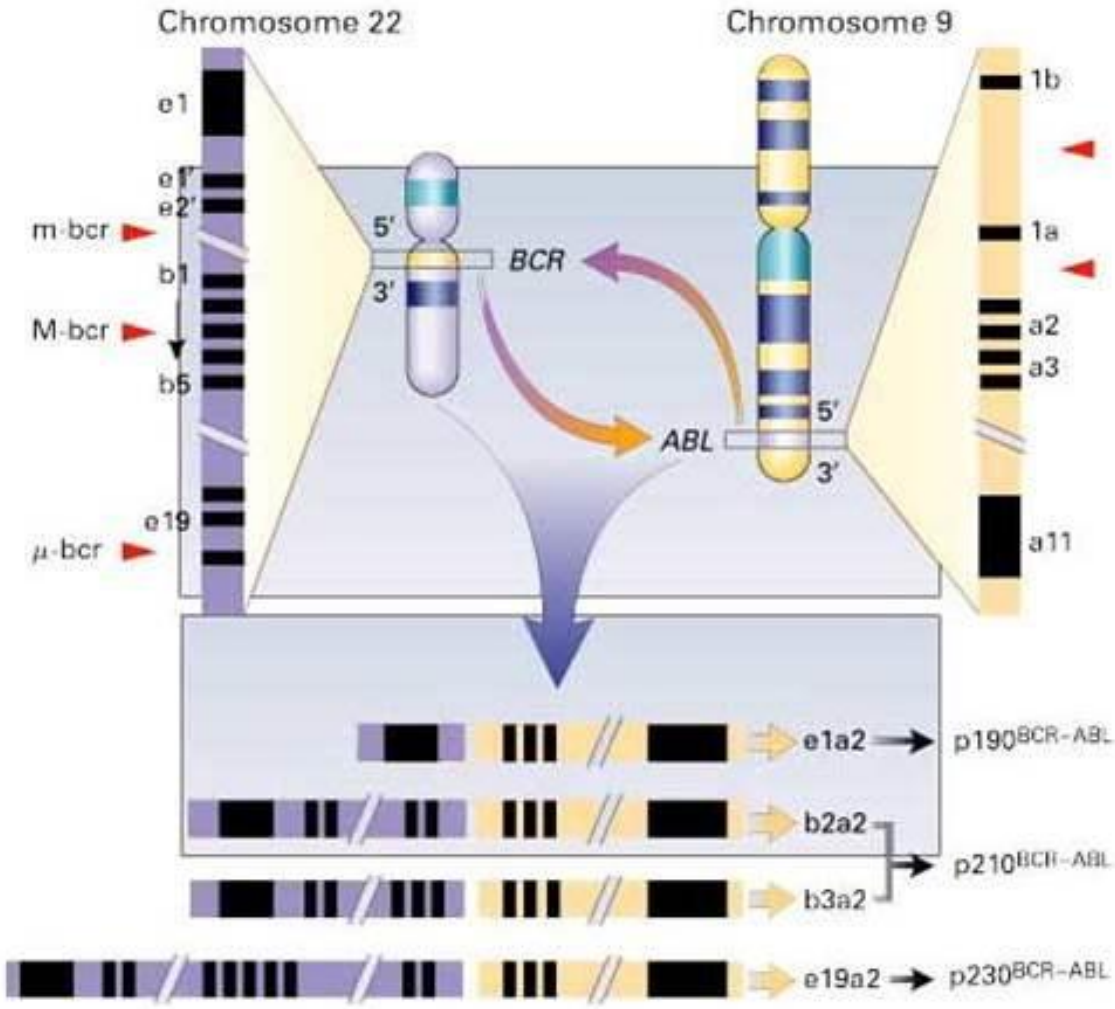

Figure 4: Indicating the translocation of t (9;22) (q34;q11) in CML and locations of the breakpoints in the *ABL* and *BCR* genes and structure of the fusion proteins derived from the various breaks. Adapted from (34).

In contrast to *ABL*, breakpoints within *BCR* localize to 1 of 3 so called breakpoint cluster regions (bcr) (Figure. 4). In most patients with CML and in approximately one third of patients with Ph-positive acute lymphoblastic leukemia (ALL), the break occurs within a 5.8-kb area spanning *BCR* exons 12-16 (also referred to as b1 to b5), defined as the major breakpoint cluster region (M-bcr) (Figure. 4). Because of this alternative splicing, *BCR/ABL* fusion gene with either b2a2 or b3a2 junction is created and transcribed into an 8.5-kb mRNA (36). A chimeric protein of 210kDa ($P210^{BCR/ABL}$) is derived from this fusion mRNA

(74). In a large number of patients, CML cells have either b2a2 or b3a2 transcripts, but in 5% of patients alternative splicing events allow the expression of both fusion products (36). Patients with b2a2 transcripts and those with b3a2 transcripts have a similar clinical feature, response to treatment and prognosis, while patients with b3a2 transcripts have higher platelet count (75).

Rarely in patients with CML (74, 76), breakpoint on chromosome 22 (situated further upstream in the 54.4-kb region) is localized between the alternative *BCR* exons e1' and e2', defined as the minor breakpoint cluster region (m-bcr) (Figure. 4). Splicing out exons e1' and e2', forms a resultant e1a2 mRNA is translated into a smaller BCR/ABL fusion protein of 190KDa ($p190^{BCR/ABL}$) (76). The CML patients that expresses $p190^{BCR/ABL}$, are characterized clinically by prominent monocytosis (36).

A third breakpoint cluster region in the *BCR* gene was identified downstream of exon 19 (e19) and exon 20 (e20), termed as micro breakpoint cluster region (µ-bcr); e19a2 fusion transcript upon translation give rise to a fusion protein of 230Kda ($p230^{BCR/ABL}$). The expression of p230BCR/ABL is associated with rare Ph-positive chronic neutrophilic leukemia variant and also with thrombocytosis (77). CML patients expressing $p230^{BCR-ABL}$ may be associated with the chronic neutrophilic leukemia variant and with thrombocytosis (77, 78). In patients with ALL and CML other junctions such as b2a3, b3a3, e1a3, e6a2 (79) or e2a2 (80) have been reported in occasional cases.

Interestingly, if in the genomic fusion transcript ABL exon1 is retained it never becomes a part of the chimeric mRNA. Thus, during processing of the primary mRNA, ABL exon1 must be spliced out; the mechanism underlining this apparent peculiarity is unknown (36). Based on the observation that the ABL part in the fusion protein is almost always constant while the *BCR* portion changes greatly, one may presuppose that transforming principle is carried by *ABL* whereas the phenotype of the disease is dictated by the distinct sizes of the *BCR* sequence (36). In support of this concept, uncommon cases of ALL express a *TEL-ABL* hybrid gene (81, 82), indicating that the *BCR* moiety can in principle be changed by the other

sequence of *TEL* and still leads to leukemia. CML like disease in mice is induced by all the 3 major *BCR/ABL* fusion proteins, but they differ in their ability to cause lymphoid leukemia (83). In contrast to p190 and p210 *BCR/ABL* tyrosine kinases, transformation to growth factor independence by p230BCR/ABL is incomplete (84), which is constant with relatively benign clinical course of p230 +ve chronic –neutrophilic leukemia (76).

On the basis of epidemiological studies it is well know that exposure to ionizing radiation (IR) is a risk factor for CML (85, 86). Morever, selective induction of the *BCR/ABL* fusion transcript in hematopoietic cells is obtained by exposure to high dose of IR in vitro (87). In human lymphocytes (88) and CD34+ (89) cell the physical distance between the *BCR* and the *ABL* genes is shorter than might be expected by chance; this kind of physical proximity may favor translocation events involving the 2 genes.

In the blood of some healthy individuals *BCR/ABL* fusion transcript of M-bcr and m-bcr are detectable at low frequency (90, 91). It is still not clear why Ph +ve leukemia does not develop in these people. It could be that an immune response suppresses or eliminates *BCR/ABL*–expressing cells or that translocation occurs in cell committed to terminal differentiation that are thus discarded (36). Indirect evidence that such a mechanism may be relevant comes from the observation that certain HLA types (HLA-B8 and HLA-A3) protect against CML.(92).

1.7 BCR/ABL directed Malignant Transformation

1.7.1 Features of the BCR/ABL protein

There are many regions that had been identified by mutational analyses and that are important for cellular transformation. In *BCR* they include a coiled–coil motif contained in amino acids 1-63 (93), the phosphoserine–threonine-rich sequences between amino acids 192-242 and 298-413 (94) and the tyrosine at position 177 (95). In *ABL* they include the SH1,

SH2, and actin-binding domains (36). However, it is critical to note that some essential features depend on the experimental system. For example *BCR/ABL* SH2-deleted mutant are ineffective to cause transformation in fibroblast, although they hold the capacity to transform Ba/F3 and FDC-P1 cell lines to factor independence and are leukemogenic in animals (96).

1.7.2 Deregulation of the ABL tyrosine kinase

Under physiological conditions the ABL tyrosine kinase activity is tightly regulated. The SH3 domain plays a major role in suppressing the tyrosine kinase activity because mutations or deletions of this region (97) activates the kinase. In order to repress the kinase activity both CIS and TRANS mechanisms have been proposed. On SH3 domain several proteins can bind (98). Inhibitory function of SH3 domain was activated by Abi-1 (99), Abi-2 (Abl interactor proteins 1 and 2) and even more interesting, activated ABL proteins promote the proteasome-mediated degradation of Abi-1 and Abi-2.

Another candidate inhibitor of *ABL* is Pag/Msp23. Due to exposure to oxidative stress (e.g. ionizing radiation) on cells, this small protein get oxidized and dissociates from *ABL*, leading to activation of kinase activity (100). Alternatively, the SH3 domain may bind internally to the proline-rich region in the center of the ABL protein, leading to a conformational alteration that hinders interaction with substrates (36, 101). Physiological suppression of the kinase activity of the *ABL* SH3 domain was abolished by fusion with *BCR* sequence 5'.

Various substrates (example: Crkl, Crk, Talin, Shc, Bap-1,PI3 kinase, Ras-GAP and Paxilin etc.) can be tyrosine phosphorylated by *BCR/ABL* (36). Due to autophosphorylation, there is a marked increase of phosphotyrosine on *BCR/ABL* itself, which led to formation of binding site for the SH2 domains of other proteins (36).

Substrates of *BCR/ABL* can be grouped on the basis of their physiological role: 1) adapter molecule as Crkl and p26DOK, 2) proteins with catalytic function as the nonreceptor tyrosine kinase Fes or the phosphatase Syp and 3) proteins linked with the organization of the cell membrane and the cytoskeleton (such as talin and paxillin). It is important to note that the election of substrate by *BCR/ABL* rely upon the cellular context. For example, *Crkl* is the main tyrosine-phosphorylated protein in CML neutrophils (102), while phosphorylated $p62^{DOK}$ is largely found in early progenitor cells (103).

In physiological conditions the effect of tyrosine kinases is regulated by tyrosine phosphatases, which keep the cellular phosphotyrosine level low. Syp and PTP1B (104) are the two tyrosine phosphatases which form complexes with *BCR/ABL* and also both appear to dephosphorylate *BCR/ABL*. Interestingly, PTP1B level rises in p210 BCR/ABL expressing K562 and Rat-1 cells or also in a kinase dependent manner, indicating that the cell attempts to limit the impact of BCR/ABL tyrosine kinase activity. Overexpression of PTP1B impaired the *BCR/ABL* induced transformation in fibroblasts (105).

1.8 Biologic properties of *BCR/ABL*–positive cells

Several mechanisms have been involved in the malignant transformation induced by *BCR/ABL*, especially altered adhesion to stroma cells and extracellular matrix (106), constitutively active mitogenic signaling (107) and decreased apoptosis (108).

1.8.1 Altered adhesion properties

Hematopoiesis takes place in close association with the marrow environment. Normal hematopoietic progenitors adhere by a variety of receptors on stroma and extracellular matrix components along with fibronectin (109). This type of adhesion through intergrins to fibronectin assist hold progenitors to microenvironment, although it also directly alter the

proliferative behavior of normal hematopoietic progenitors (109). In CML, progenitor cell exhibits decreased adhesion to bone marrow stroma cells and extracellular matrix (106). Proliferation is negatively regulated by adhesion to bone marrow stroma cells. CML cells escapes this regulation because of their abnormal adhesion properties (110). Interferon- α (IFN-α) appears to reverse the adhesion deformity (111).

An adhesion-inhibitory variant of β1 integrin is expressed in CML cells, that is not identified in the normal progenitors (112). Integrins are capable of triggering normal signal transduction from outside to inside upon binding to their receptors, but in CML progenitor cells this transfer of signal that generally inhibits proliferation is impaired. This is due to involvement of *ABL* in the intracellular transduction of such signals, due to the presence of a large pool of BCR/ABL protein in cytoplasm this process may be further disturbed (112).

In *BCR/ABL* transformed cells, out of all tyrosine phosphorylated proteins, Crkl is the most prominent one (102), and is involved in the regulation of cellular mobility (113) and in integrin-directed cell adhesion (114) by association with other focal adhesion proteins such as paxillin, the focal adhesion kinase Fak, p130 Cas (115) and Hef1 (116). The expression of α6 integrin mRNA is upregulated by *BCR/ABL* tyrosine kinase, this may be another possible mechanism by which *BCR/ABL* may have effect on integrin signaling (72).

1.8.2 Signalling Pathways activated by *BCR/ABL*

Before fusing to *BCR*, the *ABL* protein physiologically shuttles in between cytoplasm and the nucleus. However, after fusing with *BCR*, the fusion protein is retained within the cytoplasm; at this point it interacts with various proteins which are involved in oncogenic pathway (117). *ABL* tyrosine kinase activity is constitutively activated due to juxtaposition with the *BCR*, thus facilitating dimerization or tetramerization and consequent autophosphorilation (117). This led to increase in the phosphotyrosine residue on *BCR/ABL* and as a result of this, SH2

domain serves as binding sites for other protein (94, 95). A summary of the Signaling pathways activated in the *BCR/ABL*+ve cells is represented in Figure. 5.

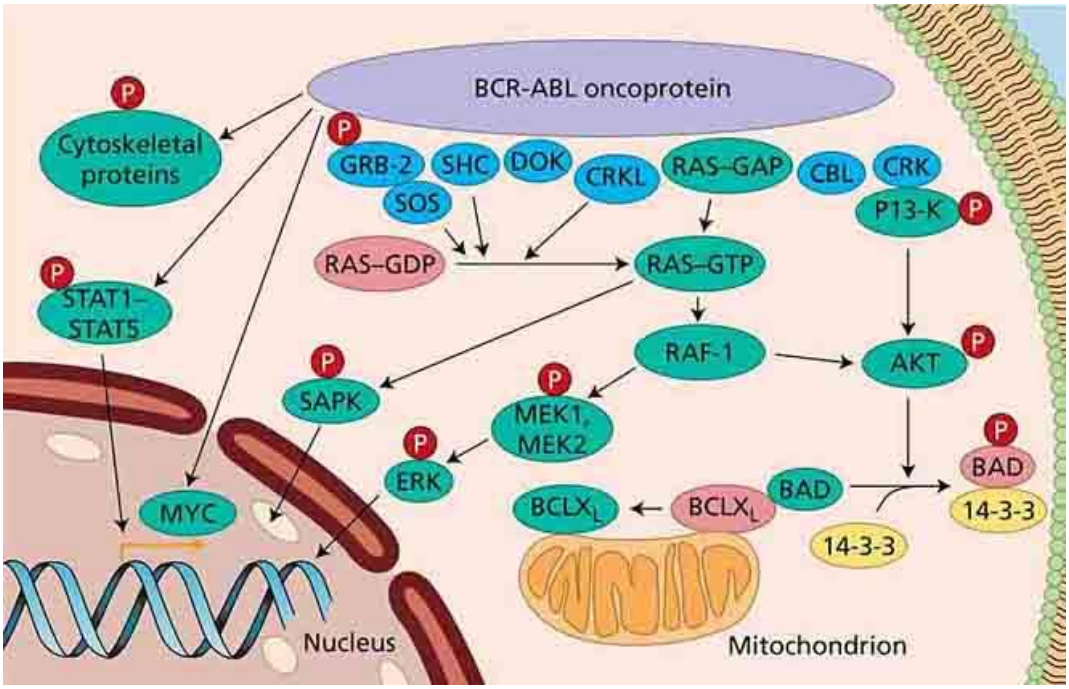

Figure 5: BCR/ABL mediated signal transduction pathways. Schematic representation of pathways (STAT, RAS/RAF/1-MEK1, PI3 kinase/AKT) that might be involved in conveying the BCR/ABL signal to the cell nucleus in the clinical setting. Letter P is used to mark the molecules known to be phosphorylated by activated *BCR/ABL*. Adopted from (118).

The *Ras* and the *mitogen-activated protein kinase (MAPK)* pathways

Association between *BCR/ABL* and Ras has been well defined. Autophosphorylation of *BCR/ABL* Tyrosine 177 serves as a docking site for the adopter molecule Grb-2 (Growth Factor Receptor-Bound Protein-2) (95). Upon activation, Grb2 binds to the Sos (Son of Sevenless) protein that stabilizes Ras in its active GTP-bound form. Both Shc and Crkl are the two other adapter molecules by which Ras is also activated and both are substrates of

BCR/ABL, binding to the fusion protein through their SH2 (Shc) or SH3 (Crkl) domains (102, 119). The relevance of Crkl involvement for Ras activation is, however, controversial since it appears to be confined to fibroblasts (120). Moreover, for the transformation of myeloid cells, direct binding of Crkl to *BCR/ABL* is not necessary (121).

Downstream of Ras in Ph +ve cells, Raf gets activated. Due to stimulation of cytokine receptors RAS get stimulated and serine-threonine kinase activated RAF gets subsequently recruited to the cell membrane (122). Raf initiates a signaling cascade of protein phosphorylation of Mek1/Mek2 and Erk. Phosphorylated Erk moves from the cytoplasm into the nucleus were it leads to the phosphorylation of a number of transcription factors and ultimately causing activation of gene transcription (123).

The Jak-Stat pathway

In normal cells, after cytokine binding to receptors, nuclear translocation of STATs occurs and is mediated by activation of the receptor associated JAK kinases. By contrast, in CML, direct activation of STATs occurs without prior phosphorylation of JAK (Janus kinase) proteins (124). Although STATs has pleotropic physiologic functions (125), activation of STAT5 in *BCR/ABL* transformed cells appears to be primarily anti-apoptotic, through up-regulation of the anti-apoptotic molecule BCL-xL (126, 127).

Studies conducted in v-ABL–transformed B cells, gave the early sign for involvement of the Jak-Stat pathway (98). Since then it is reported that several *BCR/ABL* +ve cell lines (124) and primary CML cells (124, 128) have constitutive phosphorylation of Stat transcription factors (Stat1 and Stat5). Therefore STAT5 activation appears to contribute to p210-BCR/ABL transformation (129) while p190 protein directly activate specific STAT6 as opposed to p210 (124).

The PI3 kinase pathway

In Ph +ve leukemia PI-3 kinases (PI3K) are regulated by *BCR/ABL*, and this interaction is functionally significant for the *BCR/ABL* +ve cells proliferation and survival (130). *BCR/ABL* generates the formation of multimeric complexes with PI3K, CBL and the adaptor molecule c-CRK or CRKL (114). Significance of PI-3K for *BCR/ABL* activity was established, by inhibiting PI3K anti-proliferative effect in the *BCR/ABL* dependent cells (130).

Serine–threonine kinase *AKT* emerged to be the next relevant substrate in this cascade (131). Various reports suggested that AKT, as a survival-promoting serine-threonine protein kinase, was activated by IL-3 in a PI3K-dependent manner and also identifies the pro-apoptotic protein BAD as a key major substrate of AKT (132). Interleukin-3 (IL-3) causes phosphorylation of BAD (inactive form), and due to this it is not more able to bind anti-apoptotic proteins such as BclXL and it is trapped by cytoplasmic 14-3-3 proteins. Taken together this indicates that BCR/ABL fusion protein may be able to mimic the physiologic IL-3 anti-apoptotic signal in a PI-3 kinase dependent manner (132). In response to growth factor signals and to *BCR/ABL*, both Ship (133) and Ship-2, (134) 2 inositol phosphatases are getting activated. This indicates that *BCR/ABL* appears to have a major effect on phosphoinositol metabolism, that may shift again the balance towards the physiological growth factor stimulation (36).

The MYC pathway

In many human malignancies overexpression of MYC have been demonstrated (36). MYC belongs to basic helix-loop-helix lucin zipper (bHLH-LZ) family, it is a transcription factor and the majority of its targets are still unknown. Activation of MYC appears to be independent of the Ras pathway, however *BCR/ABL* activates MYC through its SH2 domain (135). In vitro inhibition of MYC with dominant–negative construct (obtained by SH2 deletion) can suppress *BCR/ABL* leukemogenesis (134). Results obtained from *BCR/ABL*–transformed murine

myeloid cells (136) and v-ABL–transformed cells (137) suggested that signal is transduced through RAS/RAF, cyclin-dependent kinases (CDKs) and E2F transcription factors that eventually activate the MYC promoter (137). Depending on the cellular context, it is suggested that MYC constitute both proliferative and apoptotic signal (138, 139). In CML cells the apoptotic arm of MYC might be counterbalanced by other mechanisms, such as PI3K pathway (36). There are several lines of evidence suggesting that MYC is often overexpressed in blast crisis in comparison with the chronic phase, thus linking MYC to CML progression (135).

