FREQUENCY OF BCR-ABL1 TRANSCRIPTS IN CHRONIC MYELOID LEUKEMIA

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Recommendation

This is to certify that the research work entiled "**FREQUENCY OF BCR-ABL1 TRANSCRIPTS IN CHRONIC MYELOID LEUKEMIA"** has been carried out by **Ms. Roji Raut** under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is her original findings. I/we, hereby, recommend this thesis for final evaluation.

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To Whom It May Concern

Ms. Roji Raut, who enrolled in Master of Science in Central Department of Biotechnology, Tribhuvan University, Kathmandu, Nepal, conducted successfully a part of her M.Sc. thesis work entitled "**FREQUENCY OF BCR-ABL1 TRANSCRIPTS IN CHRONIC MYELOID LEUKEMIA"** in Medi Quest Laboratory Clinic Pvt. Ltd. for the partial fulfillment of her academic programme. The work conducted by Ms. Roji Raut was mutually supervised by me and her supervisor Prof. Dr. Krishna Das Manandhar.

As an external supervisor, I wish her for successful submission of her thesis.

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Dedicated

To

My beloved Parents

And

Our respected teachers

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Glossary Synonyms

ABL1 : Abelson murine leukemia viral oncogene homolog 1

ALL : Acute Lymphoid Leukemia

AML : Acute Myeloid Leukemia

AP : Accelerated phase

BAP-1 : BCR associated protein-1

BC : Blast crisis

BCR **:** Break Point Cluster Region

BM : Bone marrow

BP : Blast phase

CalB: Calcium-binding domain

CBC : Complete Blood Count

CCA : Clonal chromosome abnormalities

CcyR : Complete cytogenetic response

CDBT: Central Department of Biotechnology

cDNA : complementary DNA

CHR : Complete hematologic response

Chr9 : Chromosome 9

CLL : Chronic Lymphocytic Leukemia

CML : Chronic Myeloid Leukemia

CNS : Central Nervous System

CP : Chronic phase

CT : Computed Tomography

DNA : Deoxyribonucleic acid

ELN : European Leukemia Network

FDA : Food and Drug Administration

FGFR1 : Fibroblast Growth Factor Receptor 1

FISH : Fluorescence *in situ* hybridization

FOXO : Forkhead O

GEF : Guanine nucleotide Exchange Factor

GMQE : Global Model Quality Estimate

Grb2 : Growth factor receptor bound protein 2

HCT : Hematopoietic Cell Transplantation

HSC : Hematopoietic stem cells

IM : Imatinib Mesylate

JAK2 : Janus Kinase 2

kDa : Kilo Dalton

MAPK : Mitogen-Activated Protein Kinase

M-bcr : Major breakpoint cluster region

m-bcr : Minor breakpoint cluster region

µ-bcr : micro breakpoint cluster region

MNC : mononuclear cell

mRNA : messenger RNA

NMR : Nuclear Magnetic Resonance

OS : Overall Survival

PB : Peripheral Blood

PCR : Polymerase Chain Reaction

PCyR : Partial cytogenetic response

PDGFRA : Platelet Derived Growth Factor Receptor Alpha

PFS : Progression-Free Survival

Ph chromosome : Philadelphia chromosome

Ph+ : Philadelphia chromosome-positive

PI3K : Phosphatidylinositol 3-kinase

P-loop : Phosphate binding loop

RET: Ret Proto-Oncognene

rIFN : Recombinant Interferon-alfa

RNA : Ribonucleic acid

RQ-PCR : Real-time quantitative polymerase chain reaction

RT-PCR : Reverse transcriptase polymerase chain reaction

SBVS : Structure-based virtual screening

TKI : Tyrosine kinase inhibitor

v-ABL : Abelson murine leukemia viral oncogene

WBC : White blood cell

WHO : World Health Organization

3D : Three dimensional

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Abstract

FREQUENCY OF BCR-ABL1 TRANSCRIPTS IN CHRONIC MYELOID LEUKEMIA.

Background: Chronic myeloid Leukemia (CML) is among the largest group of leukemia cases in Nepal. Underlying pathology in CML is the translocation between ABL1 gene and BCR gene. The breakpoint in the BCR and ABL1 gene may vary leading to the formation of various types of fusion transcripts viz. p210, p190, p230 and other rare variants. Literatures suggest p210 is the most common transcript type seen in CML. Eventhough rare, the variants such as p190 and p230 have also been reported. However, studies are lacking to see the transcript type of CML patients in Nepal. Molecular diagnosis of CML is mostly done in the referral laboratories in India. In this study, we tried to analyse the frequency of different BCR-ABL1 transcripts (p210, p190, p230) in Nepalese CML patients in a laboratory within Nepal. In addition, the response of different transcripts towards drugs being used was also analysed via protein-ligamd interaction. A total of 45 samples were studied using real-time and conventional method and virtual screening approaches were explored to study drug interactions.