1.8.3 Inhibition of apoptosis

Factor-dependent human (140) and murine (141) CML cell lines expressing BCR/ABL do not undergo apoptosis after growth factor withdrawal, an effect critically dependent on tyrosine kinase activity, that correlates with the activation of RAS (107, 142). Moreover, several studies showed that *BCR/ABL* +ve cell lines are resistant to apoptosis induced by DNA damage (108). The fundamental biological mechanism is still not understood.

To prevent apoptosis, *BCR/ABL* might block the release of cytochrome C from the mitochondria and thus the activation of caspases (143, 144) maybe through the Bcl-2 family of proteins. In Baf/3 and 32D cells it has been shown that BCR/ABL lead to up-regulate Bcl-2 expression in a Ras or PI3K dependent manner (131, 145). In addition to the above mentioned pathways, Bclx is also get transcriptionally activated by STAT5 in Ph +ve cells (126). Another link between *BCR/ABL* and the restriction of apoptosis could be the inactivation of the pro-apoptotic protein BAD through its phosphorylation through active kinases such as .AKT and RAF-1 (146, 147). Hence *BCR/ABL* initiates multiple signal with proliferative and anti-apoptotic effects.

1.9 The Human BCR Promoter

The expression of the *BCR/ABL* fusion gene is driven by the human *BCR* promoter (148). The isolation and characterization of BCR promoter has been done for a region of 1.1 kb immediately 5' to the transcription starting site (148). Accordingly to Shah et al (149), functional *BCR* promoter seems to be more restricted to a region of about 1Kb 5' of *BCR* exon 1. *BCR* promoter functions seems not to be changed by the t (9:22) translocation (149). Marega M et al (150) showed that both BCR and BCR/ABL are regulated by an "in-trans" molecular mechanism which regulates both the genes during myeloid differentiation.

By DNase protection assay 10 putative protein binding sites (PBSs) were identified along the *BCR* promoter (Figure. 6) (148-150). From *in silico* studies, six of these (10 PBS) putative PBSs are localized in the region between -1443 to -1202 bp from the ATG site, which appears to be critical for its function, as suggested by Marega M et al (150). The *BCR* promoter sequence has high GC content (78%) (149) and contains a TTTAA box (148).

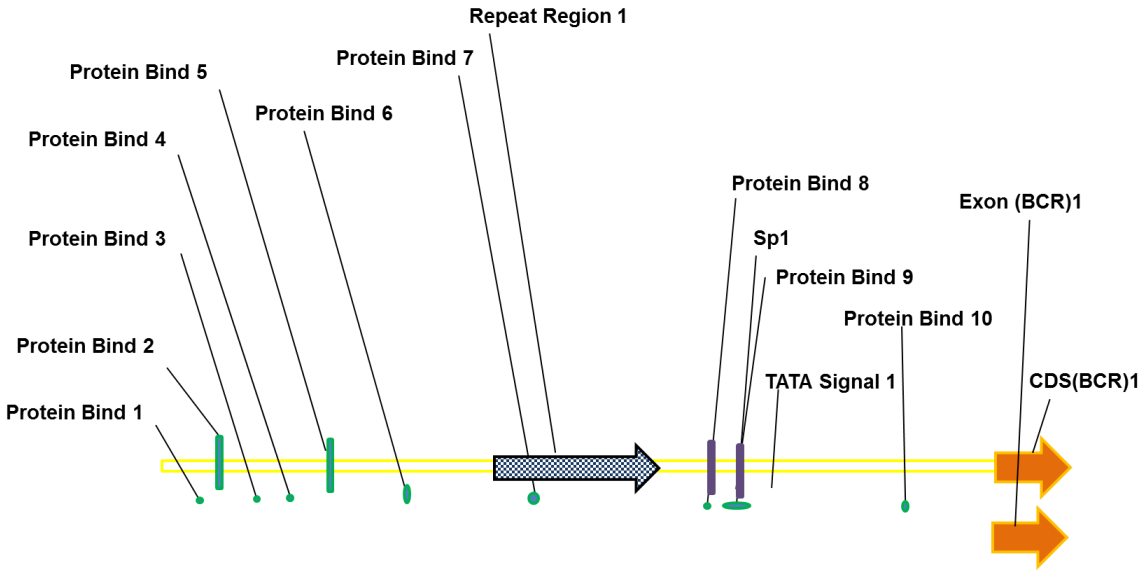

Figure 6: Schematic representation of *BCR* Promoter.

The nucleotide sequence analysis identified a repeated sequence: the 3' coding and splice donor region of *BCR* exon 1 is present in the 5' region of the gene in an inverted orientation (148). From the mouse DNA analysis, only a part of repeat region is detected in the mouse genome, hence it reveal that the repeat sequence is a relatively novel addition to the gene in evolutionary terms (148).

It seems that the inverted 5’ repeat have some function in the *BCR* promoter. The complement of the sequence 5’ CACGATGGTGGCCTCTGACACGA 3' is also protected across DNase I digestion by protein factors in both K562 and A498 extracts (148). Interestingly, the sense strand of the 3’ repeat situated in the 3’ coding and splice donor region of exon 1 is also protected, despite this sequence contains one base-pair difference compared to the 5' repeat (148).

1.10 Features implicated in CML progression

The mechanisms responsible for CML progression and transformation are complex and only partially understood. Various major features have been identified as crucial in the progression from CP to BC CML.

1.10.1 BCR/ABL expression

The expression of *BCR/ABL* is important not only in sustaining CML but also in the progression of this disease. In comparison with CP the *BCR/ABL* mRNA and protein levels are higher in BC (151, 152). The Majority of CML CD34+ progenitors may have *BCR/ABL* transcript levels up to 200-fold higher comparing to their more differentiated counterparts (153).

When *BCR/ABL +ve cells* were injected subcutaneously into syngeneic mice, cell lines with higher level of *BCR/ABL* positivity induced tumors, while low expressing clones led to tumor

formation only after prolonged latency (154). This is indicating that *BCR/ABL* is a critical mediator of disease latency (155). Reason behind the increase of *BCR/ABL* transcript level is not well known. It may result from a selective pressure which favours the expansion of highly proliferative and poorly differentiated leukemic clones. In BC CML, elevated *BCR/ABL* transcript levels have been detected in the CD34+ granulocyte-macrophage progenitor (GMP) subpopulation compared with GMP in CP CML (156). A recent study by MAREGA M et al (150) suggested that BCR is physiologically down-regulated up on myeloid maturation from HSCs to CMPs and GMPs, and that this mechanism is conserved in healthy donors and in CP-CML for both BCR and BCR/ABL. This result suggests that the two genes may be under a similar transcriptional control. In the same study the author showed that in BC this downregulation is impaired during myeloid maturation, is in fact not visible a significant difference in expression levels of both BCR and BCR/ABL. The in-trans mechanisms that physiologically downregulates the two genes during myeloid maturation is thus lost in BC transformation. Despite this recent findings, the molecular basis of this phenomenon has not been completely elucidated.

1.10.2 Other genetic alterations associated with CML progression

Various cytogenetic and molecular changes occur in vast majority of CML patients during progression to blast crisis.

Isochromosome i (17q) is detected in approximately 20% of BC cases. This abnormality leads to loss of 17p and has been thought to be associated with loss of a copy of the p53 tumor suppressor gene, which is located on chromosome 17 (157). However, the remaining p53 allele does not appear to be mutated in these cases, so a direct link of p53 inactivation and a molecular mechanism of disease progression has not been confirmed yet (157). However, decrease of the total p53 level might upset the complex integration of genetic

repair and apoptosis and contribute to progression (158). Alternatively, there may be critical but yet unexplored genes on 17q contributing to CML disease progression (158).

Trisomy 8 is seen in approximately 40% of BC cases. Because MYC is located at 8q24, it may be expected that trisomy of chromosome 8 is associated with c-Myc overexpression and that this is pathogenetically involved in disease progression. *In vitro* inhibition of c-Myc with dominant-negative c-Myc molecules or c-Myc antisense oligonucleotides, can suppressed BCR/ABL-dependent transformation or leukemogenesis (135). Trisomy 8 is a typical characteristic of cases of clonal evolution in patients with CML treated with imatinib that are in cytogenetic remission (thus, these clones have trisomy 8, but not the Ph). Hence, these cases with trisomy 8 look to have a benign course, indicating that trisomy 8 itself may not be leukemogenic (159).

Translocations of known oncogenes has been found in approximately <5% of myeloid CML-BC. The most notable of these recurrent translocations are t(3;21))(q26;q22) and t(7;11))(p15;p15), associated with expression of the *AML-1/EVI-1* and *NUP98/HOXA9* fusion proteins, respectively (160, 161). Both EVI-1 and HOXA9 are transcription factors and their irregular expression lead to differentiation arrest in the case of AML-1/EVI-1, and increased proliferation in the case of *NUP98/HOXA9*. In mouse models, *BCR/ABL* and *AML-1/EVI-1* co-expression causes rapid induction of an acute myelogenous leukemia, whereas mice that received transplants or are transfected with *NUP98/HOXA9* develop a myeloproliferative disease that progressed into an acute leukemia after a latency of 4 to 9 months (162, 163).

Double Ph chromosome has been reported in more than 30% of BC patients; however, the role of the double Ph chromosome in disease progression is still unclear (164). Additional deletions of the derivative chromosome 9 occur in 5% to 10% of chronic phase patients; these patients respond poorly to interferon, because this deletion erase critical interferon receptor genes (164, 165). The clinical impact of additional cytogenetic and molecular genetic aberrations seems to depend on the therapeutic regimen used (166). For example, chromosome 8 trisomy is more common after busulfan (44%) than hydroxyurea therapy

(12%) (166). The frequency of the common secondary changes seen after interferon-alpha treatment or especially after bone marrow transplantation seems to be lower than in the group treated with busulfan (166).

Using Single-nucleotide polymorphism (SNP) arrays, it has been shown that patients with CP CML carry 0.47 copy number alterations per case (range, 0-8), while 7.8 copy number alterations (range, 0-28) per BC CML case were spotted (167). This indicates that multiple genomic alteration accumulate during progression to BC CML.

In addition to the increase of BCR/ABL level, BCR/ABL independent mechanisms may play a critical role in CML progression. For example, activation and or overexpression of the SFKs, HCK, LYN, and FYN have been linked to imatinib resistance and CML progression (168, 169). BCR/ABL retrovirus-transduced bone marrow from mice deficient of LYN−/−HCK−/−FGR−/− (all three Src kinases) efficiently induced CML but not B-ALL in recipients. This suggests that all three Src kinases may be essential in the pathogenesis of Ph+ B-ALL and lymphoid BC-CML (170, 171).

1.10.3 Arrest of differentiation

Steadily interruption of the differentiation program is a distinctive feature of CML progression. Transcription factors that are regulating the expression of several differentiation-related genes are modulated by *BCR/ABL* (172, 173). CCAAT/enhancer-binding protein-α (CEBPα) is a transcription factor that is essential for normal granulocyte differentiation and is found expressed in normal bone marrow cells and also in CP CML samples. In contrast, CEBPα expression is vanished in the BC (174). *BCR/ABL* mediates suppression of CEBPα expression at the mRNA level, by interacting and stabilizing the poly (rC)-binding proteins heterogeneous nuclear ribonucleoprotein E2 (hnRNP E2), inhibiting the translation of CEBPα transcript (173). In CP-CML the expression of hnRNP E2 is low or undetectable, but becomes easily detectable in BC-CML samples. Transplantation of *BCR/ABL* expressing

CEBPα+/+ fetal liver cells consistently phenocopied CP-CML like diseases in transplanted mice, whereas recipients of *BCR/ABL* expressing CEBPα-/- failed to induce myeloid diseases but instead induced an immature, lethal transplantable erythroleukemia (175).

Deletion or loss of function of IKZF1 gene, that encodes for a transcription factor essential for the early lymphoid lineage commitment, have been linked with CML progression, especially to aggressive lymphoid BC-CML (167). In addition, 84 % of patients with *BCR/ABL* +ve ALL, shows monoallelic deletion of exons 3 to 6 of IKZF1, (167). IKZF1 mutation are linked with poor prognosis in both Ph+ and negative patients (176), with an increase in the relapse rate.

Infrequently development of BC CML phenotype involves the interaction of *BCR/ABL* with translocations, leading to the formation of various dominant negative transcription factors following a 2-hit paradigm as NUP98-HOAXA9 (163) or AML-EV1 (177). Although *AML1-EVI1* blocks differentiation, NUP98-HOXA9 subverts the normal balance of symmetric and asymmetric renewal division, favoring the former and causing preferential growth of immature precursors and facilitating the transition to BC CML (178).

1.10.4 Genomic instability and DNA repair

The progression of CML from chronic phase to blast crisis is associated with genetic instability, but the molecular mechanisms behind these chromosomal abnormalities are still unknown. Only few studies have directly addressed the relationship between *BCR/ABL* expression and levels/activity of proteins involved in DNA repair, particularly the repair of DNA double-strand breaks (DSBs) (179).

BCR/ABL has been shown to enhance the chronic oxidative DNA damage, DSBs in S and G2/M cell phases, and mutagenesis caused by endogenous reactive oxygen species (ROS) and exogenous genotoxic treatment (180, 181). *BCR/ABL* triggers unfaithful DSB repair pathways, homologous recombination repair (HRR), non-homologous end-joining (NHEJ)

and single-strand annealing (SSA) to repair the lesions induced by ROS and γ-irradiation (182, 183). The majority of mutations involved are G/C to A/T transitions and A/T to G/C transversions in clinically relevant amino acid substitutions in the coding regions of multiple genes (including the kinase domain of BCR/ABL), causing IM resistance. The phenomenon described above is demonstrated in BCR/ABL +ve cells and not in *BCR/ABL* –ve cells (184). Altogether, these results suggested that these series of events contribute to genomic instability of Ph +ve leukemias: *BCR/ABL* --> ROSs --> oxidative DNA damage --> DSBs in proliferating cells --> unfaithful HRR and NHEJ repair (182, 185).

The most important and the preferred pathways used by the human cells to repairs DSBs is DNA-PK-dependent pathway (nonhomologous end-joining recombination) (186)). In *BCR/ABL* expressing cells, the levels of DNA-dependent protein kinase catalytic subunit (DNA-PKCS) were markedly down-regulated (187). Down-regulation of DNA-PKCS levels was associated with a higher frequency of chromosomal abnormalities after exposure of *BCR/ABL*-expressing cells to ionizing radiation (IR) and increased radiosensitivity (187).

The same group also reported that the expression of the p210 BCR/ABL fusion protein in primary CML samples and in established cell lines leads to a down-regulation of BRCA1 protein (188), a protein involved in the surveillance of genome integrity (189, 190). Down-regulation of BRCA-1 was more evident in cell lines expressing high levels of *BCR/ABL* and was linked with an increased rate of sister chromatid exchange and chromosome aberrations after DNA damage (188).

Another group showed that *BCR/ABL* significantly enhances the expression/activity of RAD51, a protein that play a central role in homologous recombination repair (HRR) (181). Expression of *BCR/ABL* leads to increase the efficiency of HRR in a RAD51-dependent manner, furthermore increasing resistance to apoptosis caused by drugs like mitomycin C and cisplatin, which encourage DSBs (181). However on the basis of the above knowledge of enhanced high-fidelity HRR promoted by RAD51, it look unreasonable that the enhanced

expression/activity of RAD51 in *BCR/ABL*–expressing cells may be linked with genomic instability.

1.10.5 Stem cell self-renewal ability

The term self-renewal refers to the division without differentiation and is normally a feature associated with long-term HSC (191). Various self-renewal pathways have been implicated in CML transformation. The WNT/β-catenin axis is one important player in the stem cell pathway and its involvement in leukemogenesis is widely recognized (192-195). Beta-catenin activation leads to its nuclear accumulation and to activation of a pro-survival program, including c-MYC and Cyclin D expression (196, 197).

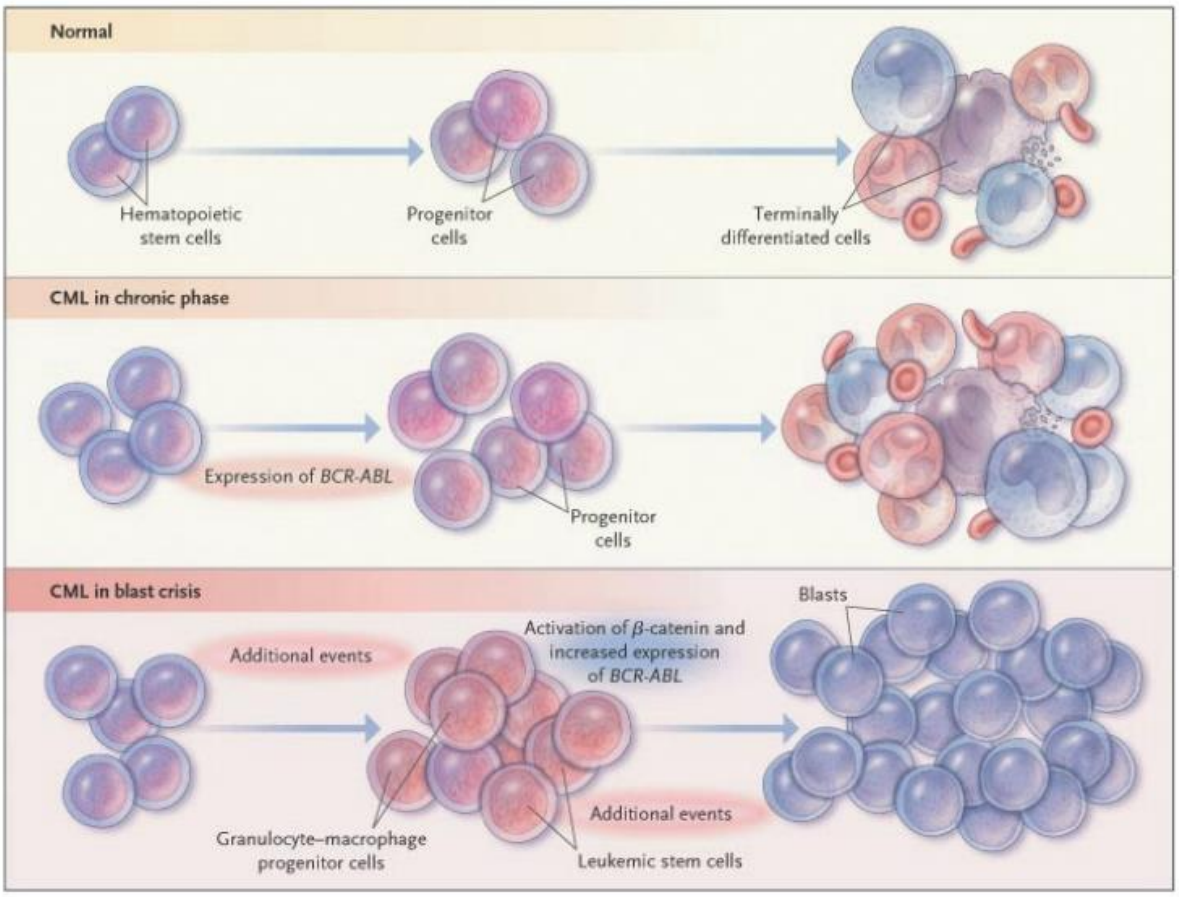

Figure 7: Representing the role of activated β-catenin in the progression of CML in comparison with normal myeloid maturation. Adopted from (156).

BC progression has been associated with beta-catenin accumulation in GMPs cells, enforcing the self-renewal potential of myeloid progenitors (Figure. 7) (156, 198). In our laboratory it has been shown that BCR/ABL is able to stabilize beta-catenin at the protein level, through phosphorylation of tyrosines 86 and 654 (Figure. 8). This phosphorylation is able to inhibit the binding of beta-catenin with the degradation complex (Axin/GSK3beta/APC), thus enabling beta-catenin accumulation, nuclear translocation and transcriptional activation (199). Furthermore, it has recently been shown that beta-catenin could also be hyper-activated in BC thanks to the expression of an in-frame deletion of GSK3-beta (200).