Methodology: During the study period, a total of 45 cases of suspected CML patients were included. Total RNA was extracted and reverse transcribed to cDNA using standard protocols. qPCR was performed with primers and probes targeting p210, p190 and p230 transcripts. The protein-ligand study was carried out using docking tools. All the results were statistically analysed.

Results: Almost all the cases, 44/45 cases (97.7%) were positive for p210 transcript only. CML was found to be more common in males with M:F ratio of 2.44 : 1. The disease was more prevalent in Kshetri community. The disease was more common in middle aged population with median age of 43 years at diagnosis. Transcript type variation made no impact to drug affinity in CML patients.

Conclusion: p210 transcript is the most prevalent transcript type in our study. Hence p210 must be targeted in the suspected cases of CML. Transcript type made no effect in drug use however larger studies may be informative regarding the frequency of other rare variants in Nepalese patients and developing effective treatments.

Keywords: BCR-ABL1, Chronic Myeloid Leukemia, Nepalese population, Real-time PCR, Transcripts, Docking.

Chapter 1

1. Introduction

1.1. Background

Blood is synthesized in the bone marrow as a hematopoietic stem cell. Hematopoietic stem cells are pleuripotent, meaning they can give rise to both myeloid and lymphoid blood cells (Figure 1). If hematopoietic stem cells develop into myeloid cells, it will mature into an erythrocyte, a thrombocyte or a leucocyte like a monocyte or a granulocyte that include: neutrophils, eosinophils and basophils. If a hematopoietic stem cell divides into lymphoid cell it will mature into some other kind of leucocytes: T cell, B cell or a natural killer cell which are referred to as lymphocytes. Once the various blood cells form they leave the bone marrow and travel around the blood or settle down in tissues or organs like the lymph nodes and the spleen. A chromosomal abnormality in the hematopoietic stem cells that are destined to become leucocvtes leads to chronic leukemia.

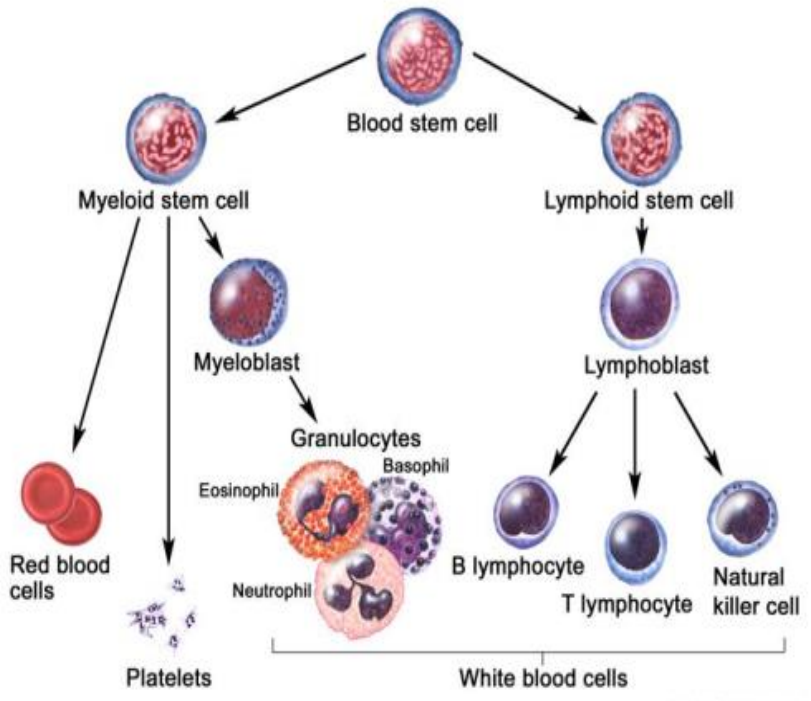


Figure 1. Haematopoiesis. A blood stem cell goes through several steps to become a red blood cell, platelet, or white blood cell. *Source: https://www.ncbi.nlm.nih.gov/books/NBK65740/*

There are two types of chronic leukemia: chronic myeloid leukemia (CML) which is characterized by a particular translocation that affects the granulocytes and chronic lymphocytic leukemia (CLL) which is caused by a variety of chromosomal mutations that affects lymphocytes, in particular B cells. Both CML and CLL cause cells to mature partially and that's a key difference from acute leukemia where cells don't mature at all. As a result, these premature leucocytes don't work effectively which weakens the immune system. In addition the chromosomal changes alter the cell's normal cell cycle. As a result the cells

start to divide too quickly and in CLL the cells don't die when they should. And in both the situations there are too many of these premature cells. So overtime, premature leucocytes accumulate in the bone marrow, which ultimately spreads into blood. Some of these cells settle down in the organs and tissues across the body but others keep circulating into the blood. With a bunch of extra cells in the blood, the healthy cells get "crowded out" and it's tough for them to survive with the extra competition for nutrients. This causes cytopenias or a reduction in the number of healthy blood cells like anemia, thrombocytopenia and leukopenia.

1.1.1. Incidence

The American Cancer Society estimates that 15% of all new instances of leukemia in United States will be Chronic Myeloid Leukemia in 2021. It also states that 9,110 new cases (5,150 in men and 3,960 in women) will be diagnosed with CML and that may lead to deaths of around 1,220 people (680 men and 540 women). One in every 526 people in United States is expected to have CML in their lifetime. (*Key Statistics for Chronic Myeloid Leukemia*, n.d.) While the occurrence rate of US was 1.9 per 100,000 men and women in 2019(*Cancer Stat Facts: Leukemia — Chronic Myeloid Leukemia (CML)*, n.d.), the annual incidence of CML fluctuates between 0.4/100,000 people and 1.75/100,000 people in different countries. CML is infrequent in children under the age of 14 (0.7 per million children per year) and increases with age (Fuente et al., 2014) Furthermore, with a male/female ratio of 1.2–1.7, CML is more frequent in men than in women (Berger et al., 2005). To the best of our knowledge, no studies have been conducted to characterize the global distribution and burden of CML. CML is diagnosed at an average age of 64 years. People aged 65 and up account for nearly half of all cases. Adults are more commonly affected by this kind of leukemia, whereas children are rarely affected.(*Key Statistics for Chronic Myeloid Leukemia*, n.d.) (Lin et al., 2020)

1.1.2. Stages

CML is divided into three stages.

Chronic Phase: The majority of the time, a person learns that he or she has CML during the early "chronic" phase, but it can also happen at a later stage. In this phase a person has less than 10% immature white blood cells (blasts) in blood or bone marrow. In this stage, people may have few or no symptoms. Treatment as soon as possible can restore normal blood cell levels and alleviate any symptoms. Infections and bleeding (from cuts or bruises, for example) are infrequent during this stage. The purpose of treatment is to keep the patient in the chronic stage as long as possible.

Accelerated Phase: During this phase, the person has more than 10% but less than 20% blasts with high percentage of basophils. The amounts of blood cells grow higher or lower

than normal throughout this phase. In addition, "blasts," or immature blood cells, are found in the blood and bone marrow. People in this stage may feel exhausted, develop anemia due to a lack of red blood cells, and have an enlarged spleen. Symptoms and blood levels may not react to treatment as well as they should. The chromosomes of leukemia cells may have aberrant alterations.

Blast Phase: More than 20% of the blasts in blood or bone marrow are present during this phase. Blast cells frequently spread outside the bone marrow. The blood counts are abnormally low. Tiredness, fever, loss of appetite, bleeding, shortness of breath, and an enlarged spleen are all possible symptoms. This is a high-intensity period. This indicates that the cancer is rapidly spreading(“Chronic Myeloid Leukemia (CML): Phases,” n.d.).

1.1.3. Molecular Mechanism of CML

Chronic myeloid leukemia is a case of specific chromosomal abnormality resulting from the reciprocal translocation of chr9 and chr22. Chronic myeloid leukemia is characterized by the translocation of v-ABL (Abelson murine leukemia viral oncogene from chr9 with housekeeping gene BCR (breakpoint cluster region) on chr22 t(9;22).

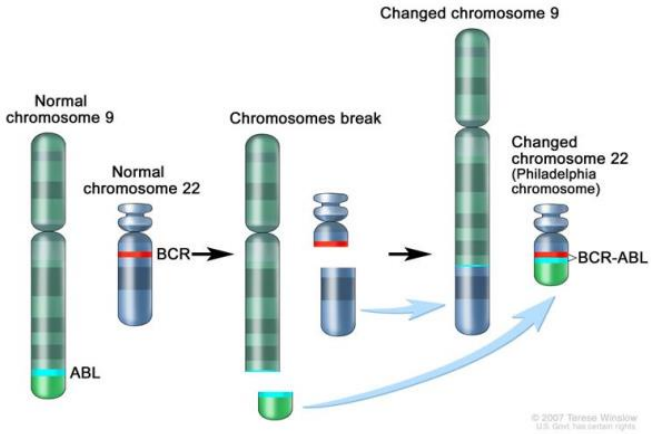


Figure 2. Translocation of BCR and ABL genes to form Philadelphia chromosome. A piece of chromosome 9 and a piece of chromosome 22 break off and trade places. The BCR-ABL gene is formed on chromosome 22 where the piece of chromosome 9 attaches. The changed chromosome 22 is called the Philadelphia chromosome. *Source: https://www.ncbi.nlm.nih.gov/books/NBK65740/*