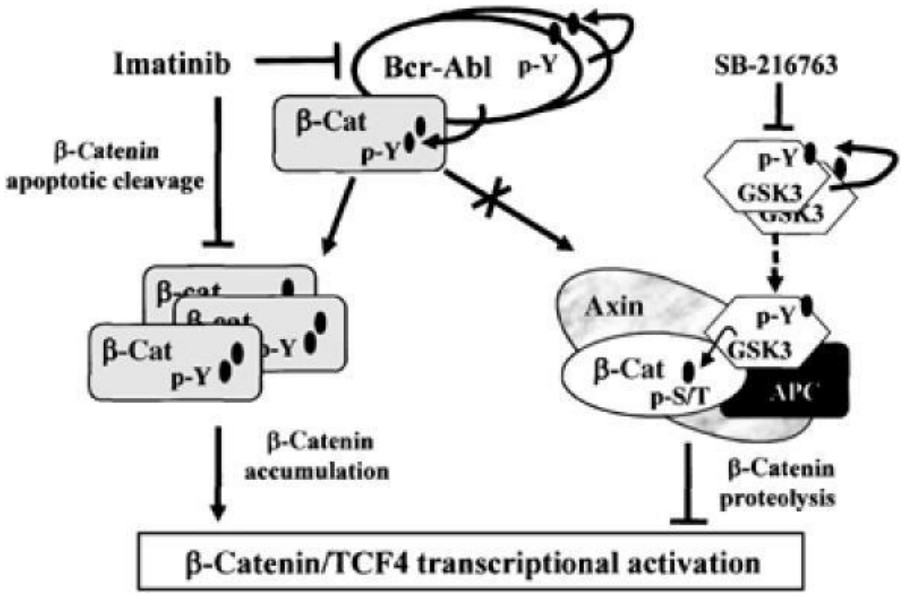

Figure 8: Representing a model about activation of β-catenin/TCF4 transcription in BCR/ABL positive CML cells. The above figure outlines the effects of BCR/ABL, SB-216763 and imatinib on β-catenin protein stabilization and nuclear signaling. Adopted from (199).

1.10.6 Inactivation of tumor suppressor genes

One of the most typical tumor suppressor mutations associated with CML progression is p53, which is mutated in 25% to 30% of patients with myeloid BC. In addition, exon 2 of the INK4A/ARF locus is found deleted in 50% of cases of lymphoid BC (201). This latter event results in the deletion of both p16 and p14/ARF, two proteins that normally check G1/S cell cycle by inhibiting the G1-phase cyclin D-Cdk4/Cdk6 and p53 up-regulation, respectively (201). Because ARF increase p53 levels interfering with the action of MDM2 (principle negative regulator of p53), homozygous deletion at the p16/ARF locus observed in lymphoid blast crisis might symbolize a functional equivalent of p53 mutation in myeloid BP (201). In addition p53 pathways play a major role in response to imatinib therapy. p53 is selectively activated by imatinib treatment in BCR/ABL expressing cells as a consequence of *BCR/ABL* kinase inhibition. Inactivation of p53, which frequently lead to CML progression, impedes the response to imatinib in vitro and in vivo without preventing *BCR/ABL* kinase inhibition. Hence, in some CML-BC patients mutations affecting the p53 pathway may contribute to imatinib resistance (202).

The cell cycle regulatory effects of the p105 retinoblastoma protein (RB) was abrogated/attenuated indirectly due to loss of p16INK4A; however, the Rb gene itself is inactivated by mutation, deletion, or loss of expression in approximately 18% of accelerated or blast crisis CML cases, especially those associated with a megakaryoblastic or lymphoblastic phenotype (203, 204).

The RUNX family of transcription factors play a vital role in regulating the expression of genes involved in differentiation and cell cycle progression (205), and it has also been involved in imatinib response and disease persistence in patients with CML. It has in fact been shown that BCR/ABL+ve cell lines with stable or inducible expression of RUNX1 or RUNX3 were protected from Imatinib-induced apoptosis and that Imatinib therapy can select for RUNX-1 expressing cells in-vivo (206).

Another gene involved in CML progression is the tumor suppressor PP2A (207). PP2A play a vital role in regulating proliferation, survival and differentiation and it is also involved in activation of protein tyrosine phosphatase 1 (SHP1), which catalyzes *BCR/ABL* dephosphorylation and proteosomal degradation. *In vitro* and in human leukemia, it has been demonstrated that *BCR/ABL* can up-regulate SET, a negative regulator of PP2A (207). Hence, progression may set up a feedback loop through which increasing BCR/ABL increases SET, decreasing PP2A, which further establish the persistence of *BCR/ABL (207)*. Treatment of *BCR/ABL* +ve cells and mouse models with forskolin, a pharmacologic activator of PP2A, results in decreased *BCR/ABL* leukemic potential, hence indicating a promising therapeutic target to arrest or revert CML progression (207).

1.11 Definition of treatment response in CML

The aim of CML therapy is the recovery of total blood counts to normal values, reduction and elimination of the Ph chromosome and *BCR/ABL* gene expression. Advancement toward these goals can be determined by measuring the hematologic, cytogenetic and molecular responses, respectively.

- Hematologic response: a complete hematologic response (CHR) means that the laboratory values return to normal levels, with a white blood cell count <10,000/mm3, a platelet count <450,000/mm3, the presence of <5% myelocytes plus metamyelocytes, the presence of <5% basophils, the absence of blasts and promyelocytes in peripheral blood, and the absence of extramedullary involvement (208, 209). With tyrosine kinase inhibitor (TKI) therapy, nearly all patients with CML in chronic phase achieve a CHR. Achievement of CHR within 3 months from the start of therapy is an optimal response, as stated by European LeukemiaNet (210).

- Cytogenetic response: a complete cytogenetic response (CCyR), based on the cytogenetic analysis of standard karyotype with 20 metaphases, shows the absence of BCR/ABL +ve cells (Table 2) (210).

Table 2: Category of cytogenetic responses.

Response	Category	Criteria
Cytogenetic responses	Complete	No Ph chromosome-positive metaphases
	Major	0% to 35% Ph chromosome-positive metaphases
	Partial	1% to 35% Ph chromosome-positive metaphases
	Minimal	36% to 95% Ph chromosome-positive metaphases

- Molecular response (MR): The majority (83%) of patients with CML treated with TKI therapy obtain a complete cytogenetic response. In-order to detect the minimal residual diseases more sensitive measurements are necessary (211). In molecular monitoring, real time quantitative polymerase chain reaction (QPCR) is used for detecting the presence of *BCR/ABL* mRNA. In the IRIS trial the major molecular response (MMR) is defined as a 3-log reduction in BCR/ABL transcripts from a standardized line based on the median value of BCR/ABL/BCR expression present at diagnosis (212, 213). Anyway, it has to be considered that assay sensitivity varies from center to center and from sample to sample. The 3-log reduction has thus been defined as $\leqslant$0.1% BCR/ABL on an International Scale (IS) (213). From this definition the terms CMR(4), CMR(4.5), and CMR(5) are used to indicate BCR/ABL

levels of ⩽0.01% (≥4-log reduction from IRIS baseline), ⩽0.0032% (≥4.5-log reduction from IRIS baseline) and ⩽0.001% (≥5-log reduction from IRIS baseline) respectively (214).

1.11.1 Therapy Options for CML

Since 1981 interferon alpha (IFNa) has been extensively studied as treatment for patients with CML. Prior to the development of TKI, interferon alpha was the non-transplant treatment of choice for most patients with CML. INFa was distinctly superior to the primarily leukostatic hydroxyurea (215), but only 20-30% of patients achieved CCyR after treatment with IFNa (216). In response to antigenic stimuli (such as viral infection & malignant diseases) eukaryotic cells produces interferons. Interferons are glycoproteins that have pleiotropic effect, including immunomodulatory, antiviral, antiproliferative and antiangiogenic activities. In comparison with any other interferon, interferon alpha has been used more extensively in the treatment of patients with both solid and hematological malignant tumors (217). The precise mechanism of action of IFNa in CML is not known. Its regulatory effect on the leukemic clone is through enhancement of hematopoietic cell-microenvironmental cell interactions, restoring defective cytoadhesion and inhibition of proliferation of CML colony-forming cells (217, 218). With the development of TKI and the profound increased toxicity of INFa compared with TKIs, this latter drug has been used much less commonly for the CML (219). In 1990s allogeneic hematopoietic stem cell transplant (HSCT) was very popular, but this technique was curative exclusively when carried out at early phase of the disease. HSCT was advantageous to the younger patients with an appropriate donor. Various factors that determine the outcome of the HSCT are: age of patients, the stage of the disease, time since diagnosis, type of donor, source of stem cells, post-transplant management of infections and graft versus host disease (220). Best outcomes are obtained in younger patients in chronic phase disease with matched related donors (220, 221). Survival outcome following HSCT in chronic phase patients is nearly 80%, in accelerated phase it is about 40%-50%, and 20% in

patients who receive it after blast crisis sets in (222). After the outcome of TK inhibitors the therapeutic landscape for CML has undergone a paradigm change. Even in younger patients, HSCT is no longer the choice for first-line treatment. Currently, HSCT is recommended for patients in the advanced stage of disease or who develop resistant mutations that fail to respond to second line TK inhibitors as well (222, 223).

Imatinib Mesylate (Imatinib) (also known as Gleevec, Glivec, ST1571 and formerly Ciba-Geigy compound CGP57148B) is a potent and selective inhibitor of *BCR/ABL* kinase activity and was discovered by Novartis Pharmaceutical Corporation, NJ, USA. It acts via competitive inhibition of ATP at the ATP-binding site (ATP pocket) of the tyrosine kinase domain of both the normal ABL and BCR/ABL protein, hence resulting in the inhibition of phosphorylation of proteins involved in cell signal transduction. In addition it also blocks c-KIT tyrosine kinase, as well as platelet-derived growth factor receptor (PGDFR) (223). Imatinib entered in clinical practice in 2001 (224) is currently established as first line therapy for CML patients (225). Despite its definite efficacy, it does not completely eliminate *BCR/ABL* expressing cells (226).

The International Randomized Study of Interferon and Imatinib (IRIS) clinical trial demonstrated the dramatic effectiveness of Imatinib relative to interferon-α: newly diagnosed CP-CML patients treated with Imatinib had an 8-years overall survival rate of 85% (227).

The research study Imatinib Long-Term (side) effects (ILTE), demonstrated for the first time that CML patients in CCyR after two years of Imatinib treatment, have a mortality rate similar to that of the general population The study was conducted in 27 centers in Europe, North and South America, Africa, the Middle East, and Asia (228). An additional long term single institution analysis (229) showed again that in newly diagnosed CML patients treated with Imatinib, survival is indistinguishable from that of the general population. The results of these studies are extremely important. In fact the recent availability of 2nd and 3rd generation TKI for both first or second line CML treatment renders the clinical management of this disease more intricate. Despite the positive results obtained with Imatinib treatment, resistance to this drug

can occur. Resistance can be divided in two categories: primary resistance is the failure to achieve any of the landmark responses established by the European LeukemiaNet (ELN) or National Comprehensive Cancer Network (NCCN) guidelines, while secondary resistance define patients who have previously achieved and subsequently lost their response in accordance with those guidelines (230).

Imatinib resistance can be multi-factorial; however at the time of relapse, most of the patients show the reappearance of a fully active BCR/ABL protein, mainly due to the presence of point mutations occurring in the BCR/ABL gene (231-240). In particular, inside the Kinase domain of the BCR/ABL protein, these mutations can cause amino acid substitutions, consequently hindering Imatinib binding and resulting in a loss of sensitivity to the drug by a direct or indirect mechanism. Based on the regions where the mutations are located, they can interrupt the critical contact points between the BCR/ABL protein and the drug or it may induce conformational change, resulting in the impairment of binding of imatinib to the protein.

The patients who failed or were intolerant to imatinib emphasized the need of additional options. Presently there are four additional TKI available drugs for CML treatment: dasatinib, nilotinib, bosutinib and ponatinib, with dasatinib and nilotinib already approved for first-line CML treatment (241, 242).

Dasatinib (Sprycel, Bristol-Myers Squibb) is a highly potent second generation *BCR/ABL* kinase inhibitor; it is also known to inhibit the Src family of kinases, which might be essential in crippling the critical cell signaling pathways (243). Src kinase inhibition by dasatinib might be advantageous in imatinib resistance disease, where dasatinib has been shown to directly inhibit 21 out of 22 *BCR/ABL* imatinib resistant mutant forms (244, 245). In addition dasatinib also inhibits c-KIT, platelet-derived growth factor receptor and EphA2 (246).

Nilotinib (Tasigna, Novartis Pharmaceutical Corporation, NJ, USA) is another second generation TKI. It is a structural analog of imatinib; however its affinity for the ATP binding

site on *BCR/ABL* is up to 50 times more potent in vitro (247). Interaction between the drug and kinase binding site has been improved (compared with imatinib) by the inclusion of alternative binding group to the N-methyl-piperazine moiety (248). Like Dasatinib, Nilotinib is advantageous on the imatinib resistance diseases.

Bosutinib (SKI-606; Wyeth) is a dual inhibitor of Src/Abl tyrosine kinase and has shown a potent activity against chronic myeloid leukemia (CML). In contrary to dasatinib, bosutinib does not inhibit PDGFR or KIT. In addition, after treatment with bosutinib (BOS) the phosphorylation of cellular proteins, including STAT5 is inhibited and phosphorylation of the autoactivation site of the Src-family kinases LYN and /or HCK is also reduced (249). The phase I/II clinical trials has evaluated the efficacy and safety of BOS at well tolerated doses in imatinib-resistant CML or acute lymphoblastic leukemia patients (250).

Ponatinib is a potent TKI of both unmutated and mutated *BCR/ABL*. Phase I/II clinical trials has demonstrated that ponatinib has significant efficacy in CML intolerant or resistant to dasatinib and/or nilotinib and more interestingly, it showed activity also against the T315I mutation, which causes resistance to all the other TKIs (251, 252).

Accordingly to Carlo Gambacorti Passerini and Piazza R, imatinib represents the best choice for first-line treatment of CP-CML, thanks to its therapeutic activity, safety profile and availability of independent and long-term studies on treated patients (253).

DCC-2036 is a multiple tyrosine kinases inhibitors that bind to the “switch pocket” that guide the shift between the active and inactive states of the ABL kinases and can act against the T315I mutation (254). Phase I clinical trial for this drug is going to finish.

Recently, Redaelli and Mologni et al (255) compared the activity profile of DCC-2036 and Ponatinib to the other TKI in a panel of 24 clinically relevant BCR/ABL mutants. Their results showed that the availability of several drugs and of their different activity profiles against specific mutations may become a useful tool for clinicians for the specific and effective treatment of drug-resistant CML patients.

AIM

1) In the first part of the project I investigated the molecular mechanisms associated with the progression of Chronic Myeloid Leukemia (CML) to blast crisis (BC), the advanced phase of this disease. Until now the molecular events responsible for the evolution to BC are poorly understood and the prognosis for these patients remains unfavorable. My aim was to identify the main genetic alterations associated with CML progression, thus defining new potential targets to improve the therapeutic intervention for this tumor.

The genomic DNA obtained from peripheral blood or bone marrow of 11 BC patients and the corresponding chronic phase (CP) samples were analyzed through a Whole Exome Sequencing (WES) methodology. Through this approach I was able to identify mutations specific of BC, discarding mutations present also in the corresponding CP sample. In particular I highlighted the presence of BC specific and recurrent alterations (found in more than one patient). The frequency of such mutations were also analyzed in a larger cohort of chronic/blast phases samples in order to identify their frequency and specificity in the blast phases populations. Thanks to specific software developed in our laboratory (Comparative Exome Quantification analyzer or CEQer) (256), WES data were also used for the identification of acquired Copy Number Alterations (CNA) during blast crisis evolution.

This project, based on the availability of very informative samples (matched CP/BC) and on the accessibility to High Throughput Sequencing Technologies, helped in clarifying the main molecular mechanisms associated to CML progression.

2) The second part of this thesis project was focused on the identification of transcription factors (TFs) specifically binding on BCR promoter and which may play a significant role in regulating the expression of BCR and BCR/ABL in CML cellular models. In fact ,after the oncogenic translocation t(9;22), the BCR promoter drives the production of the BCR/ABL mRNAs. Despite of all the research performed in this field, the molecular mechanisms involved in the regulation of BCR promoter are mostly unknown.

First of all *in silico* analysis was performed to identify putative binding sites of transcription factors on BCR promoter through Jasper database (http://jaspar.genereg.net/), and TRANSFAC Biobase (http://www.biobase-international.com/product/transcription-factor-binding-sites). Chromatin Immunoprecipitation assays were used to confirm *in silico* data. To determine the role of selected transcription factors on BCR and BCR/ABL expression, the BCR/ABL +ve CML cell lines were a) transfected with eukaryotic expression vectors for the specific TFs or b) the specific transcription factors were silenced by short hairpin RNA (shRNA). The mRNA and protein expression level of BCR and BCR-ABL were detected by real time PCR and Western blotting. BCR promoter activity was also assessed by luciferase reporter assay. The role of the identified transcription factor in tumoral progression were investigated through in-vitro assays.

2. Materials and Methods

2.1 Patient samples: Peripheral blood and Bone marrow of eleven matched CP and BC CML samples were collected at diagnosis after informed consent. All samples showed the BCR/ABL fusion gene. Leukemic myeloid cells were obtained by separation on a Ficoll-Plaque Plus (GE Healthcare, UK) gradient from BM or from the buffy coat fraction of PB samples and by the lysis of red cells with 5 volumes of lysis buffer (155mM NH4Cl, 10mM KHCO3 and 0.1mM EDTA). Myeloid cells were assessed using surface markers (CD33, CD13, or CD117) with fluorescence-activated cell sorting (FACS) analysis. Only samples with a population of myeloid cells >80% were further evaluated.

2.2 Whole-Exome Sequencing (WES): All the exome libraries were generated starting from 1µg of genomic DNA (gDNA) extracted with PureLinkTM Genomic DNA Kit (Invitrogen, Life technology, Grand Island, NY). Only pure and non-degraded gDNA (A260/280 ratio between 1.8 and 2.0 and A260/230 ratio major than 2.0) was used. gDNA was fragmented to a size of 500-100bp using Bandelin Sonopuls sonicator (Cycles: 50; Processing time: 10sec; Pulsation: 20%; Power (amplitude): 10 %;) and then processed according to standard Illumina TruSeqTM DNA Sample Preparation Kit (Catalog #: FC-121-1001) protocol, with selection on 2% agarose gel of a 200-300bp fragment. Multiplexed genomic libraries were then pooled and enriched for exome sequences using two rounds of hybridizations with capture probes of target regions provided in the Illumina TruSeqTM Exome Enrichment Kit (Catalog #: FC-121-1008). The libraries were subsequently sequenced on an Illumina Genome Analyzer IIx with paired-end reads 76bp long using Illumina TruSeqTM SBS kit v5 (Catalog #: FC-104-5001).

2.3 WES data analysis: Image analysis and base calling were performed using the Illumina Real Time Analysis Software RTA v1.9.35. The binary bcl files were converted to qseq by using the Off-Line Basecaller OLB v1.9.0. Qseq files were deindexed and converted

in the Sanger-FastQ file format using in-house scripts. FastQ sequences were aligned to the human genome database (GRCh37/hg19) using the Burrows–Wheeler-based BWA alignment tool (257) within the Galaxy framework (258-260). The alignment files in the SAM format were analysed by SAM tools (http://samtools.sourceforge.net/, (261)). Uniquely mapped reads, with a mapping quality higher than 30 were generated and mapped in proper pair were accepted for the downstream analysis. Duplicated paired-ends reads were excluded from the analysis, the results were then converted in the Pileup format. The generated Pileup data from matched samples (CP and BC), were cross-matched between the BC and CP exomes was performed with dedicated in-house C# software. The percentage of reads matching the reference human genome was over 90%, with mean exon coverage of over 60-fold and a percentage of exons with a mean coverage ≥ 20x of over 70% for both the CP and BC samples.

2.4 Comparative Exome Quantification Analysis: WES data were analyzed with Comparative Exome Quantification analyzer (CEQer) software, a new graphical, event-driven tool for copy number alterations/allelic imbalance coupled analysis from WES data (256). By using case-control matched exome data, CEQer performs a comparative digital exonic quantification in order to generate copy number alterations (CNA) data and couples this information with exome-wide Loss of Heterozygosity (LOH) and allelic imbalance (AI) detection. These data are used to build mixed statistical/heuristic models allowing the identification of CNA/AI events.

2.5 Validation of mutations: To validate the somatic point mutations identified by whole-exome sequencing, two primers, upstream and downstream of the mutation, were designed using Vector NTI software (as reported in the Table 3 and 4), and used in a polymerase chain reaction (PCR) (FastStart High Fidelity PCR System, Roche Applied

Science, Mannheim, Germany) to amplify a region of 200–500 bp. These amplicons were then sequenced by Sanger Sequencing (Eurofins Genomics, Ebersberg, Germany) and the presence of the mutation was identified using Chromas 2 Software.

Table 3: PCR and sequencing primers used to validate the somatic mutations identified by whole exome sequencing.