The fusion results in Philadelphia chromosome and BCR-ABL gene which is transcribed to BCR-ABL mRNA and translated to BCR-ABL protein which is capable of autophosphorylation of tyrosine kinases. These tyrosine kinases are like ON/OFF switch for various cellular functions including cell division. When the BCR-ABL fusion protein turns on the tyrosine kinases, it forces myeloid cells to keep dividing quicker than they should which causes the buildup of the premature leucocytes in the bone marrow that eventually spill into the blood. The premature leukocytes then move into the liver and spleen causing swelling of those organs or “hepatospleenomegaly”. Since these CML cells

divide quicker than they should, there is high chance that further genetic mutation can happen. And if that happens CML might progress into more serious acute leukemia, which is also called blast crisis because the totally immature cells of acute leukemia are referred to as blasts. A lot of cases of these blast crisis include formation of trisomy of chromosome number 8 with the doubling of Philadelphia chromosome.

1.1.1. CML transcripts

The BCR gene in Chr22 constitutes 23 exons and the ABL in Chr9 constitutes 11 exons. Generally, three types of BCR/ABL transcripts are formed based on the breakpoint in the BCR gene and ABL gene. Breakpoints in the ABL gene's introns 1 or 2 and the BCR gene's major breakpoint cluster region (M-bcr), generally occurs forming the major transcript. The breaks occur either between exons 13 and 14 (b2) or 14 and 15 (b3) resulting either b2a2 or b3a2 mRNA. The end outcome of this genetic rearrangement is p210BCR/ABL, a cytoplasmic fusion protein of 210 kDa that is required for CML malignancy and responsible for the phenotypic abnormalities seen in chronic phase CML. (Salesse & Verfaillie, 2002) Besides, break in exon e1 leads to e1a2 typically termed as minor transcript and break in exon 19 leads to e19a2, micro transcript. The minor and micro transcripts encode 190KDa and 230KDa proteins respectively. Atypical BCR/ABL transcripts, such as those featuring ABL exon a3 instead of a2, are less common causes of CML (Salesse & Verfaillie, 2002).

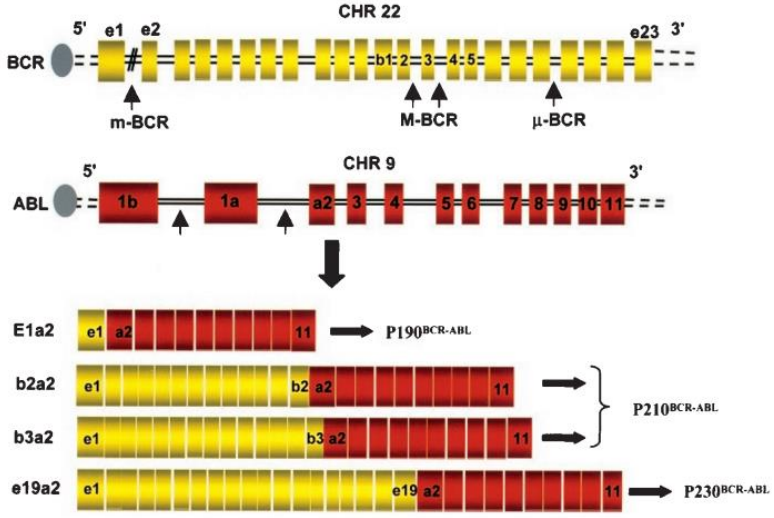


Figure 3. Locations of the breakpoints in the ABL and BCR genes and structure of the chimeric BCRABL mRNA transcripts, *Source: Salesse and Verfaillie, (2002)*.

1.1.2. Diagnosis of CML

Generally the cases of CML go unnoticed and are asymptomatic to be diagnosed early. But with the progression of disease, the person may feel tired, weight loss, drenching night sweats, fever and pain or feeling of fullness below ribs on left side. With these symptoms, the person may consult a physician who will perform the following multiple tests and procedures to diagnose the condition:

1.1.2.1. Physical Examination

In most cases, splenomegaly or enlargement of spleen is the characteristic feature of CML caused by the deposition of excessive immature cells in the spleen. This can further be aided by different imaging tests such as Computed Tomography (CT) scan or Ultrasound to get a clear picture of the spleen.

1.1.2.2. Blood Counts

Generally a complete blood count (CBC), is performed to identify a person with CML. The spleen enlargement is often clinically manifested by the increase in immature White blood cells (Deshpande, et.al. 2020) count exceeding 250,000/µL, which is generally 5000 to 10000/µL in a normal person. The red blood cell count is generally reduced with variable no. of platelets counts based on the severity. .

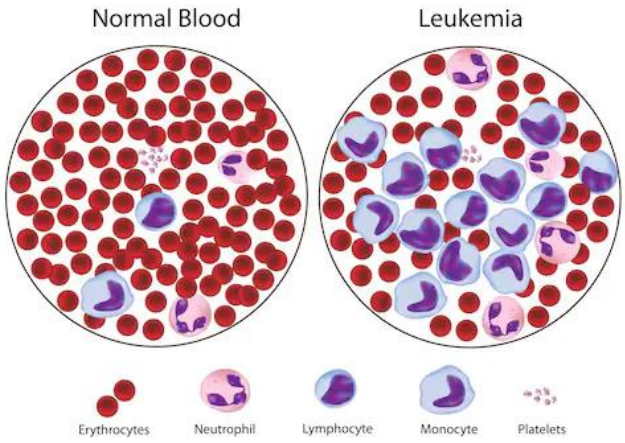


Figure 4. Comparision between normal and leukemic blood under microscope. The normal cells consist higher erythrocytes and lesser granulocytes. The leukemic cells contain higher no. of monocytes and lymphocytes. *Source: Deshpande et.al., 2020.*

In most CML patients, the Philadelphia chromosome is the significant characteristic feature. During Karyotyping, the number, shape, size and arrangement of chromosomes can help determine presence of Philadelphia chromosome thereby CML.

1.1.2.4. Fluorescent InSitu Hybridization

Fluorescent Insitu Hybridization is one effective way for identification of Philadelphia chromosome more sensitive than karyotyping. It uses blood or bone marrow samples and can be used to detect BCR-ABL gene for diagnosis and monitoring treatment.

1.1.2.5. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) tests are the most sensitive among all the available tests for BCR-ABL identification. These tests can be performed in blood or bone marrow samples and may even be used for quantification of the Philadelphia chromosome to

determine the level of severity or monitor the mutation levels at treatment. The tests are even used for determination of variation of transcript types using fluorescent probes.

1.1.3. Treatment and Resistance

Prior to the discovery of BCRABL1, cytotoxic medicines including busulfan and hydroxyurea were used to treat CML (Salesse & Verfaillie, 2002). Although successful chemotherapy might lower the white blood cell count, prevent problems from hyperleukocytosis, and control the clinical signs of CML, it had no effect on the disease's natural history, as most patients would eventually move to the blast phase(Faderl, Talpaz, Estrov, & Kantarjian, 1999). When compared to cytotoxic medicines, the introduction of recombinant interferon-alfa (rIFN-) offered a considerable survival advantage (Inst, 1997). The majority of patients who received rIFN- monotherapy obtained hematologic remission; however, only a small percentage of patients (13 percent to 27 percent) attained complete cytogenetic remission (Bonifazi et al., 2001). Furthermore, the majority of patients were unable to tolerate rIFN- due to its side effects (Hehlmann et al., 1994). The only proven curative treatment for CML was allogeneic hematopoietic cell transplantation (HCT) (Silver et al., 1999).

However, because to a high rate of morbidity and death, as well as a scarcity of HLA-matched donors, only a tiny number of CML patients can benefit from HCT (Baccarani et al., 2006). As the complex mechanisms of CML become clearer, researchers are focusing their efforts on creating drugs that target the abnormal BCR-ABL tyrosine kinase. The first BCR-ABL tyrosine kinase inhibitor (TKI) to be utilized for CML treatment was imatinib mesylate (IM, STI-571, or Gleevec) (Kinases, Therapeutic, & Leukemia, 2002). However, point mutations in the ABL kinase domain or overexpression of BCR-ABL have been linked to imatinib resistance in individuals with advanced illness in numerous investigations (Sawyers et al., 2002)(Gorre et al., 2001). As a result, several other new TKIs, including as dasatinib (BMS-354825, Sprycel), nilotinib (AMN107, Tasigna), and bosutinib (SKI-606), were developed and evaluated in patients with BCR-ABL positive CML to overcome imatinib resistance(Gorre et al., 2001)(Shah et al., 2004)(Shah et al., 2004).