GENE	FORWARD PRIMERS (5'--3')	REVERSE PRIMERS (5'--3')
KCNH3	CAGGTGTCCAGGCAAGAGTG	CTGCTCTCGATCTCCCGCTGGC
	CCGTACCACATTCGTGTCCAAG	GAGCCTCCAGTACCAATCTCAG
RTP2	TCCAGTGAAAGAATCATTGCCTATC	GCTGGCACCTGGCAGTCTG
	GGCAGGACTGAGGAAGGAGAAC	GCTGGAGGAGGAGGTGACCAC
FAT4	CATTGGCACAAACGTGATATC	ATGTAACTCTCTGTTAGCCTTTGAC
FUT3	CTAGCAGGCAAGTCTTCTGGAGG	CCCAGCAGAAGCAACTACGAGAG
RUNX1	GCTTTGAGTAGCGAGAGTATTGAC	GGTAACTTGTGCTGAAGGGCTG
SMARCA4	CCCGCAGATCCGTTGGAAGTAC	GCCTACAGCACGCTACAGCCTCTAG
	CTCAACACGCACTATGTGGCACC	GCAGACTCTCACCAGGCACCCAG
UBE2A	CATGCGGGACTTCAAGAGGTTGC	CAATCACGCCAGCTTTGTTCTAC
PTPN11	GGTGATTTGTTGGCAAGTGAGGG	GCATGGCAGTTCTTCAATCTGGCAG
	GTTTCCTTCGTAGGTGTTGACTGCG	GTTGTCTATCAGAGCCTGTCCTCC
FAM123C	CGAGAGGAAGAGACACGAGGTCAC	CACGGAGGTGACACTCTGGATGC
	CACGGAGGTGACACTCTGGATGC	CTCTGGTCCTGTGTGCTGGCTGAG
LAMA2	GTAGTACCCGAATATAAGGTGTTACAG	CTTCATCATCTTTCCTACAAGTAAACTG
	CAGCTCCTTTCTGAAATACATTGTAC	GGTTCACTCACCTTACAACCAGTG
GRIN3A	GACTTGTCCTTTGATACTCCTCCAG	CCATGACACCCTAGCAGGTAGTCTG
	GTTCCGAGATACATTTAGGGAGTCTG	CAGATGAAGACAGCACCTTGAGAGC
NRAS	CCT AGA TTCTCA ATG TCA ACAACC	ACTGGGTACTTA ATC TGTAGCCTCC
	GCTCTATCTTCCCTAGTGTGGTAAC	GGGACA AACCAG ATA GGCAGAAATG
DEFB119	CCTGACTCAATAGCCTCTCCTGC	GCTAGGAAGACAGAAGGGTGAG
	GGAGCTGTTGTGGAC ATCTGAGG	GGA CTGGGTCTGGCATTACTC
IKZF1	GATCAA ATTGACCCAGCCAGTG	GTGAGACTTCTGTGTGTGTATGTGC
	CTTTCTCGTAGCATCGTCCTCATG	GTTAGCCAGCAAGGACACAATC
C9orf98	CCTGTCATTAAATGCTTTCCTGTG	GATGGAGCACGGGAAGTAGCACC
	CTTATGCCTCTGGTCATCTCTGG	GCTGGTCTGTGTCCTGAGCTGC
CASK	GGTATTACTTCAGTTGTTGATGTTGC	GAAACTGGA ATAATGAACATTCTAGTC
	GTGTTCAGACAAAGTGTA GAAGGTG	CAA CAGAAGAATTGCATTTGTCC
PCLO	CATGGCATGTTGGAAGTCATCC	GGGATCTTC TCTTG CACAGGAC
	CTTCAGGCA ATGAGTGGCTTGATC	CTCCAGACCTTCCTCTGTCTATGG
PPT1	CTCTTCCTATGTCTCCAGCAATG	CCTCAGGTAGTCCACCCACCTC
MDH1B	CCAGATACTCAGAATGTTCCTAGAGG	GAGACCCAAGACCTGGCATCTC
	CAGTGTTCTTCCTAG CTGCTTTG	CAAGGAGGTGTTCACTCTGGAG
GPR98	GTTCTCCACAGGGCTGCCTCC	GAGTCACTATGTTCCAGGTACAGTG
CEL	CCAGCA ACCAACGTGACCTAG	CCAGGATAA AGAACGGAAATGTGG
LRP4	GACACAACCTCCTCC ACGTTGC	GCCACTCTTCTGGTACTGATGC
CYP2B6	GACTCAGAGCCTTCTTCCAACTTC	CTCCAGTTTCGTCTGTCTCTGTC
BCR	GAGCAGGTGGGAGGGAGCAG	CACAGGGCTGACGCAACGAAC

Table 4: The primers used for DNA amplification of UBE2A.

GENE	FORWARD PRIMERS (5'--3')	REVERSE PRIMERS (5'--3')
UBE2A_g1	CTCTCTCTGCTCTCAGGTTGGTTC	ATTCCACTCAAGCCTTTAGCAG
UBE2A_g2	CTTTCCTCTCTACCCTGTATCTTTG	TCTAGGACAAGACAGCCACAGAC
UBE2A_g3	CTGATTTCCTGGATAATAGGGCAGC	AAGGAAGATGGAAACAGCACAACAG

2.6 Cell Lines: The BCR/ABL +ve CML cell lines K562, LAMA-84 and KCL22 were cultured in RPMI-1640 medium supplemented with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin G, 80 µg/ml gentamicin and 20 mM HEPES in a 5% CO_2 incubator at 37°C.

The 293T human embryonic kidney cell line was maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin G, 80 µg/ml gentamicin and 20 mM HEPES.

2.7 *In silico* analysis: To establish the transcription factor involved in the regulation of BCR expression, *in silico* analysis of BCR promoter was done using Jasper database, available on http://jaspar.genereg.net/ and Patch™ tool of TRANSFAC® database which is a product of BIOBASE Biological (262, 263).

2.8 Chromatin Immunoprecipitation (ChIP): K562 cells were crosslinked with 0.4% formaldehyde (Sigma, St. Louis, MO, USA) for 10 min at room temperature and the reaction was stopped by adding glycine to final concentration of 0.2M, centrifuged at 1500rpm for 5min and the supernatant was discarded. Fixed cells were rinsed twice with ice cold PBS containing protease inhibitor cocktail (PMSF, Aprotinin, Pepstatin A - Sigma-Aldrich Corp. St. Louis, MO USA) and nuclear pellet was obtained by centrifugation. Obtained nuclear pellets were then resuspended in 300µl SDS Lysis buffer (10mM Tris-HCl pH 7.4,

100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 0.1% SDS, 1% Triton X-100, 10% Glycerol) containing protease inhibitor cocktail. Lysate were incubated on ice for 10 minutes and then sonicated using Bandelin Sonopuls sonicator (fragment size of chromatin between 200-1000bp and confirmed by standard 1.5% agarose gel analysis) and centrifuged at 13000 rpm for 10 min. The sonicated chromatin was diluted 10 times in ChIP Dilution Buffer (1% SDS, 0.1M NaHCO3) containing protease inhibitors. 50 µL of each samples were stored at -20°C and used as the INPUT. The sonicated chromatin was pre-cleared for 1 hour, rotating at 4°C, with 80µL of Salmon Sperm DNA/ProteinA agarose (Upstate, Lake Placid, USA), before the overnight incubation with 4 µg of α-COUP-TF1 (SC-30180X, Santa Cruz Biotechnology,INC) or α-CTCF (ab70303, abcam, Cambridge, UK) or α-MYC (SC-764X,Santa Cruz Biotechnology,INC) or α-MAX (SC-197X,Santa Cruz Biotechnology,INC) antibodies. The bound material was recovered after 2 hours incubation, rotating at 4°C, with 60 µL of Salmon Sperm DNA/ProteinA agarose. The agarose beads bound chromatin was washed, for 5 minutes, once in Low Salt Buffer (pH 8.1) (0.1% SDS, 1% Triton X-100, 2mM EDTA, 10mM Tris-HCl, 150 mM NaCl), once in High Salt Buffer (pH 8.1) (0.1% SDS, 1% Triton X-100, 2mM EDTA, 10mM Tris-HCl, 500 mM NaCl), once in LiCl Buffer (pH 8.1) (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl) and once in TE (pH 8)(10mM Tris-HCl, 1mM EDTA). ChIPed material was eluted by two 15 minute incubations at room temperature with 250 µl Elution Buffer (SDS 1%, 0.1 M NaHCO3). Chromatin was reverse-crosslinked by adding 20 µl of NaCl 5M and incubated at 65°C for 4 hours minimum. Purification of DNA was done by using a DNA purification kit (QIAquick PCR Purification Kit, QIAGEN). Primers for the Q-PCR analysis of ChIP samples are as follows.

Primers used for COUP-TF1, CTCF, MYC and MAX binding (region between -1424 bp to -1199 bp from ATG codon) on the BCR promoter

BCR-FOR	5'-GGGAAGTTCTGAGTCAGGTCG-3'
BCR-REV	5'-TGAGTAACCAATGCCAGCACCC-3'

Primers for MYC and MAX binding at PBS 3 and 4

2BCR-MYCMAX-FOR	5'-GAGGACTGCTGCGAGTTCTGCC-3'
2BCR-MYCMAX-REV	5'- GACTCCCTGGTCCATAAAGACC-3'

Positive control primer for COUP-TF1 (264)

mFabp7ChIP FOR	5'-CTAACCCAGTGGCCTGAGCA-3'
mFabp7 ChIP REV	5'-TGGGCAGATGGCTCCAAT-3'

Positive control primer for CTCF (265)

CTCF positive FOR	5′-GGGGTACCCCTGGCGTTGCCGCTCTGAATGC-3′
CTCF positive REV	5′-CCGCTCGAGCGGAGGGACTGAGCTGGACAACCAC-3′

Positive control primer for MYC (266)

NPM1_MycFOR	5'-CTCGTGAGCCAGGGATGCT-3'
NPM1_MycREV	5'-CCCTAGTGCTACCAGCCTCTTAAC-3'

Positive control primer for MAX (267)

MARS-MAXFOR	5'-AAGTGCGACTTGCCCTAAAA-3'
MARS-MAXREV	5'-CCATGCAGCTGGGACTACA-3'

2.9 Real-time Quantitative PCR: Total RNA was extracted by Trizol reagent following standard procedures (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 1 µg of total RNA, using Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). The total RNA obtained from transfectant cells were pretreated with DnaseI (Life technologies, Carlsbad, CA, USA) to avoid contamination from genomic DNA. QPCR was performed using TaqMan® Brilliant II QPCR Master Mix (Agilent technologies, La Jolla, CA, USA) for TaqMan assays or with Brilliant III Ultra-fast SYBR Green QPCR Master Mix (Agilent technologies, La Jolla, CA, USA) for SYBR Green real-time PCR on a Stratagene-MX3005P (Stratagene, La Jolla, CA, USA) under standard condition. All the QPCR experiments were performed in triplicate. The housekeeping gene β-glucronidase (GUSB) was used as an internal reference. For MYC and MAX expression analysis we used

TaqMan® Gene Expression Assays from Life technologies consisting of a pair of unlabeled PCR primers and a TaqMan® probe. TaqMan PCR was performed according to the manufacturer's specifications. The assay identification numbers were as follows: **MYC**-Hs00153408_m1, **MAX**-Hs00811069_g1 and **MAX**-Hs01105524_g1. For BCR, BCR/ABL and GUSB the sequences of primers and probes were as follows:

Primers Sequences for TaqMan Q-PCR

BCR

BCR taqman for	5'TTCTCCCTGACATCCGTGG3'
BCR taqman Rev	5'ACCTCCAGGGTGCAGTACAGAT3'
BCR probe	5'FAM-TGAAACTCCAGACTGTCCACAGCATTC3-TAMRA3'

BCRABL

BCR/ABL taqman for	5'TCCGCTGACCATCAATAAGGA3'
BCR/ABL taqman Rev	5'CACTCAGACCCTGAGGCTCAA3'
BCR/ABL probe	5'FAM-CCCTTCAGCGGCCAGTAGCATCTGA-TAMRA3'

GUS

GUS taqman for	5'GAAAATATGTGGTTGGAGAGCTCATT3'
GUS taqman Rev	5'CCGAGTGAAGATCCCCTTTTTA3'
GUS probe	5'FAM-CCAGCACTCTCGTCGGTGACTGTTCA-TAMRA3'

2.10 Antibodies and Western Blot Analysis:

Western blotting was performed using the following antibodies**:** rabbit anti-MAX (SC-197X) (Santa Cruz Biotechnology, CA, USA), mouse anti c-Myc (SC-40) (Santa Cruz Biotechnology, CA, USA), rabbit anti-c-Abl (K-12) (Santa Cruz Biotechnology, CA, USA), rabbit anti PARP-1 (E102) (abcam, Cambridge, UK) and anti-actin (Sigma-Aldrich, St Louis, MO). The cells were harvested, washed in PBS at 4°C, resuspended in lysis buffer (0.025 M Tris (pH 8.0), 0.15 M NaCL, 1% NP-40, 0.01 M NaF, 1mM EDTA, 1mM DTT, 1mM sodium orthovanadate and protease inhibitors) and incubated on ice for 30 minutes. Lysates were then clarified by centrifugation at 13,000 × g for 15 minutes at 4°C. The protein concentration of cell lysates was determined using the

Bradford protein assay following standard procedures (Bio-Rad Laboratories Hercules, CA, USA). Equal amount of total protein lysates were gel separated by SDS-polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Palm Springs, CA) and the gel were blotted onto Hybond ECL nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden) following a standard protocol and probed with selected antibodies. Proteins were visualized by chemiluminescence (Thermo Scientific, Rockford, IL, USA) with Image Station 440 CF (Eastman Kodak Company).

2.11 Site-direct mutagenesis:

Mutation in BCR promoter at the MYC::MAX binding site was introduced by the following protocol. Specific primers were designed and used to mutagenize the starting template of the recombinant plasmid pGL3/BCR promoter (Full BCR promoter) longest form and 1200 bp BCR promoter with the Pfu Ultra High Fidelity enzyme (Agilent, Santa Clara, CA, USA). The product was digested with DpnI (Roche, Indianapolis, IN, USA) and 2 µl was used to transform the competent TOP10 bacterial strain (Life Technology, Monza, MB, Italy). The presence of the deletion was subsequently confirmed by Sanger sequencing.

Primers Sequences for mutagenesis:

MYCMAX-34-FOR-D 5'GAGGTAGGTGGTGGGGCTTGGCTGTTCCAGGACTGCAGGACTG3'
MYCMAX-34-REV-D 5'CAGTCCTGCAGTCCTGGAACAGCCAAGCCCCACCACCTACCTC3'

2.12 Lentivirus-mediated Short hairpin RNA (shRNA) against MYC:

Lentiviruses were packaged by co-transfection with the packaging plasmids pCMV-dR8.91, vesicular stomatitis virus glycoprotein (VSVG) and MYC-shRNA (268) (kind gifts from Dr. Robert N Eisenman) FUGW-H1-Scrambled control shRNA (FUGW-H1, plasmid 40625 from Addgene, Cambridge, MA, USA) using jetPRIME Polyplus (Polyplus-transfection S.A, New-York, NY, USA) into 100mm plates 293FT cells (derived from human embryonic kidney cells transformed with the SV40 large T antigen). Transfected cells were cultured in D-MEM

medium containing 10% FBS supplemented with 0.1 mM MEM Non-Essential Amino Acids, 1 mM sodium pyruvate and 2 mM L- glutamine. At 48 h post-transfection, medium containing viruses was collected and filtered through a 0.45 µm filter.

To generate MYC silenced K562, LAMA-84 and KCL22S cells with lentiviruses, we transduced 5* 10^4 cells by spin infection in lentviral supernatants supplemented with 4 µg/ml polybrene (Sigma-Aldrich) and 20% RPMI 1640. After 48 h, the lentiviruses-infected cells (MYC-shRNA and non-targeted-shRNA control) were resuspended in complete medium. Lentiviruses-infected cells expressing MYC-shRNA and non-targeted-shRNA control fused with GFP were sorted using a FACSAria flow cytometer (BD Bioscience, San Jose, CA, USA).

2.13 MAX cloning and Transfection: Two primers spanning the whole MAX cDNA (NM_145112.2) and introducing artificial HindIII and EcoRI sites at the 5' and 3' ends of the coding region (respectively) were used for amplification. The amplicon was cloned into the pCDNA3 basic vector.

MAX FOR	5'AATAAAGCTTGAAATGAGCGATAACGATGAC3'
MAX REV	3'AATAGAATTCCCCGAGTGGCTTAGCTGGCCT5'

K562 cells were suspended in RPMI 1640 without FBS and antibiotics, at a concentration of 10^7 cells per ml. A volume of 0.450 ml was transferred to a sterile electroporation cuvette (Bio-Rad Gene Pulser cuvette, 0.4 cm), and incubated on ice for 2 minutes in presence of 10µg of plasmid (pWZL Blast myc, plasmid 10674 from addgene, pCDNA3 max vector and pCDNA3 empty vector). Then cells were electroporated (270 V, 975 µF) using a GenePulser XCell (Bio-Rad, Hercules, CA). After receiving the electric pulse cells were kept on ice for 2 minutes and then transferred in RPMI 1640 with FBS (10%). Transfected cells were selected with antibiotics (K562MYC and K562MYC::MAX – 5 µg/ml blasticidin) (InvivoGen, San Diego, California, USA) (K562MAX and K562Empty **–** 1 mg/ml geneticin) (Life technologies, Carlsbad, CA, USA).

2.14 Luciferase assays: HEK-293 cells were infected with the lentviral vector encoding either MYC-shRNA or the control shRNA. After confirmation the silencing of MYC by QPCR and Western blotting these cells were then co-transfected with Renilla and pGL3 luciferase vectors along with the indicated various BCR promoters constructs. Luciferase activity of cell lysates was determined luminometrically (after 72 hours of incubation) using the 1450 Wallac MicroBeta®luminometer (PerkinElmers, Waltham, Massachusetts, USA) by the dual luciferase reporter assay system (Promega, Madison, Wisconsin, USA) as specified by the manufacturer. The experiment was performed in triplicates.

2.15 Cell viability assay: We monitored growth of shRNA-infected (shRNA MYC and shRNA scrambled control) LAMA-84 and KCL22 cells over a 4-day period using CellTiter 96 AQ One Solution Cell Proliferation Assay Kit (Promega, Madison, WI 53711 USA). Cells (5,000 per well) were seeded in 96-well plates. Briefly, 20 µl of MTS reagent was added directly to the wells and cell plates were incubated at 37°C for a minimum of 1 hour. Absorbance was measured at 490 nm on a Model 680 Microplate Reader (Bio-Rad Laboratories Ltd; Hemel Hempstead, Herts, UK). Background absorbance was subtracted using a set of wells containing only the medium.

2.16 Statistical analysis: All the statistical analyses (unpaired two-tailed T-test) were performed by the GraphPad Prism (GraphPad, CA, USA) statistical package.

3. Results

3.1 - Identification of the molecular lesions associated with CML progression

3.1.1 Whole-Exome Sequencing (WES) of CML

Next-generation sequencing (NGS) technologies have led to an exponential increase about the knowledge of the genetic causes in both extremely rare diseases and common human disorders. Exome sequencing - the targeted sequencing of the portion of the human genome that is protein coding-has proven to be a powerful and cost-effective tool for dissecting the genetic basis of diseases which have been proved to be unintelligible using conventional gene-discovery methods (269).

To investigate the genetic changes associated with CML progression, we decided to use high throughput approach to individual patient at two different phases: CP and BC. In particular we studied the exome of 11 matched CP and BC samples using a whole-exome sequencing approach, as specified in materials and methods. Each read of a massively parallel sequencing run is clonal and therefore derives from a single molecule of genomic DNA. Hence, the portion of sequencing reads reporting an allelic variant gives a quantitative estimate of the proportion of cells in the DNA sample carrying that mutation, assuming adequate coverage of the investigated gene.

As the NGS technologies, in general, use PCR-like amplification steps in the library preparation, multiple reads originating from the same template can be sequenced and overrepresentation of certain alleles due to amplification bias introduced during library construction tends to interfere with variant calling step (the process of mismatch variant is called calling step). Therefore in order to curtail the detection of false mutations, we had exclusively considered mutations with a frequency of at least 30%. By filtering exome data using this minimum relative coverage and by comparing exome-sequences of 11 paired CP (used as a control) and BC samples we found a total of 38 single nucleotide mutations

occurring in BC samples that were absent in the corresponding CP sample. These 38 somatic mutations identified include 28 nonsynonymous and 10 synonymous variations, as reported in Table 5. By using Vector NTI software (Invitrogen Corporation), PCR primer pairs specific for each genomic loci (as reported in the Table 3) have been designed and respective genomic loci were successfully amplified by standard PCR. The amplicons were subsequently analyzed by traditional Sanger Sequencing obtaining a validation rate (mutation percentage confirmed by Sanger sequencing) of 100%.