The introduction of these TKIs altered the prognosis and treatment options for people with newly diagnosed CML. BCRABL TKIs are currently the first line of treatment for most CML patients. However, currently multiple CML cases tend to develop resistance to TKI. While most of the study explains point mutation as the cause for resistance, this study explores the impact of transcript type variation on resistance(An et al., 2010).

1.2. Research Questions/ Research Hypothesis

- What is the frequency of different BCR-ABL1 transcripts in Nepalese Population with chronic myeloid leukemia?
- Is CML more common in specific age group, gender or ethnicity?
- Is Drug resistance to CML drugs more prevalent in one transcript type?

Null hypothesis:

Ho_1 : The frequency of p210, p190 and p230 transcripts in CML cases are similar.

Ho_2 : CML is common in all ages, gender and ethnicity.

Ho_3 : Affinity to CML drugs is independent to transcript type.

Alternative hypothesis:

$H1_1$: The frequency of p210 transcripts are greater than p190 and p230 transcripts in CML.

$H1_2$: CML affects specific age, gender and ethnicity.

$H1_3$: Affinity of CML drugs is higher in particular transcript type.

1.3. Objectives

1.1.1. General Objective

- Microscopic and molecular analysis(BCR-ABL1 transcript) of Chronic myeloid leukemia in Nepalese population.

1.1.2. Specific Objective

- Identification of Chronic myeloid leukemia by microscopy
- Real time PCR based identification of BCR-ABL1 transcripts in suspected samples.
- Determination of frequency of p210, p190 and p230 transcripts.
- Analysis of the diagnosed cases according to the age, gender and ethnicity.
- Analysis of correlation between transcript type and drug affinity.

1.4. Statement of Problem

Western literatures suggest that p210 is the most common transcript type seen in CML in upto 95% of cases. Some reports from India and Pakistan suggest only the p210 transcript type found in their reports. There is no data from Nepal on this research question. Hence this study may provide some insight regarding the transcript type among CML patients in Nepal. In addition, this study also addresses the risk of developing resistance against CML drugs with variation of transcript types.

Chapter 2

2. Literature Review

2.1. History

Figure 5.Peter Nowel (left) and David Hungerford(right) while collaborating in cytogenetics in the early 1960s. *Source: Knutsen 2011.*

The first descriptions of chronic myeloid leukemia (CML), or chronic granulocytic leukemia as it was known at the time, date back to 1845. At the time, two independent pathologists, John Bennett and Rudolf Virchow, published case reports of individuals with splenomegaly, an enlarged liver, and leukocytosis (Pai-Dhungat, 2015)(Bennett, 1845). However, little was known about the condition, if anything at all. Virchow created the term "Leukämie," which means "white blood," to describe the assumption that observed symptoms were caused by changes in normal hematopoiesis (Pai-Dhungat, 2015). Ernst Neumann diagnosed leukemia as a disease originating in the bone marrow not long after. However, it took nearly a century for fresh knowledge about the condition to emerge. After improving a method of visualizing chromosomes in mitotic cells (karyotyping), Peter Nowell and David Hungerford reported the identification of an abnormal "minute chromosome" in patients with chronic granulocytic leukemia, marking the first discovery of a link between chromosomes and cancer(Knutsen, 2011)(A.J.Clarke, J.E. Bailey, 1973). The "Philadelphia (Ph) chromosome" was later named to this chromosome. Janet Rowley discovered that the minute chromosome was the consequence of a reciprocal translocation between chromosomes 9 and 22 (t(9;22)) in 1973 utilizing chromosomal staining methods such as quinacrine fluorescence and Giemsa banding(A.J.Clarke, J.E. Bailey, 1973).

This was the first time a chromosomal translocation had been linked to cancer. Hematopoietic stem cells (HSC), which are at the apex of the hematopoietic hierarchy, are the cells that undergo this translocation (Figure 1). Nora Heisterkamp and Jim Groffen discovered the human equivalent of the Abelson murine leukemia viral oncogene (v-Abl), ABL1, in the region of chromosome 9, which translocates to chromosome 22 (Heisterkamp et al., 1983)(Bartram, 1984) a few years later. Only patients with the Ph chromosome were found to have the ABL1 proto-oncogene translocated (Minciacchi, Kumar, & Krause, 2021). Furthermore, a tiny region of up to 5.8 kb on chromosome 22 was identified, which was dubbed the "breakpoint cluster region" (BCR) since it contained all of the translocation breakpoints (Groffen et al., 1984). BCR was later given to the gene on chromosome 22 that fuses with ABL1. The majority of CML cases are caused by a BCRABL1 breakpoint, which results in a BCR-ABL1 oncoprotein of 210 kDa (p210) by fusing exons 13 or 14 of BCR with exon 2 of ABL1 (named b2a2 or b3a2, respectively) (Groffen et al., 1984). In individuals with CML, subsequent studies revealed the presence of a chimeric mRNA arising from the fusion of the two genes, BCR and ABL1 (Ghalesardi et al., 2021). In the same year, Konopka and coworkers discovered that the Ph+ human leukemia cell line K562 had an altered version of the c-ABL protein (p210) with tyrosine kinase activity (Konopka, Watanabe, & Witte, 1984). This protein was discovered to be the result of a fusion gene resulting from the t(9;22) translocation (Nicol, 1947), which caused hematopoietic cells to undergo cellular transformation (Nicol, 1947)(Daley & Baltimore, 1988). Retroviral transduction of mouse bone marrow with the BCR-ABL1 fusion resulted in a CML-like myeloproliferative disease in mice (Andreas Hochhaus, Eigendorff, & Ernst, 1990), demonstrating the fusion protein's carcinogenic potential and important role in CML. In conclusion, BCR-ABL1 emerged as a key factor in the pathogenesis of CML, with the production of its oncoprotein leading to clonal growth of hematopoietic cells carrying this fusion gene. CML and its discoveries have served as a model for a variety of cancers.

2.2. Characteristics of CML

BCR–ABL1 positive cells are genetically unstable and prone to developing multiple and heterogeneous genomic abnormalities, leading to the transformation of the leukaemic phenotype from chronic to acute, and thus progression from chronic (CP) to accelerated (AP) and blast (BP) phases [5, 6]. The WHO and European Leukemia Net (ELN) definitions of CML stages are compared in Table below:(*Hochhaus et al., 2017*)

Table 1: Characteristics of CML. Clinical and haematological criteria for the definition of AP and BP according to WHO and ELN.

	Accelerated phase		Blast phase	
	WHO	ELN	WHO	ELN
Spleen	Persisting or increasing splenomegaly unresponsive to therapy	–	–	–
WBC count	Persisting or increasing WBC count (> 10 x 10^9/L) unresponsive to therapy	–	–	–
Blast cells[a]	10%–19%	15%–29%	≥ 20%	≥ 30%
Basophils[a]	> 20%	> 20%	–	–
Platelet count	> 1000 x 10^9/L uncontrolled by therapy < 100 x 10^9/L unrelated to therapy	– Yes	– –	– –
CCA/Ph+	Any new clonal aberration during therapy Additional clonal chromosomal abnormalities in Ph cells at diagnosis that include 'major route' abnormalities (second Ph, trisomy 8, isochromosome 17q, trisomy 19), complex karyotype or abnormalities of 3q26.2	Present	–	–
Extramedullary involvement[b]	–	–	Present	Present
'Provisional' response-to-TKI criteria	Haematological resistance to the first TKI (or failure to achieve a complete haematological response[c] to the first TKI) or Any haematological, cytogenetic or molecular indications of resistance to 2 sequential TKIs or Occurrence of 2 or more mutations in BCR–ABL1 during TKI therapy			

The criteria of AP are different, reflecting the difficulty of making the diagnosis of this transitory phase. The criteria of BP differ only for the percent of blast cells. Only one of the listed criteria is sufficient for the diagnosis of AP or BP.
[a]In peripheral blood or in BM.
[b]Excluding liver and spleen, including lymph nodes, skin, CNS, bone and lung.
[c]Complete haematological response: WBC < 10 x 10^9/L; platelet count < 450 x 10^9/L, no immature granulocytes in the differential and spleen non-palpable.
AP, accelerated phase; BM, bone marrow; BP, blast phase; CCA/Ph+, clonal chromosome abnormalities in Ph+ cells; CNS, central nervous system; ELN, European LeukemiaNet; Ph, Philadelphia; TKI, tyrosine kinase inhibitor; WBC, white blood cell; WHO, World Health Organization.

BCR is a Break point cluster region representing gene found in Chr22 (22q.11.23), starting at 23180509bp and ending at 23318037bp from pter (according to GRCh38/hg38-Dec_2013)("BCR (Breakpoint cluster region)," n.d.). BCR gene constitutes 23 exons of 130kb spanning 5'centromer to 3' telomere("BCR (Breakpoint cluster region)," n.d.), with alternate exon 1 and exon 2 coding for a 1271-amino-acid protein(Peiris, Li, & Donoghue, 2019). The BCR protein has a complex structure with several domains. A potential serine/threonine kinase domain, a growth factor receptor bound protein 2 (Grb2) binding site, a BCR associated protein-1 (BAP-1) interaction site, and two SH2 domains make up the first exon. Exons 3–8 have a core guanine nucleotide exchange factor (GEF) domain, followed by exons 19–23 including a RacGap domain and a PSD95, Dlg1, Zo-1 (PDZ) domain binding motif(Chen, Niu, Xu, Wu, & Shi, 2007). BCR has an anti-parallel coiled-coil oligomerization domain that is important for the fusion partner's kinase activity (Xun, Saghi, Harvey, Malashkevich, & Kim, 2002)(McWhirter, Galasso, & Wang, 1993). The BCR oligomerization domain is the sole therapeutically targetable domain in BCR that has been discovered thus far.