We identified a limited number of acquired mutations in BC when compared with the CP sample (average=4 mutations/patient). By using this approach we found recurrent, somatic, single nucleotide mutations in Runt-related transcription factor 1 (*RUNX1)* and Ubiquitin-conjugating enzyme E2A (*UBE2A*) in 2 out of 11 BC samples. No additional recurrent mutations were observed, even when lowering the accepted frequency below 30%. RUNX1 is a transcription factor that regulates the differentiation of hematopoietic stem cells into mature blood cells. RUNX1 mutations were previously identified as recurrent abnormalities in myelodysplastic/myeloproliferative neoplasms, including promoting acute myeloid leukemia transformation in CML (270, 271). The second recurring mutation affected *UBE2A*. The same mutation was identified in patient #3(D114V) and #10(I33M) with mutation frequencies (obtained by analyzing exome data) of 93% and 39% respectively.

UBE2A is an X -chromosomal gene. *UBE2A* mutation was homozygous in patient #3(D114V) (male), but in patient #10(I33M) (female) the same mutation was heterozygous.

Out of the 38 variants identified, 23 ranked ≥1 in the Gene Ranker cancer scoring system (http://cbio.mskcc.org/tcga-generanker). Genes with a score >1 are considered oncogenic and include *FAT4, FUT3, SMARCA4, UBE2A, ABL1, PTPN11, LAMA2, NRAS, IKZF1, CASK, PPT1, CASK, PPT1, CEL, CYP2B6, BCR, EPB41L3, FGFR4, BARD1, BSN, KRT7, ROBO2* and *XPO1*. When comparing the average Gene Ranker score in the BC population, its value (9.85±6.34) (P value = 0.0188) is significantly higher than that of mutations identified in a group of 22 CP patients (3.4±3.17).

Table 5: List of somatic mutations identified in 11 CML-BC patients by whole exome sequencing analysis. * signify a stop codon. “Fraction” indicates the portion of total reads that were mutated. The “absolute coverage” column indicates the coverage obtained for the nucleotide where the mutation was identified. Due to poor quality and insufficient sample for patient **#5 and #8 we did not identified any mutation.**

GENE	GENE DESCRIPTION	MUTATION	LOCUS	MUTATION FRACTION (%)	ABSOLUTE COVERAGE	MUTATION FRACTION IN CONTROL (%)	ABSOLUTE COVERAGE IN CONTROL	PATIENT#
KCNH3	Potassium voltage-gated channel subfamily H member 3	Ala314Val	12,49937814-49937815,1,C/T	54.90	51	0	40	1
RTP2	Receptor-transporting protein 2	Ala190Val	3,187416394-187416395,1,G/A	41.38	116	0	101	1
FAT4	Protocadherin Fat 4	Arg169Trp	4,126242657-126242658,1,C/T	53.25	77	0	42	2
FUT3	fucosyltransferase 3	Arg354Cys	19,5843790-5843791,1,G/A	38.36	146	0	38	2
RUNX1	Runt-related transcription factor 1	Lys194Asn	21,36231801-36231802,1,T/A	67.50	120	0	46	2
SMARCA4	ATP-dependent helicase SMARCA4	Ala945Thr	19,11132616-11132617,1,G/A	55.0	20	0	23	3
UBE2A	Ubiquitin-conjugating enzyme E2 A	Asp84Val	X,118717099-118717100,1,A/T	93.3	30	0	30	3

ABL1	Proto-oncogene tyrosine-protein kinase ABL1	Phe486Ser	9,133755487-133755488,1,T/C	50.0	64	0	93	3
PTPN11	Tyrosine-protein phosphatase non-receptor type 11	Gly503Val	12,112926887-112926888,1,G/T	32.88	73	0	92	4
FAM123C	Protein FAM123C	Arg709His	2,131521770-131521771,1,G/A	40.58	69	3	69	4
LAMA2	Laminin subunit alpha-2 Precursor	Pro1025Ser	6,129621915-129621916,1,C/T	44.26	61	0	55	4
GRIN3A	Glutamate [NMDA] receptor subunit 3A Precursor	Arg1024*	9,104335733-104335734,1,G/A	39.29	84	0	82	4
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5
NRAS	GTPase NRas Precursor	Gln61Arg	1,115256528-115256529,1,T/C	32.73	110	0	56	6
DEFB119	Beta-defensin 119 Precursor	Arg42His	20,29976969-29976970,1,C/T	46.97	66	3	32	6

IKZF1	DNA-binding protein Ikaros	Asn159Ser	7,50450291-50450292,1,A/G	41.38	58	0	32	6
C9orf98	Putative adenylate kinase-like protein C9orf98	Arg125His	9,135730271-135730272,1,C/T	65.22	69	4	41	6
CASK	Peripheral plasma membrane protein CASK	Lys250Arg	X,41519773-41519774,1,T/C	47.45	137	7	74	6
PCLO	Piccolo Presynaptic Cytomatrix Protein	Leu4239Trp	7,82544276-82544277,1,A/C	32.35	34	0	22	7
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8
PPT1	palmitoyl-protein thioesterase 1	Val168Ala	1,40557017-40557018,1,A/G	41.38	29	0	23	9
MDH1B	malate dehydrogenase 1B	Ala272Val	2,207619827-207619828,1,G/A	47.25	91	1	70	9
MDH1B	malate dehydrogenase 1B	Ala272Thr	2,207619828-207619829,1,C/T	47.31	93	0	71	9

GPR98	G protein-coupled receptor 98	Arg1745Cys	5,89971181-89971182,1,C/T	47.06	170	0	178	9
CEL	carboxyl ester lipase	Glu216Gln	9,135942014-135942015,1,G/C	44.26	61	0	55	9
LRP4	low density lipoprotein receptor-related protein 4	Asp449Asn	11,46916334-46916335,1,C/T	55.00	80	0	64	9
CYP2B6	cytochrome P450, family 2	Arg145Trp	19,41510299-41510300,1,C/T	39.69	194	0	134	9
BCR	breakpoint cluster region	Phe615Val	22,23610684-23610685,1,T/G	44.30	149	0	128	9
ASXL1	Putative Polycomb group protein ASXL1	Gly641_fs	20,3102243-3102244,1,-/G	37	NA	NA	NA	10
EPB41L3	Erythrocyte Membrane Protein Band 4.1-Like 3	Pro963Leu	18,539627-539628,5,G/A	37	,,	,,	,,	10
FGFR4	Fibroblast growth factor receptor 4	Val262Met	5,17651936-17651937,8,G/A	39	,,	,,	,,	10
RUNX1	Runt-related transcription factor 1	Asp171Gly	21,3623178-3623179,1,T/C	15	,,	,,	,,	10

UBE2A	Ubiquitin-conjugating enzyme E2 A	Ile33Met	X,11870890-11870891,8,A/G	39	,,	,,	,,	10
ABL1	Proto-oncogene tyrosine-protein kinase ABL1	Glu255Val	9,13373835-13373836,4,A/T	28	,,	,,	,,	11
BARD1	BRCA1-associated RING domain protein 1	Gly527_fs	2,21561725-21561726,9,C/CGGTT	28	,,	,,	,,	11
BSN	Bassoon Presynaptic Cytomatrix Protein	Arg3264His	3,4969905-4969906,9,G/A	26	,,	,,	,,	11
EFCAB4B	EF-Hand Calcium Binding Domain 4B	Val643Met	12,373659-373660,7,C/T	31	,,	,,	,,	11
KRT7	Keratin, type II cytoskeletal 7	Arg339Trp	12,5263928-5263929,8,C/T	41	,,	,,	,,	11
MUDENG	MU-2/AP1M2 domain containing	Asp289Asn	14,5774704-5774705,7,G/A	38	,,	,,	,,	11
ROBO2	Roundabout, axon guidance receptor, homolog 2	Pro1055_fs	3,7765702-7765703,7,T/TC	24	,,	,,	,,	11
XPO1	Exportin 1	Glu571Lys	2,6171946-6171947,2,C/T	32	,,	,,	,,	11

The analysis of transitions (purine-purine or pyrimidine-pyrimidine interchanges) and transversions (purine-pyrimidine interchanges) in the nonsynonymous alterations showed that transitions were present in 73.7% of the cases and transversions in 26.3%, as shown in Figure. 9.

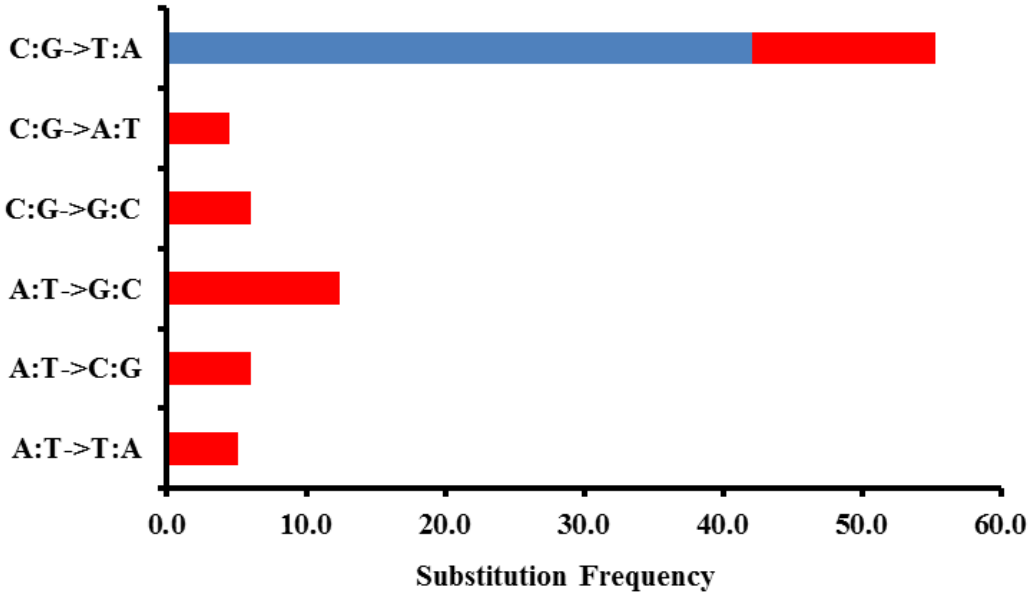

Figure 9. Distribution of substitutions in the 6 different classes of mutations. The red bars indicate the frequency of substitutions in the 11 CML samples for the six possible mutation classes. The blue bar indicates the proportion of C:G>T:A mutations occurring in the first base of a CpG dinucleotide.

3.1.2 CEQer analysis

CEQer (Comparative Exome Quantification analyzer) is an efficient and easy to use graphical, event-driven tool for copy number alterations/allelic imbalance (CNA/AI) coupled

analysis from exome sequencing data (256). This tool was developed in our laboratory by (256).

In line with previous reports, indicating that chromosomal instability increases upon progression from CP-CML to BC (272), CEQer analysis of 9 matched BC/CP exome revealed the acquisition of CNA events during BC evolution (Figure. 10, Figure. 11). Interestingly, in 3/9 cases, a large (patient#1) or complete (patient#4 and #9) deletion involving chromosome 7 was detected (Figure. 10). This was not surprising, because partial or complete single-copy loss of chromosome 7 is one of the most recurring event encountered upon progression to BC (166). In patient #1 and #9, the loss of chromosome 7 was accompanied by a partial loss of chromosome 9 involving the *CDKN2A/p16*^{Ink4a} oncosuppressor locus. In particular, in patient #1 chromosome 9 is characterized by a partial loss of a large pericentromeric region, spanning 54 Mbases and involving the CDKN2A/p16 locus. In patient #9 CEQer analysis showed the loss of a single copy of the entire short arm of chromosome 9, and the loss of two copies of a 3.5 Mbases of the region extending from MLLT3 to ELAV1 genes and comprising CDKN2A/p16. (Figure. 10). In addition to the above mentioned alterations, in patient#9 the analysis showed also the loss of part of the short arm of chromosome 19.

In patient #4 a complex set of CNA events occur in chromosome 17 (Figure. 11). Here, a large deletion spanning 19.2 Mbases and involving the vast majority of the short arm of chromosome 17 comprising the locus of the TP53 oncogene was detected. This region was followed by a copy neutral, pericentromeric region spanning 24.3 Mbases and by a 35.1 Mbases copy gain region, comprising almost the whole long arm of chromosome 17. Within the copy neutral pericentromeric region, a second, relatively short copy gain region spanning 2.0 Mbases and extending from the *WSB1* to the *CRYBA1* gene was reported. In line with CNA data, LOH/AI (loss of heterozygosity/allelic imbalance) analysis revealed a pattern of ‘copy number loss’ LOH within the deleted region, while a cluster of ‘single copy gain’ AI events was reported in the copy gain regions (Figure. 11). In patient #7 the analysis showed

two copy number losses on chromosome 12 and 16. Unfortunately, the low coverage obtained from WES data for this patient do not allow for a deeper examination of the regions involved in CNA events.

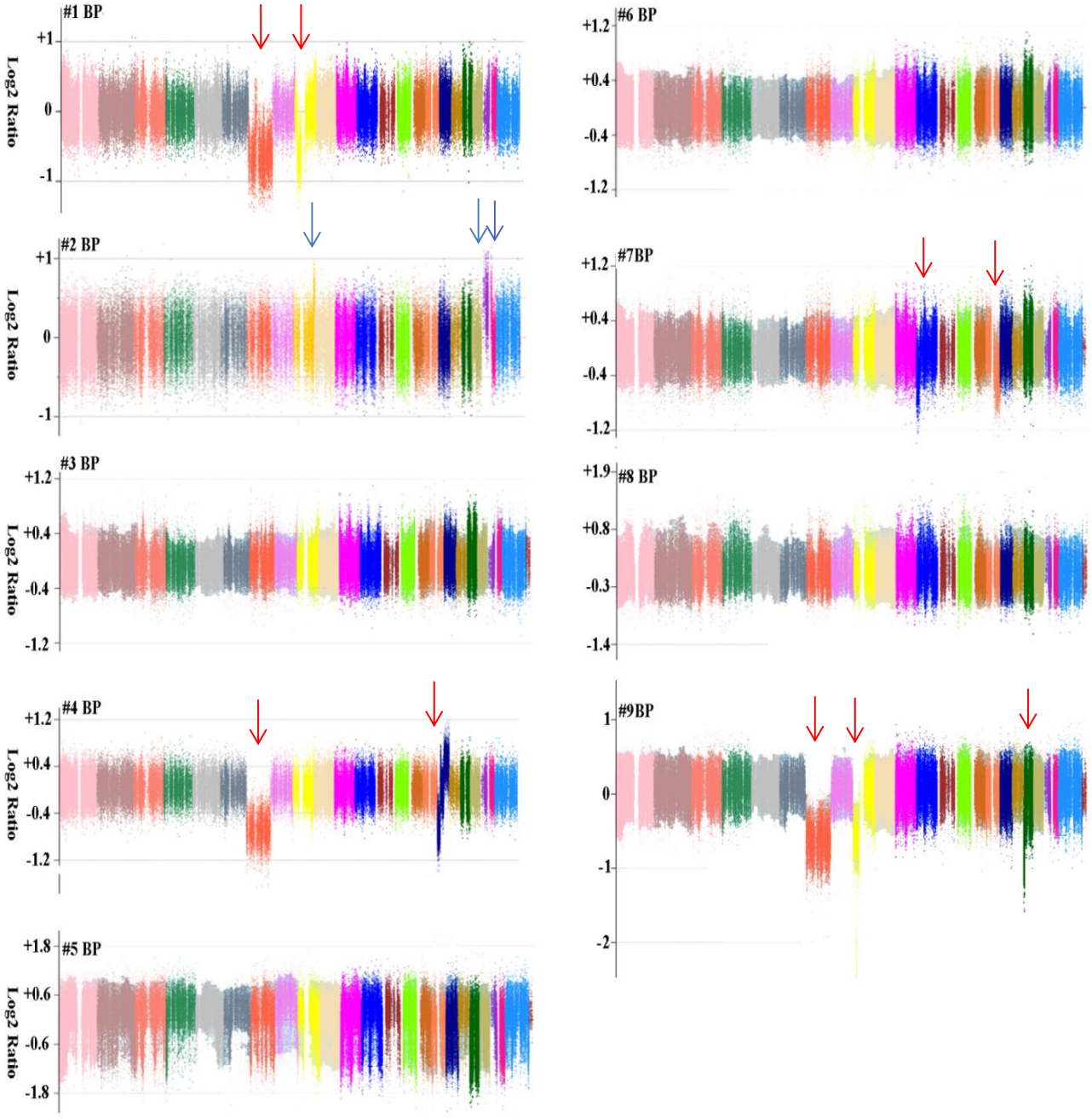

Figure 10: CNA analysis of 9 CML BC samples. Genome view of patient #1 to #9 analyzed by CEQer. Genome views of coverage data are represented as Normalized Log2 Ratios.

Individual colors represent specific chromosomes from 1 (left) to X (right). The red and blue arrows indicate the copy loss and copy gain CNA regions respectively.

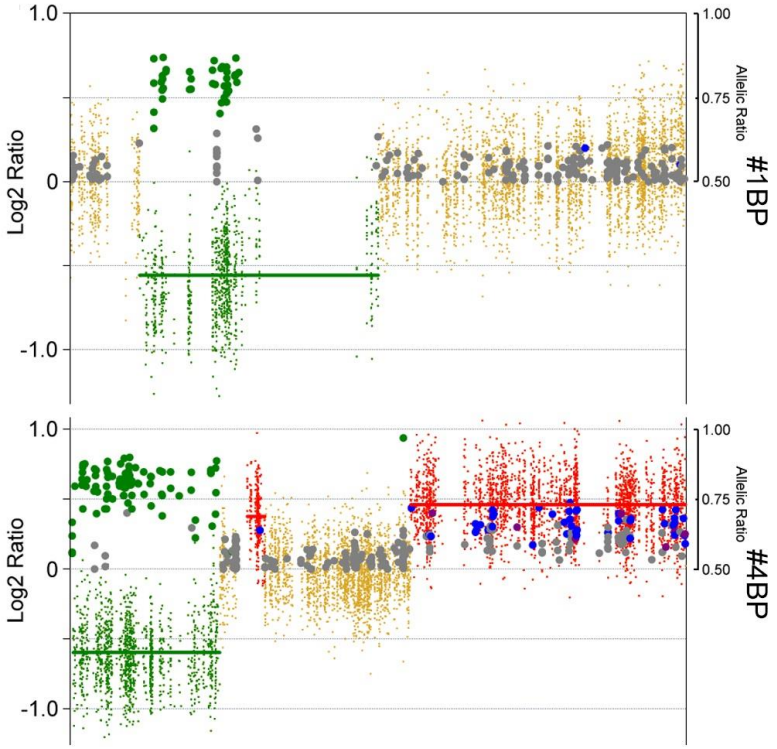

Figure 11: Individual views of patient #1-chromosome 9 and of patient#4-chromosome 17. Dark-yellow dots indicate copy neutral exons; green dots copy loss and red dots copy gain CNA regions. Thick red and green horizontal bars identify copy gain and loss regions, respectively. Large gray circles indicate heterozygous positions in the control dataset whose heterozygosity is conserved in the case. Large green, blue and dark-red circles indicate copy loss, +1 copy gain and +2 copy gain LOH/AI events, respectively. The position of LOH/AI markers on the y axis indicates the corresponding allelic ratio (256).

In patient #2, two apparent copy gains were detected, occurring in chromosome 9 and 22 (Figure. 12A). In depth analysis of these events revealed that chromosome 9 copy gain started at *ABL* exon 2 and involved the whole distal fraction of chromosome 9 long arm (Figure. 12B); chromosome 22 copy gain involved the whole short arm and pericentromeric

regions and ended at *BCR* exon 14 (Figure. 12C). Notably, chromosome 9 and 22 CNA start and end were identical to the breakpoint exons of the patient #2. *BCR/ABL* fusion and the genes involved in the copy gain abnormality perfectly superimpose with the genes present in the Philadelphia chromosome, suggesting that the two CNAs are instead a single copy gain of the whole Philadelphia chromosome comprising the *BCR/ABL* fusion oncogene. In the same patient (#2) the analysis showed also a duplication of the entire chromosome 21 (where RUNX1 is located). Interestingly, for this patient WES data showed the acquisition of a RUNX1 mutation (Lys194Asn). Deep analysis of these data indicates that the chromosome 21 which is duplicated is the one carrying the RUNX1 mutated allele.

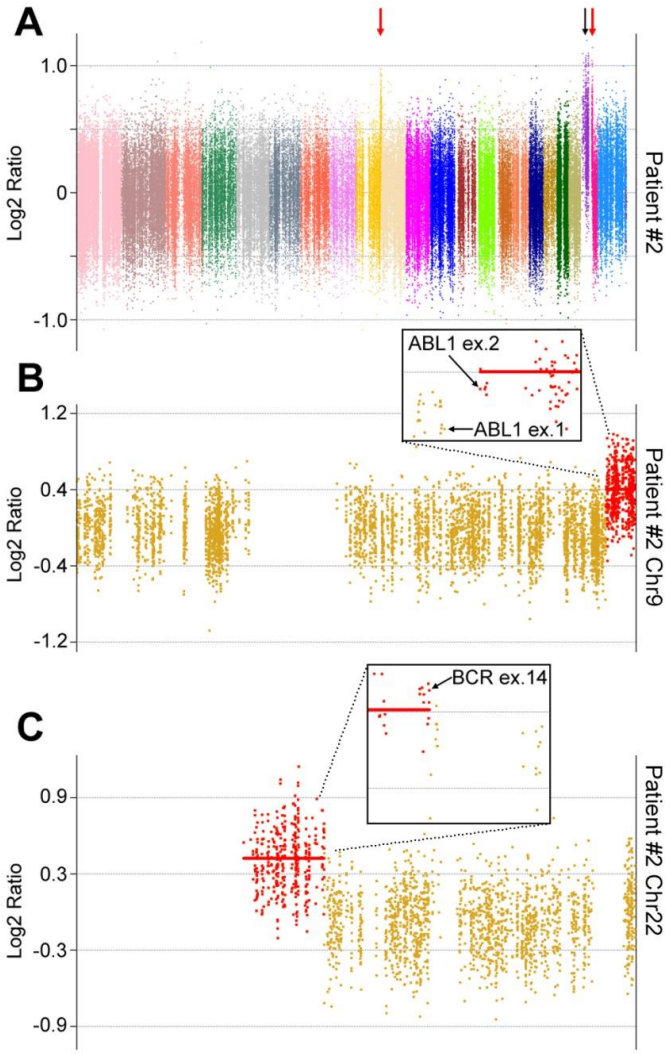

Figure 12: CNA analysis of Patient #2. **(A)** Genomic view of patient #2. Coverage data are represented as Normalized Log2 Ratios. Individual colors represent specific chromosomes from 1 (left) to Y (right). **(B)** Individual views of chromosome 9 and chromosome 22. **(C)** Dark-yellow dots indicate copy neutral exons, red dots copy gain CNA regions. Thick red horizontal bars identify copy gain regions. Boxed panels represent magnified views of the CNA boundaries: individual genes/exons delimiting the copy gain CNA region (s) are indicated (256).

3.1.3 Frequency and specificity of UBE2A mutation in CML-BC samples

The identification of novel and recurrent somatic *UBE2A* mutations in two different CML-BC cases (Figure. 13) motivated us to define the frequency of mutated *UBE2A* in a larger cohort of CP and BC CML patients through standard Sanger sequencing.

We thus amplify, through three different amplification reactions of genomic DNA, the entire *UBE2A* coding sequence (Table 3). In this new analysis, (Table 4) 1 of 11 BC (9%) and 0 of 8 CP samples have been found positive for *UBE2A* mutation. In total, we found 3 of 27 BC (11%) and 0 of 19 CP (0%) cases positive for *UBE2A* mutation.

After identification of the recurrent, somatic *UBE2A* mutation in CML patients, we focused our study on the characterization of this mutation by using COSMIC dataset (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/).

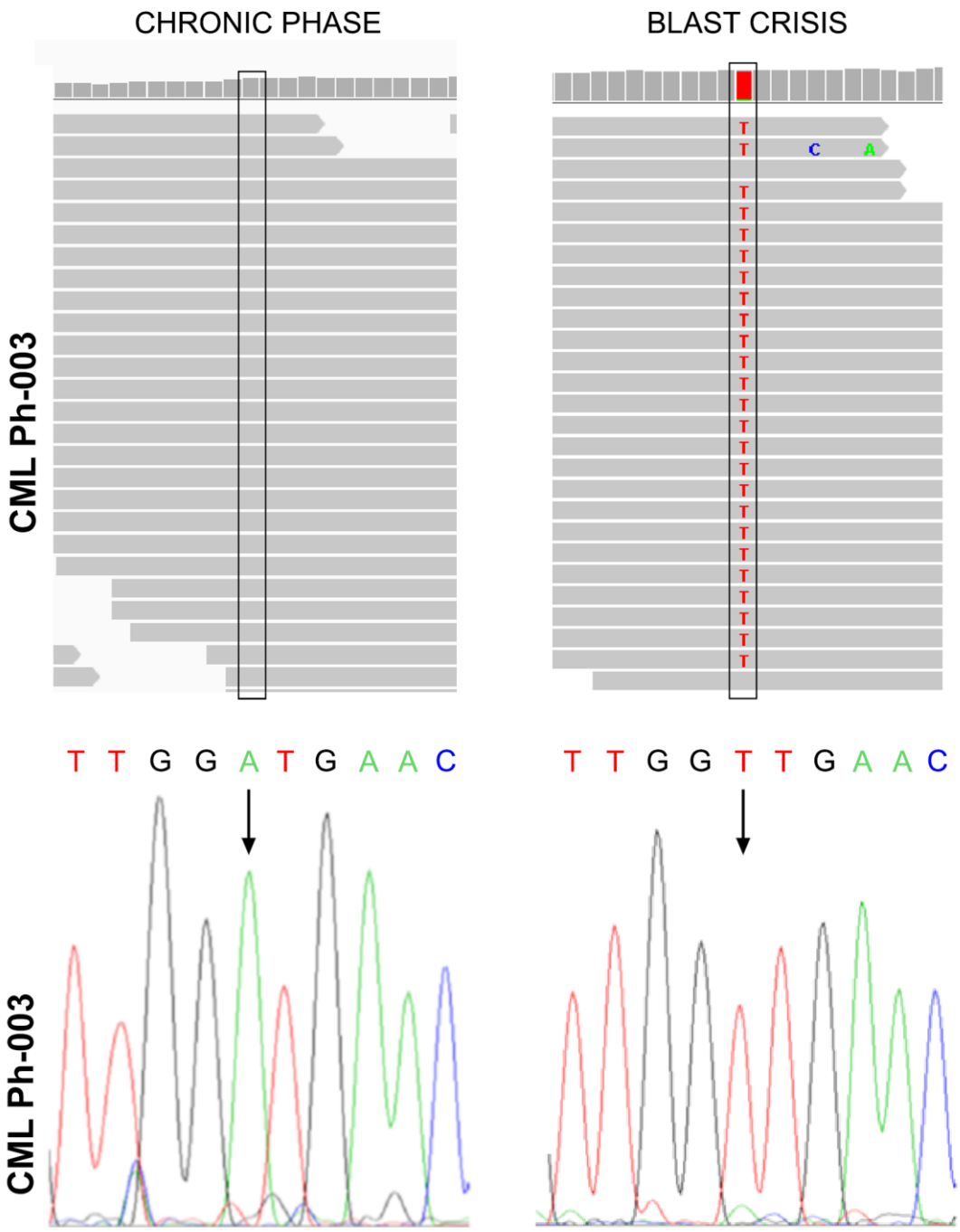

Figure 13: UBE2A somatic substitution causing Asp84Val mutation. **a)** Detail of the whole-exome sequencing reads from patient Ph-003 showing an A>T substitution in 93% of the reads in the blast crisis (right panel) sample but not in the chronic phase genomic DNA (left panel). The mutation is shown in black. The intensity of the color is proportional to the corresponding read quality. The black boxes highlight the position of the mutated nucleotide.

In the lower panels, the *UBE2A* genomic locus, the wild-type nucleotide sequence and the corresponding amino acid sequence are shown. **b)** Confirmation of the whole-exome sequencing results obtained using Sanger Sequencing in patients CML Ph-003. Chronic phase sample (lower panel), Blast phase sample (lower panel) the black arrows point to the mutated nucleotide.

According to cosmic data set (as reported in Table 6) *UBE2A* gene was found mutated in 4 cases of lymphoid neoplasm, with low frequency (≤1%). In addition it was also found mutated in other non-hematological malignancies such as adenocarcinoma, squamous cell carcinoma, endometrioid carcinoma and clear cell renal carcinoma always at low frequency. During this analysis we also found two mutations located at Isoleucine 33: Ile33Met/Ile33Thr in kidney carcinoma and colon adenocarcinoma (Figure. 14), where we had already identified the mutation in the subject 10 (Ile33Met).

Table 6: UBE2A mutation reported in COSMIC database.

Primary tissue	Genomic Coordinates	Cancer Type	Amino Acid Substitutions	Somatic Status	Reference
Large intestine	X:118708691..118708691	Adenocarcinoma	R6Q	Confirmed Somatic	http://www.sanger.ac.uk/cosmic
Lung	X:118708716..118708716	Squamous cell carcinoma	K14N	Variant of unknown origin	“
Lung	X:118708873..118708873	Adenocarcinoma	E18D	“	“
Lung	X:118708874..118708874	Adenocarcinoma	D19Y	“	“
Lung	X:118708884..118708884	Adenocarcinoma	A22D	“	(273)
Urinary tract	X:118708895..118708895	NS	G26R	“	http://www.sanger.ac.uk/cosmic
Haematopoietic and lymphoid	X:118708898..118708898	Diffuse large B cell lymphoma	A27P	Confirmed Somatic	(274)
Large intestine	X:118708917..118708917	Adenocarcinoma	I33T	Variant of unknown origin	(275)
Kidney	X:118708918..118708918	Clear cell renal cell carcinoma	I33M	Confirmed Somatic	(276)
Lung	X:118709339..118709339	Adenocarcinoma	P43T	Variant of unknown origin	http://www.sanger.ac.uk/cosmic
Haematopoietic and lymphoid	X:118716553..118716553	Follicular lymphoma	Y52D	Confirmed Somatic	(277)
Kidney	X:118716565..118716565	Clear cell renal cell carcinoma	S56G	Confirmed Somatic	http://www.sanger.ac.uk/cosmic

Large intestine	X:118716593..118716593	Adenocarcinoma	R65H	Variant of unknown origin	“
Endometrium	X:118716599..118716599	Endometrioid carcinoma	S67I	Variant of unknown origin	“
Large intestine	X:118717115..118717115	Adenocarcinoma	N89T	Variant of unknown origin	“
Lung	X:118717129..118717129	Adenocarcinoma	S94C	Variant of unknown origin	“
Prostate	X:118717163..118717163	Adenocarcinoma	R105L	Confirmed Somatic	“
Large intestine	X:118717177..118717177	Adenocarcinoma	R110C	Variant of unknown origin	“
Haematopoietic and lymphoid	X:118717178..118717178	Myelofibrosis	R110P	Confirmed Somatic	(278)
Breast	X:118717178..118717178	NS	R110L	Confirmed Somatic	http://www.sanger.ac.uk/cosmic
Lung	X:118717208..118717208	Squamous cell carcinoma	R120L	Variant of unknown origin	“
Endometrium	X:118717208..118717208	Endometrioid carcinoma	R120H	“	“
Haematopoietic and lymphoid	X:118709341..118709342	Chronic lymphocytic leukaemia-small lymphocytic lymphoma	G45fs*33	Confirmed Somatic	(279)

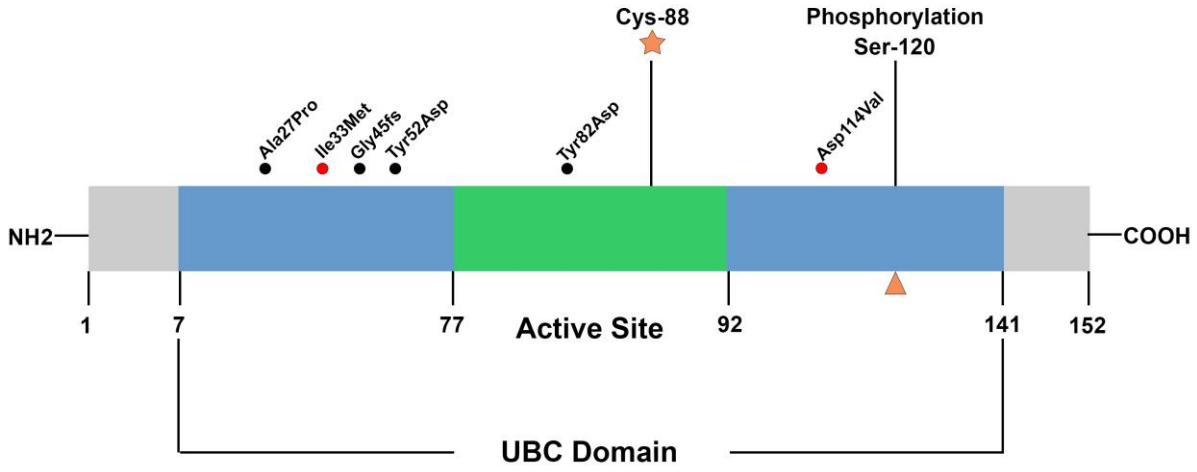

Figure 14: Distribution of some alterations on the UBE2A protein. 6 exons of the *UBE2A* transcript variant 1 encode for a protein of 152 amino acids. The *UBE2A* sequence contains ubiquitin conjugating domain (UBC Domain) (amino acid 7-141) and an active site (amino acid 77-92), a cysteine residue (Cys-88) required for the formation of a thioester bond with Ubiquitin. *UBE2A* is phosphorylated on a Ser-120 conserved residue (280). Altered amino acids identified in CML are highlighted as red circles (Ile33Met, Asp114Val) and black circles represents alterations found in lymphoid neoplasms. *UBE2A* numbering refers to the NCBI reference sequence NM_003336.3.

UBE2A Ser120 residue is an active site of phosphorylation (Figure. 14). Previous studies demonstrated that CDK-mediated phosphorylation of UBE2A on a Ser120 residue is an important regulatory event in the control of cell cycle progression (280).

3.1.4 *In Silico* Analysis of *UBE2A* mutations

We used two specific software, called SIFT (281) and Polyphen-2 (282). In order to know how these mutations could potentially alter the function of the protein. Sorting Tolerant From Intolerant (SIFT) is a web-based tool which used sequence homology from multiple sequence alignment (MSA) to predicts weather an amino acid substitution (AAS) can affect

the protein function. SIFT prediction are based on the fact that during evolution crucial positions in a protein sequence have been conserved and therefore substitutions at these positions may alter protein function (281). PolyPhen-2 (Polymorphism Phenotyping v2) predicts the possible impact of amino acid substitutions on the stability and function of human proteins using structural and comparative evolutionary considerations. It calculates Naïve Bayes posterior probability that this alteration is damaging and reports evaluates of false positive and true positive rates. A mutation is also examined qualitatively, as benign, possibly damaging, or probably damaging (283). The results for the two *UBE2A* protein variants found in our WES analysis invariably predicted a damaging alteration of the normal protein function.

3.2 - Molecular characterization of BCR promoter regulation

3.2.1 *In Silico* Analysis of BCR Promoter

In silico analysis was performed to identify putative binding sites of transcription factors (TFs) on *BCR* promoter through Jasper database (http://jaspar.genereg.net/), and TRANSFAC Biobase (262, 263). Several putative protein binding sites (PBS) for different transcription factors have been identified. Criteria for selecting the transcription factors (TF) for subsequent studies were: 1) TFs have role in haematopoiesis 2) TFs have a role in differentiation of hematopoietic progenitors 3) TFs binds to a DNase protected area of *BCR* promoter (149). The putative binding region for the selected transcription factors COUP-TF1 (Chicken ovalbumin upstream promoter-transcription factor1) and CTCF (CCCTC-binding factor) are shown in Figure. 15. MYC and MAX proteins putatively bind to BCR promoter at four different loci here indicated as PBS1 (-1354 bp to -1341 bp from ATG codon), PBS2 (-1283 bp to -1263 bp), PBS3 (- 813 bp to -801 bp) and PBS4 (-768 bp to -756 bp) (Figure. 15).

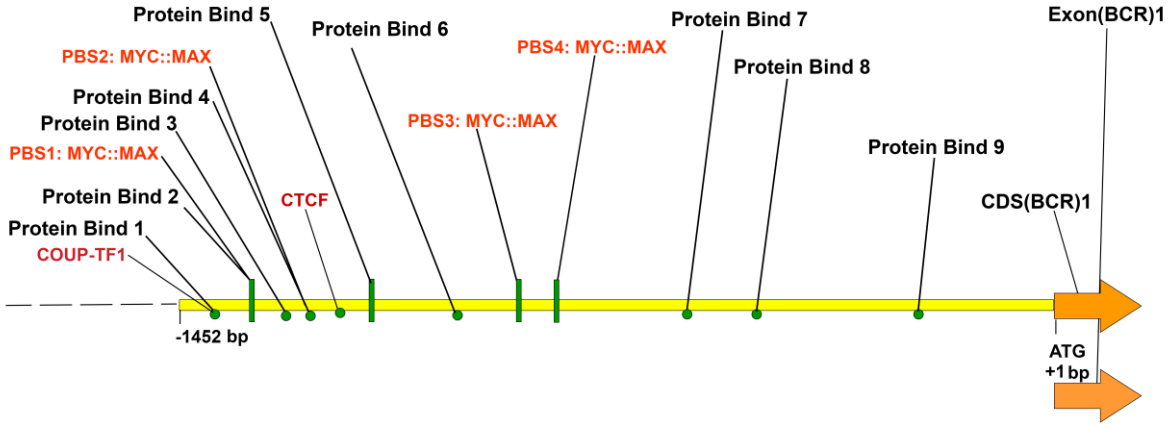

Figure 15: Schematic representation of COUP TF-1 (-1386 bp to -1380 bp from ATG codon), CTCF (-1242 bp to -1237 bp from ATG codon) and MYC::MAX (PBS1-4) binding region on BCR Promoter. The orange arrow on the right side shows the ATG site of the BCR gene and

the yellow line indicates the BCR promoter ranging up to -1452 bp from the ATG site. The green dots along the BCR promoter are the DNase protected area (148, 149) and they are annotated as Protein Bind 1 to 9. Putative COUP TF-1, CTCF and MYC::MAX binding regions are highlighted as red.

3.2.2 Chromatin Immunoprecipitation (ChIP) of transcription factors

I performed Chromatin Immunoprecipitation (ChIP) to confirm the binding of COUP-TF1, CTCF, MYC and MAX on BCR promoter to all the above mentioned sites using the K562 cell line as a CML cellular model. I subsequently analyzed the results by real-time quantitative PCR (qPCR).

By Chip assay I demonstrate that COUP-TF1 (Figure. 16 a, b) and CTCF (Figure. 16 c, d) binds on the BCR promoter to the specific sites identified previously from in-silico analysis (Figure. 15). The signal intensity for COUP-TF1 and CTCF binding on BCR promoter was significant when compared with the nonspecific IgG-Control. The fatty acid binding protein 7 *(Fabp7)* (264) promoter was used as positive control for COUP-TF1 binding. Similarly the serotonin transporter (SLC6A4) (265) promoter was used a positive control for CTCF binding.

In addition, by performing Chip assay I also confirm the binding of MYC and MAX proteins on BCR promoter at the four different loci previously identified PBS1-4. The Nucleophosmin (*NPM1*) (266) and the methionyl-tRNA synthetase (*MARS*) (267) promoters were used as positive control for MYC and MAX binding, respectively. I thus demonstrated that MYC binds to the BCR promoter (Figure. 17 a, c) at region 1 (comprising PBS1 and PBS2) and at region 2 (comprising PBS3 and PBS4) with a 2.5 and 5 fold-enrichment respectively when compared with the nonspecific IgG-control. Similarly MAX binds to the BCR promoter (Figure. 17. b, d) at the same regions with a 3 and 6 fold-enrichment respectively, when compared with the non-specific IgG control.

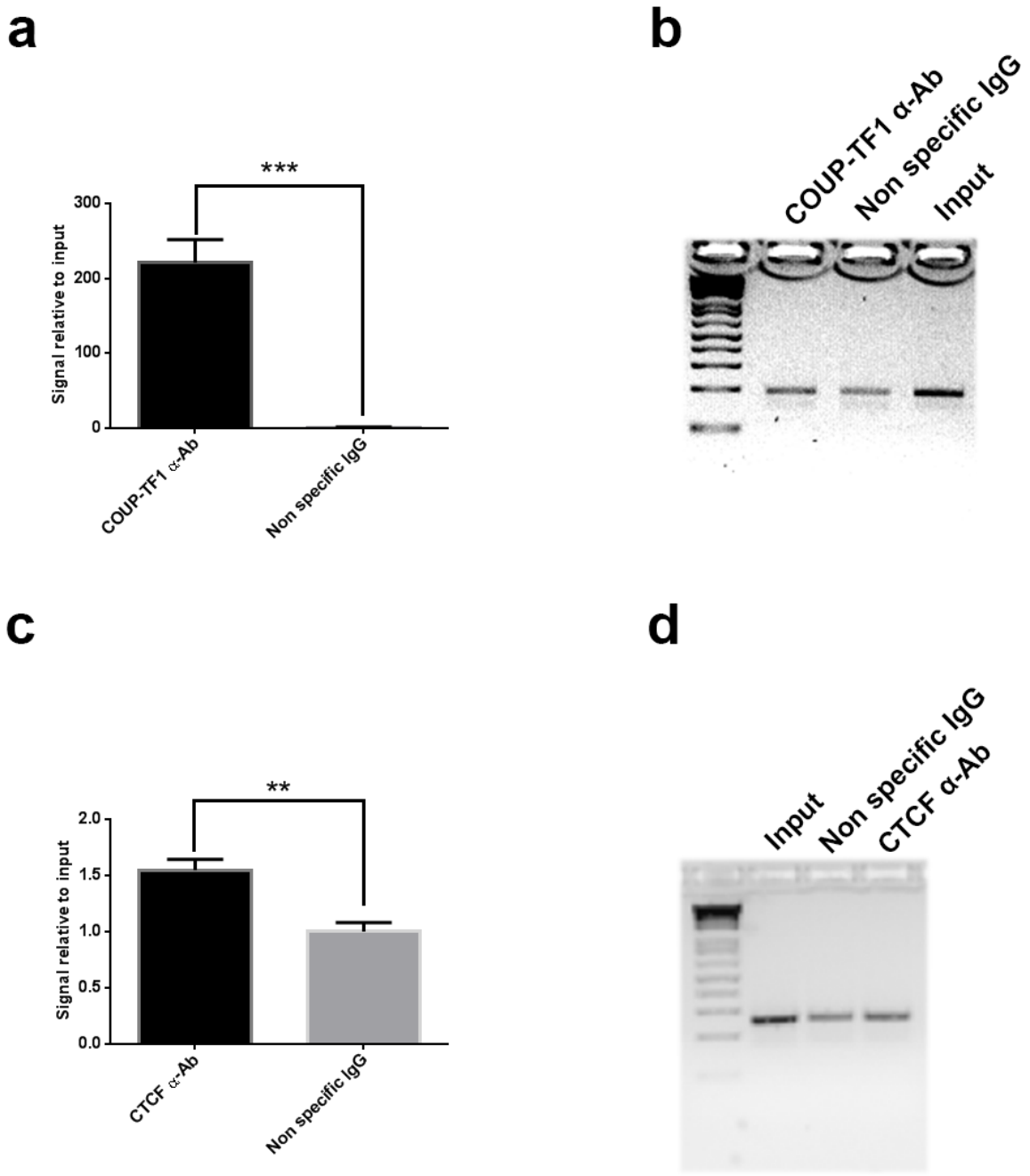

Figure 16: ChIP for COUP-TF1 and CTCF to verify the binding of these transcription factors to BCR promoter. The results of ChIP– real-time quantitative PCR assays shows that both **(a, b)** COUP-TF1 and **(c, d)** CTCF binds on the BCR promoter to the specific sites identified previously from in-silico analysis. Chip assays was performed with chromatin from K562 cells by using the indicated primary antibodies, and enriched DNA was amplified with specific

primers for the q-PCR analysis of ChIP samples. Signals were normalized to the input DNA (mean ± SD). *** P<0.0001, **: P < 0.01. Amplified genomic loci obtained after real-time quantitative PCR (qPCR) were tested on 3% agarose gel.

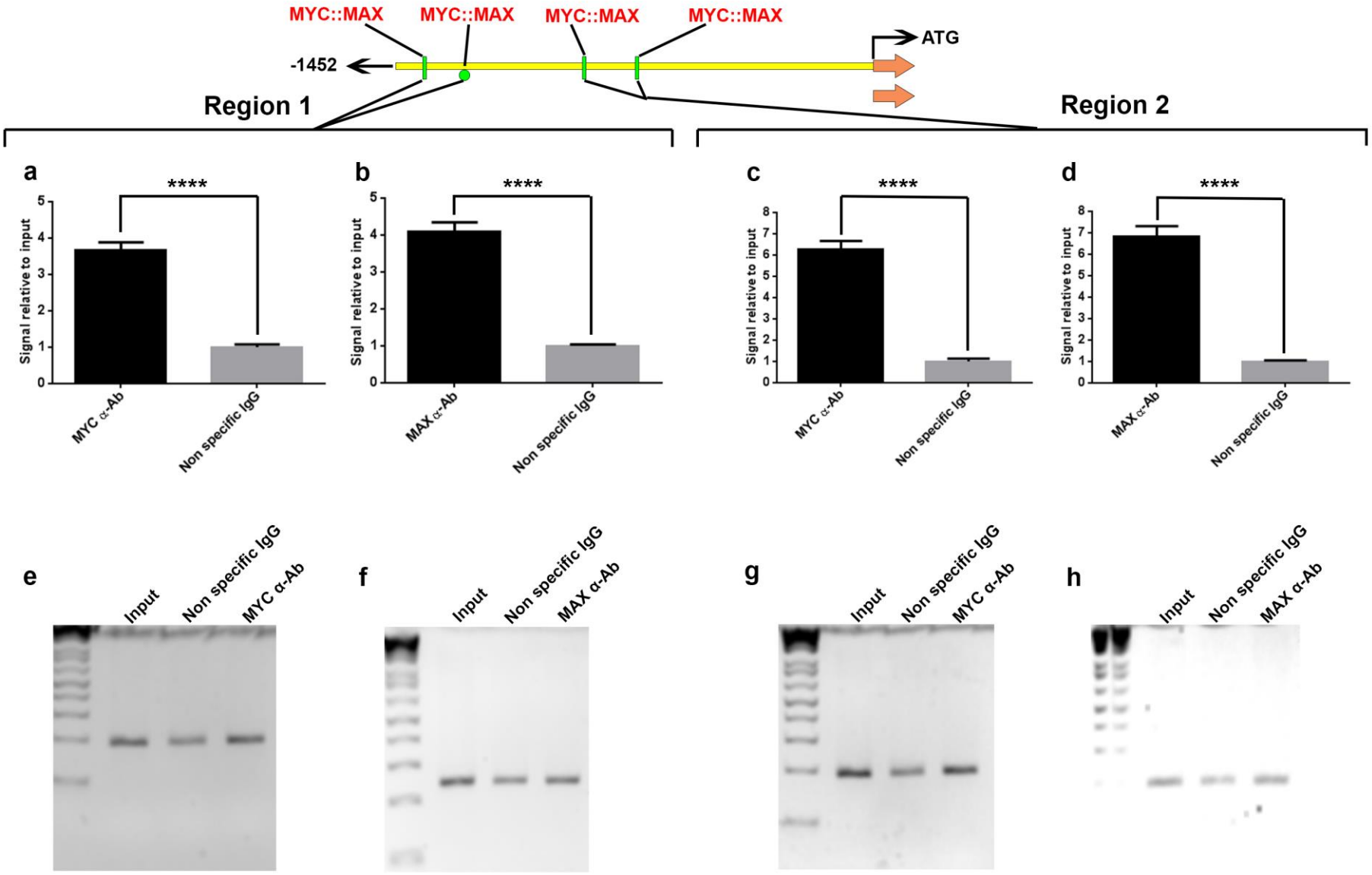
MYC::MAX
MYC::MAX
MYC::MAX
MYC::MAX
ATG
-1452
Region 1
Region 2
a
b
c
d
****
Signal relative to input
MYC α-Ab
MAX α-Ab
Non specific IgG
e
f
g
h
Input
Non specific IgG
MYC α-Ab
MAX α-Ab

Figure 17: ChIP experiments to verify the binding of MYC and MAX to BCR promoter. The results of ChIP– real-time quantitative PCR assays shows that **(a, b)** both MYC and MAX bind at region 1 (PBS1 and PBS2) and **(c, d)** at region 2 (PBS3 and PBS4). Chip assays was performed with chromatin from K562 cells by using the indicated primary antibodies, and enriched DNA was amplified with specific primers for the q-PCR analysis of ChIP samples. Signals were normalized to the input DNA (mean ± SD). *** P<0.0001. **(e, f, g, h)** Amplified genomic loci obtained after real-time quantitative PCR (qPCR) were tested on 3% agarose gel.

3.2.3 Alteration of MYC and MAX levels in CML cell lines

As many studies have indicated the involvement of MYC in CML progression (284, 285), we focused our study on MYC and its co-factor MAX for further studies. In order to determine the role of selected transcription factors on BCR and BCR/ABL expression, the K562 BCR/ABL +ve CML cell line was transfected with expression vectors encoding for MYC and MAX (pcDNA3 MAX, pWZL MYC and the control pcDNA3 Empty) both as single or double transfectants. The stable K562 transfectants (K562_MAX, K562_MYC, K562_MYC::MAX and K562 pcDNA3Empty) were obtained. Then the mRNA and protein expression levels of MYC, MAX, BCR and BCR/ABL were detected by real-time quantitative PCR (qPCR) and Western blotting (Figure. 18 and 19).

The transfection with the pcDNA3_MAX and pWZL_MYC vectors significantly increased MYC and MAX expression (*** Pvalue<0.0001) at mRNA (Figure. 18a, b, result representative of three experiments) and protein levels (Figure. 18 c) in K562 cells, compared to Empty cells, as expected.

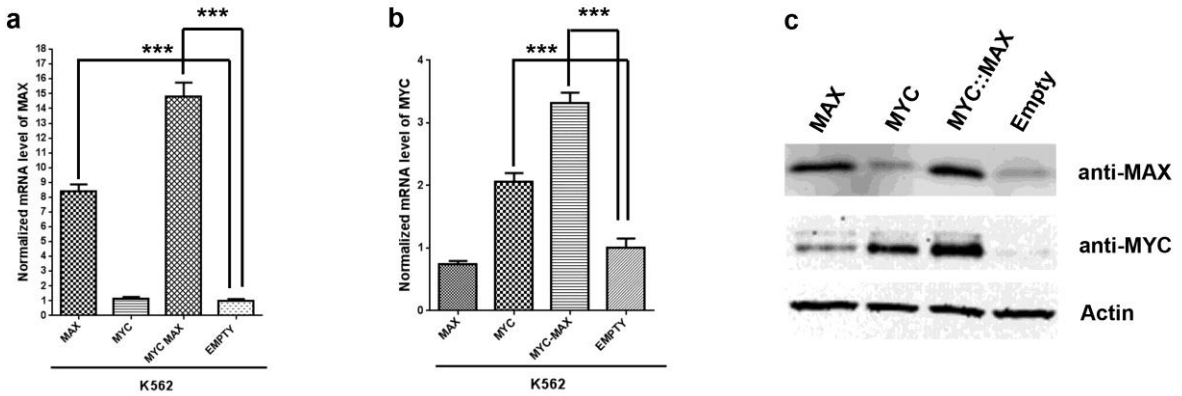

Figure 18: Overexpression of MYC and MAX in K562 cells at both mRNA and protein levels. **(a)** qPCR results shows that K562_MAX (8.38±0.493) and K562_MYC::MAX (14.81±0.939) cells had significantly higher MAX expression (*** Pvalue<0.0001) in comparison with empty

vector transfected K562 cells (1.00 ±0.120). **(b)** RT-qPCR results shows that K562_MYC (2.06±0.138) and K562_MYC::MAX (3.32±0.165) cells had significantly higher MYC expression (*** Pvalue<0.0001) in comparison with empty vector transfected K562 cells (1.00 ±0.120). Mean and s.e.m. values from three independent experiments are plotted. *** Pvalue<0.0001. **(c)** Anti-MAX and Anti-MYC Immunoblotting of K562 whole cell lysates. Actin was used as a loading control.

qPCR (Figure. 19 a, b. result representative of three independent experiments) and Western Blot analysis (Figure. 19 c) showed that, when overexpressed, *MYC::MAX* heterodimer significantly up-regulates BCR (2.01±0.33 fold; p-value: 0.0403) and BCR/ABL (2.61±0.56; p-value:0.0082) expression in comparison with K562_Empty cells. This induction reaches significant values when MYC and MAX genes are over-expressed in combination (BCR and BCR/ABL expression in K562_MAX: 0.79±0.56 and 1.27±0.18; in K562_MYC: 1.41±0.99 and 2.34±0.68, respectively).

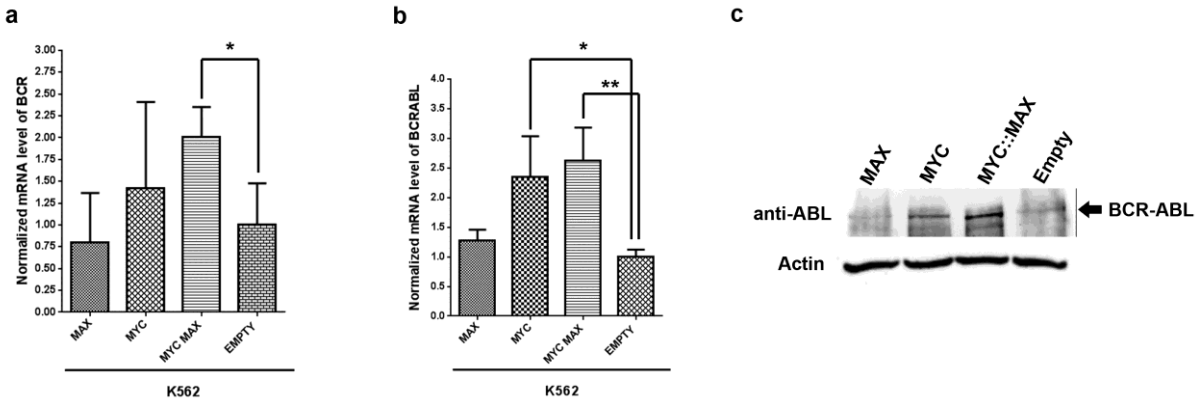

Figure 19: Effect of MYC and MAX transcription factors on BCR and BCR/ABL expression. qPCR for **(a)** BCR and **(b)** BCR/ABL in K562 MAX, MYC, MYC::MAX and Empty stable transfectants. The data shown represent the means ± SD (standard deviation) of three independent experiments. **(c)** Immunoblotting for BCR/ABL and actin on whole-cell lysates. Immunoblotting for actin was used as loading control.

I next assessed whether the silencing of MYC in various *BCR/ABL +ve cell lines* may influence BCR and BCR/ABL expression levels. Therefore I performed silencing of MYC in the BCR/ABL +ve cell lines K562, KCL22 and LAMA-84 using shRNA (kind gifts from Dr. Robert N Eisenman) (268) by lentiviral infection. As shown in the Figure. 20 a, d, g and Figure. 21 a, b, c expression of shRNA targeting MYC effectively reduced the expression of MYC both at mRNA and protein levels. This caused a significant decrease in the BCR (Figure. 20 b, e and h) and BCR/ABL expression at mRNA (Figure. 20 c, f and i) and protein levels (Figure. 21 a, b and c) in comparison with BCR/ABL +ve cell lines transduced with control shRNA.

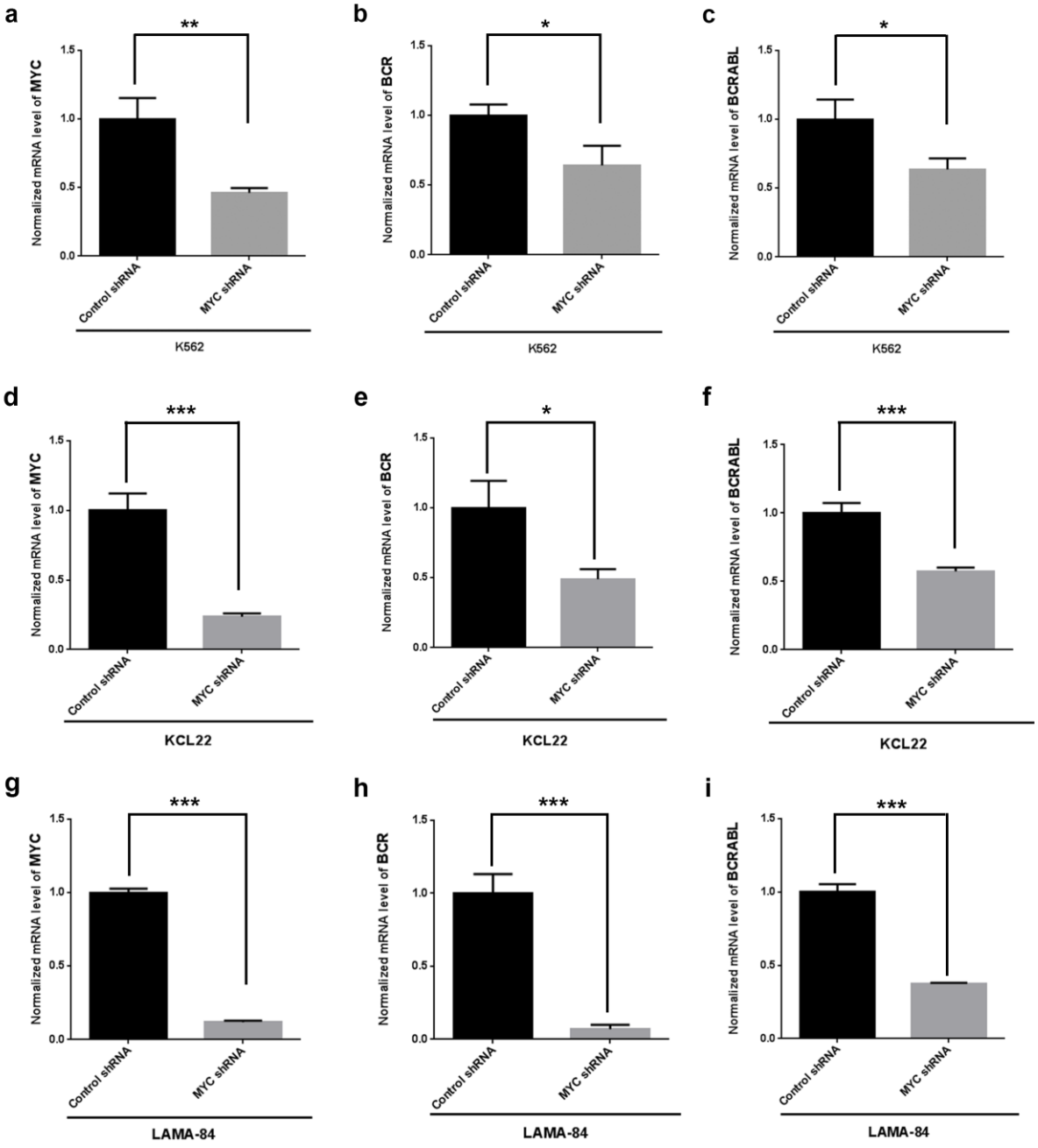

Figure 20: MYC silencing causes downregulation of BCR and BCR/ABL expression at mRNA. *BCR/ABL +ve cell lines* were transduced with lentiviruses expressing (non-targeted) control shRNA or MYC shRNA plasmid. **(a, d and g).** Silencing of MYC at mRNA level was assessed by qPCR. qPCR data also shown that, K562, KCL22 and LAMA-84 cells stably expressing MYC shRNA led to significant down regulation of **(b, e and h)** BCR and **(c, f and i)** BCR/ABL. * Significantly different from control values, as determined by the Student's t-test (***: $P < 0.001$, **: $P < 0.01$,*: $P < 0.05$).

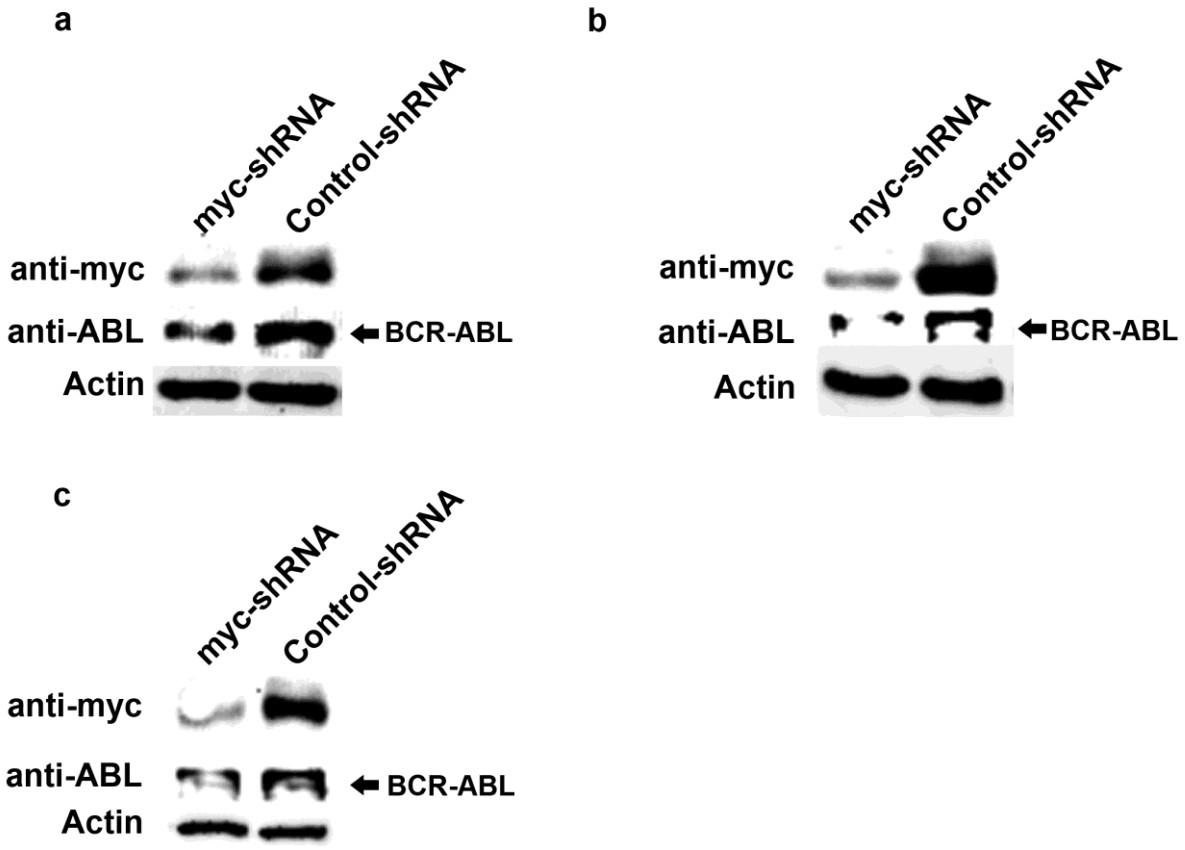

Figure 21: Silencing of MYC in a) K562 b) LAMA-84 and c) KCL22 BCR/ABL +ve CML cell lines leads to downregulation of BCR/ABL at protein level. Anti-MYC, anti-ABL and anti-Actin immunoblots. Immunoblotting against Actin was used as loading control.

3.2.4 Biological effect of MYC silencing in BCR/ABL +ve cell lines.

Previous studies have shown that MYC is an important transcription factor involved in cell proliferation and growth (286). I investigated whether shRNA mediated down-regulation of MYC levels could influence the ability of BCR/ABL +ve CML cells (KCL22 and LAMA-84) to proliferate and undergo apoptosis. MYC silencing in (Figure. 22 a**)** LAMA-84 and (Figure. 22 c) KCL22 resulted in induction of apoptosis as determined by PARP cleavage (Figure. 22 a, c). To determine whether cell viability was affected as a consequence of MYC knock-down induced by MYC-shRNA, I monitored growth of shRNA-infected LAMA-84 and KCL22 cells over a 4-day period. The viability of LAMA-84 and KCL22 cells infected with MYC-shRNA was markedly reduced ($p < 0.0001$) (Figure. 22 b, d) in comparison with LAMA-84 and KCL22 cells transduced with control-shRNA.

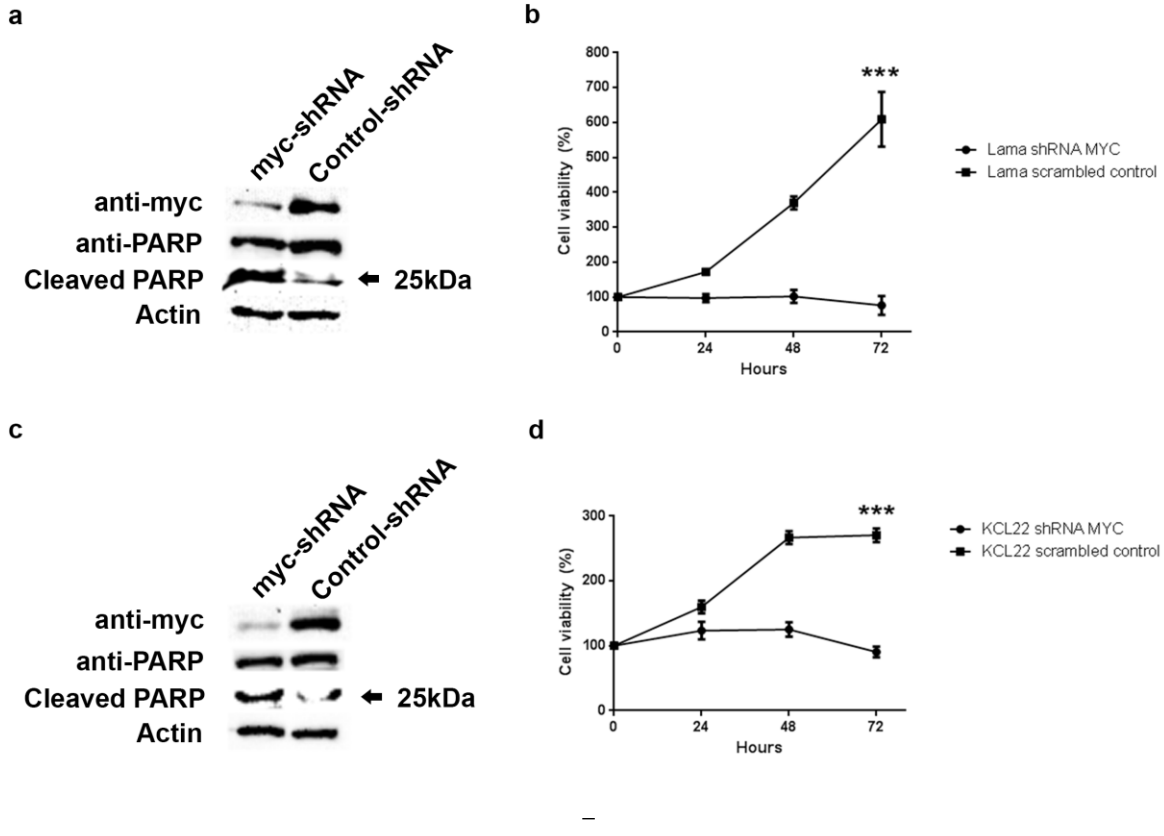

Figure 22: Induction of apoptosis and reduced proliferation in MYC-shRNA transduced BCR/ABL +ve CML cells (KCL22 and LAMA-84). **(a)** LAMA-84 and **(c)** KCL22 cell lines were

infected with lentiviruses encoding either MYC-shRNA or the control shRNA. After infection cell lysates were immunoblotted for Anti-MYC, Anti-PARP and Actin. The PARP antibody recognizes total and cleaved PARP (25kDa). Cleavage of PARP indicates activation of the apoptotic pathway. Cell viability of MYC-shRNA and control shRNA-transduced **(b)** LAMA-84 and **(d)** KCL22 cells was determined by the MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. Values represent the mean normalized percentage of survival compared to control cells (n = 5 wells; ± SD). * Indicates a significant difference compared to control values, as determined by the Student's t-test (***: $P < 0.001$).

3.2.5 Luciferase Assay

BCR activity was analyzed by luciferase reporter assay. To determine which of the four MYC binding sites is important for BCR and BCR/ABL regulation (Figure. 23), we performed Firefly luciferase assay with HEK-293 cells infected with the lentviral vector encoding either MYC-shRNA or the control (non-targeted) shRNA. After confirmation of MYC silencing by qPCR (Figure. 23 a) and Western blotting (Figure. 23 b), HEK-293 cells were co-transfected with various BCR promoter constructs:

1) “Full BCR” (-1444 bp from the BCR ATG site comprising all four MYC::MAX binding sites)

2) “Full BCR 34 Δ” (-1387 bp from the ATG starting site with 3 and 4 MYC::MAX binding sites deleted)

3) “1200bp BCR” (-1204 bp from the ATG site with 3 and 4 MYC::MAX binding sites)

4) “1200bp34 Δ” (-1147 bp from the ATG site without any binding sites for MYC::MAX)

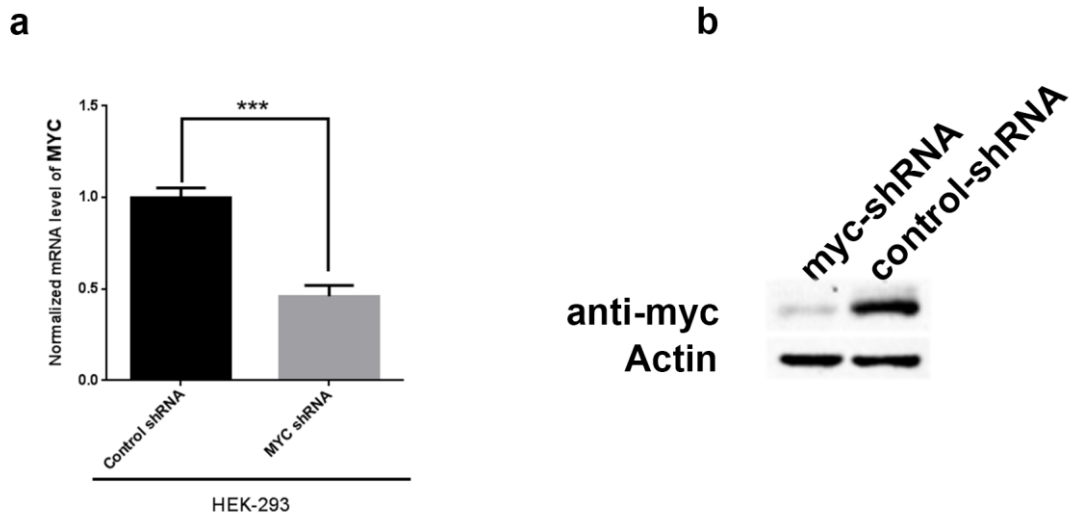

Figure 23: HEK-293 cell line was transduced with lentiviruses expressing (non-targeted) control shRNA or MYC shRNA. **(a)** Silencing of MYC at mRNA level was analyzed by RT-qPCR. Shown is a graph of Myc values normalized to HEK-293 shRNA control values **(b)** Immunoblotting for Anti-MYC and Actin on whole cell lysates.

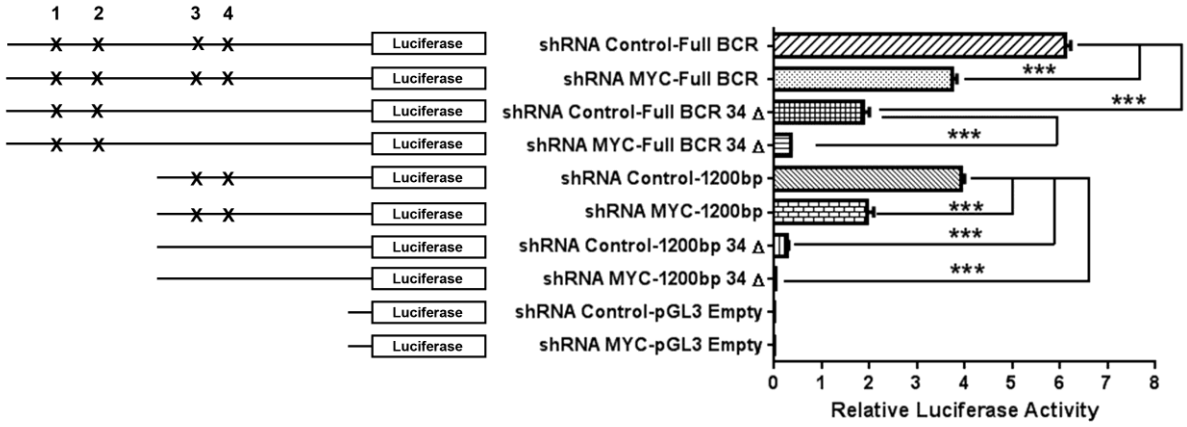

Figure 24: PBS3 and PBS4 MYC::MAX binding regions are critical for BCR and BCR/ABL regulation. Luciferase activity on HEK-293 cells transduced with control shRNA or MYC shRNA transiently transfected with Full BCR promoter, Full BCR 34 Δ, 1200bp, 1200bp34 Δ and pGL3 Empty. Putative binding sites of MYC and MAX are labelled as 'X'. Results are

presented as the average fold of Firefly luciferase activity versus Renilla. Values reported in the graph represent the average of three separate experiments. *** P<0.0001.

As expected, a significant increase (*** P<0.0001) in the luciferase activity was detected in HEK-293 shRNA control cells transfected with the full BCR promoter when compared with MYC silenced HEK-293 cells (Figure. 24). When PBS3 and PBS4 were specifically deleted (HEK-293 shRNA control-FULL BCR 34 Δ), a significant decrease of luciferase activity was observed in comparison with HEK-293 shRNA control expressing all four PBS (HEK-293 shRNA control-FULL BCR). In addition when only PBS3 and PBS4 were maintained (HEK-293 shRNA control-1200bp) a significant increase (*** P<0.0001) in the luciferase activity was seen in comparison with MYC silenced HEK-293 cells (HEK-293 shRNA MYC-1200bp) and cells transfected with BCR construct lacking all four PBS of MYC and MAX (HEK-293 shRNA control l-1200 bp 34 Δ and HEK-293 shRNA MYC-1200 bp 34 Δ). These data suggest that PBS3 and PBS4 are critical for BCR and BCR/ABL regulation.

4 DISCUSSION

Before the advent of specific Tyrosine Kinase Inhibitors (TKI), all cases in chronic phase ultimately progressed to the advanced phase of the disease (BC). Nowadays CML is mainly diagnosed in chronic phase (CP) and progression cases are limited but unfortunately they are still present. Despite the aggressiveness of BC and the poor overall survival of these patients, little is known about the molecular mechanisms responsible for progression of this disease.

Considering the poor information about progression in CML, very few recurrent genomic abnormalities have been correlated until now to these diseases. We applied the whole-exome sequencing approach to compare the whole exome-sequences of 11 paired CP (used as control) and BC sample: I found a total of 38 single mutations (average= 4 mutations/pts; range 0-8) occurring in BC samples that were absent in the corresponding CP sample. I identified a limited number of acquired mutations in BC when compared with the CP samples and with higher oncogenic potential; this because all passengers mutations present were filtered out using the CP sample as “baseline”. Therefore it seems likely that the mutations identified in the BC samples are causally linked to the progression of CML. By using this approach we found recurrent, somatic, single nucleotide mutations in *RUNX1* and *UBE2A* in 2 out of 11 BC samples.

RUNX1 (Runt-related transcription factor 1) mutation (gene ranker score of 6.5) was identified in patient#2 and #10. It acts as a key regulator of hematopoiesis, regulating the differentiation of hematopoietic stem cells into mature blood cells (287). *RUNX1* is frequently altered in leukemia through chromosomal translocation or gene mutations (288). The role of *RUNX1* in CML progression is already reported (270, 289). *RUNX1* mutation were also reported in one CP patient sample (289), thus suggesting that *RUNX1* mutation seems not strictly associated to BC.

The second recurring mutation identified was affecting the ubiquitin-conjugating enzyme E2A (*UBE2A*). It was identified mutated in patient #3(D114V) and #10(I33M) with mutation frequencies of 93% and 39%, respectively. *UBE2A* gene is located at Xq23.11 and encodes

a protein of 152 aa residues (NM_003336.3) (290). Alteration of proteins, either with single ubiquitin protein or chains of ubiquitin is a major cellular process by which abnormal and short lived proteins are degraded. Ubiquitination occurs through the chronological action of at least three classes of enzymes: E1s (ubiquitin-activating enzymes), E2s (ubiquitin-conjugating enzymes) and E3s (ubiquitin-protein ligases). *UBE2A gene* encodes a member of the E2s family. This enzyme is involved in various biological processes such as positive regulation of cell proliferation (280)**,** regulating β-Catenin stabilization (291) and post-replicative DNA damage repair (292). Protein sequence of this enzyme is entirely identical in the rat, mouse and rabbit thus indicating that *UBE2A* is highly conserved in eukaryotic evolution (293). At present, *UBE2A* gene was found mutated in 4 cases of lymphoid neoplasm, with low frequency (≤1%) (http://www.sanger.ac.uk/cosmic) (274, 277). This gene have been also found mutated at Isoleucine 33, the same aa altered in our patient 10: Ile33Met/Ile33Thr in one case of kidney carcinoma and one of colon adenocarcinoma (http://www.sanger.ac.uk/cosmic). This is the first time that recurrent *UBE2A* mutations are detected specifically in BC samples. Ongoing analysis on additional BC/CP samples and *in vitro* experiments will help to clarify the role of *UBE2A* mutations in CML progression.

The data obtained from exome sequencing were also applied to study copy number alterations events (CNA) by using a graphical, event-driven tool called CEQer (Comparative Exome Quantification analyzer), developed in our laboratory by (256). In line with previous reports, indicating that chromosomal instability increases upon progression from CP-CML to BC (272), CEQer analysis of nine matched BC/CP exomes revealed the presence of large CNA events acquired during CML evolution. We confirmed the high frequency of chromosome 7 alterations (3/9 patients) and the recurrent deletion of two critical regulator of cell cycle control in 2/9 patients (CDKN2A and p53). We also confirmed the appearance of Philadelphia chromosome duplication in one patient, as already reported in previous studies (295).

In summary, this first part of the study shows the presence of a limited number of acquired mutations in the blast crisis samples when compared with matched chronic phase. Nevertheless, despite some heterogeneity in the genetic alterations identified in BC samples, we were able to find 2 recurrently mutated genes associated with blastic transformation, *RUNX1* and *UBE2A*, with the last one never been detected in CML samples.

One of the determinant of CML progression is the unrestrained activity of BCR/ABL in hematopoietic progenitor cells (174, 179). Previous studies showed also that the expression level of BCR/ABL is critical for the differentiation block seen in CML blast crisis cells (296). The mechanisms underlying the regulation of BCR/ABL dosage in BC is not completely known. After the oncogenic translocation, it is the BCR promoter which drives the production of the BCR/ABL mRNAs (148). Jamieson et al showed that BCR/ABL mRNA level decreases during myeloid differentiation in chronic phase samples and healthy donors and that this down-regulation was undetectable in BC samples (156). Next, Marega et al showed that both BCR and BCR/ABL levels are equally impaired during blastic transformation in the GMP subpopulation, suggesting the presence of an “in trans” mechanism acting on both the promoters (150).

In this study I attempted to identify specific transcription factors which bind to BCR promoter and can play a significant role in regulating the expression of BCR and BCR/ABL, and which can correlate to the BCR/ABL upregulation observed during blastic transformation. First of all, I performed in silico analysis for BCR promoter (-1452 bp from the ATG site). Several putative protein binding sites (PBS) for different transcription factors have been identified along the entire BCR promoter sequence analyzed. Hereby, I found that MYC and MAX proteins putatively bind to BCR promoter at four different loci: PBS1 (- 1354 bp to -1341 bp from ATG codon), PBS2 (-1283 bp to -1263 bp), PBS3 (- 813 bp to -801 bp) and PBS4 (-768 bp to -756 bp) (Figure. 15). A number of previous reports had indicated the involvement of MYC in myeloid leukemogenesis. For instance, in mice where MYC expression is enforced in

the hematopoietic precursors these animals develop acute myeloid leukemia (297). Also, infection of murine bone marrow with MYC retroviruses results in myeloid but not lymphoid leukemia (298). Moreover, analysis in transgenic models shows that MYC is vital for normal differentiation of myeloid stem cells (299). By using Chromatin Immunoprecipitation assays I showed for the first time that MYC and MAX proteins can bind to BCR promoter at 4 specific sites in the BCR/ABL +ve K562 cell line (Figure. 17).

According to previous studies, MYC forms required heterodimers with the specific bHLH-LZ partner, MAX, and binds to the core DNA consensus site. Dimerization with MYC::MAX heterodimer and DNA-binding are crucial for the mitogenic and oncogenic functions of MYC (300). In line with these data, a significant upregulation of BCR and BCR/ABL was detected only when I enforced the expression of both MYC and MAX, which suggests that the formation of the MYC::MAX heterodimer is essential for MYC-driven BCR promoter activation. These findings were further strengthened by the evidence that induction of MYC silencing through lentiviral mediated shRNA delivery led to significant downregulation of BCR and BCR/ABL expression at both transcription (Figure. 20) and protein levels (Figure. 21) in various BCR/ABL +ve CML cell lines (K562, KCL22 and LAMA-84).

By using a luciferase reporter assay I showed that in MYC-silenced cells the activity of the BCR promoter was significantly downmodulated (Figure. 24). When PBS3 and PBS4 were deleted from the full BCR promoter, a significant decrease of luciferase activity was similarly observed, thus identifying these two regions as critical for BCR and BCR/ABL regulation (Figure. 24). These data suggest that PBS3 and PBS4 binding sites are critical for BCR and BCR/ABL regulation.

As stated previously, according to Marega.M et.al, BCR is physiologically down-regulated upon myeloid maturation from HSCs to CMPs and GMPs, and this mechanism is conserved in healthy donors and in CP-CML. Conversely, in BC this regulation is impaired for both BCR/ABL and BCR, which suggests the presence of an '*in trans*' deregulated transcription of both BCR and BCR/ABL promoters, associated with CML progression (150). Interestingly

MYC is often upregulated in the blast crisis through chromosome 8 trisomy or gene amplification (179, 284, 285, 301). Furthermore, MYC is a known beta-catenin target gene, which has been shown to be activated in GMP cells from BC patients (156, 199). These data suggest that an increase in MYC levels may play a causative role in the evolution from the chronic phase to blast crisis. Our results show that MYC is able to bind at BCR promoter and to regulate BCR and BCR/ABL expression through recruitment of MAX.

In conclusion, this is the first description of a new pathway putting BCR and BCR/ABL under the transcriptional control of the MYC::MAX heterodimer. Since MYC is frequently over-expressed in BC, this phenomenon could explain why BCR/ABL downregulation typically occurring upon myeloid differentiation in CP is lost in BC.

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