Exon 1 of BCR contains a potential serine/threonine kinase and two SH2 domains. BCR has been demonstrated to autophosphorylate on serine and threonine residues and can

phosphorylate both casein and histones (Maru & Witte, 1991), despite its poor similarity to other known serine/threonine kinases. Furthermore, BCR's cysteine 332 is required for its kinase activity, as mutations affecting C332 result in the loss of autophosphorylation activity (Maru & Witte, 1991). Two SH2 domains in the BCR engage with the ABL SH2 binding sites. On BCR exon 1, these SH2 domains cover amino acids 192–242 and 293–413. Through phosphorylated serine and phosphorylated threonine residues, full-length BCR binds selectively to the SH2 binding region on ABL(Maru & Witte, 1991). Furthermore, at Y177 in BCR, it is known that BCR interacts with growth factor receptor bound protein2 (Grb2). Grb2 SH2 domain interacts with Y177 on BCR and this contact is mediated by tyrosine phosphorylation. When BCR Y177 is changed to phenylalanine, the connection is disrupted, resulting in a considerable reduction in Ras pathway activation, as found in BCR-ABL. (Peiris et al., 2019)

Besides, BCR has also been found fused to Fibroblast Growth Factor Receptor 1 (FGFR1), Platelet Derived Growth Factor Receptor Alpha (PDGFRA), Ret Proto-Oncognene (RET), and Janus Kinase 2 (JAK2). BCR fusion proteins, which are cancer drivers, have basically been discovered in hematological cancers. However, the BCR gene's endogenous function is yet unknown.

2.4. ABL gene

ABL, Abelson murine leukemia virus (ABL) gene, found in chr9 (9q34.12), maps from 130713042 to 130887674 encoding 174633bp gene. ABL encodes a nonreceptor tyrosine kinase with a size of 145 kDa. Based on alternatively spliced initial exons, the ABL gene is produced as a 6- or 7-kb mRNA transcript. Exon 1a (ABL1A) encodes the N-terminal portion of the ABL protein, which is thought to be localized in the nucleus, whereas exon 1b (ABL1B) encodes the N-terminal glycine, which is myristylated and thus thought to drive the protein to the plasma membrane(Chissoe et al., 1995).

Three SRC homology domains (SH3, SH2, and SH1), as well as a C-terminal domain, make up the domains of ABL1. SH1 has ABL1 kinase activity, which is important in CML leukemogenesis, while SH2 and SH3 are involved in SH1 kinase activity control. BCR-ABL1 is constitutively active by numerous methods, whereas normal ABL1 kinase is carefully regulated. The deletion of the N-terminal sequence for myristoylation (N-cap) is one of the most important. Self-inhibition is maintained by myristoylated N-cap interacting with the C-terminal lobe; however, this may be lost following fusion with the BCR gene, resulting in kinase dysregulation.

The ABL1 kinase is found all over the body. It interacts with a variety of biological processes such as cell proliferation, differentiation, survival, retraction, migration, and cytoskeleton remodeling in response to external stimuli, cell motility and adhesion,

receptor endocytosis, autophagy, DNA damage response and apoptosis. (Wang, 2014)(Soverini et al.2018). In lymphocytes, neurons, and the intestinal epithelium, it is also involved in the regulation of specialized functions. The ABL1 kinase has a wide range of functions, including serving as a shuttle or hub in a variety of cellular settings. ABL1 interacts with a variety of different proteins involved in signaling pathways, kinases, transcription factors, and cell cycle regulators to accomplish this. Loss of autoinhibition and constitutional activation of ABL1 may occur when the mutant BCR-ABL1 protein is produced, with consequences on signaling pathways related to cell cycle and apoptosis, such as the RAS/RAF/MEK/ERK pathway, the JAK2/STAT pathway, and the PI3K/ AKT/mTOR pathway; and finally, it promotes the malignant transformation of hematopoietic cells (Soverini et al., 2018).

2.5. Structure and regulation of tyrosine kinase

The BCRABL1 protein shape and domain composition are determined by the precise genomic breakpoint. In CML cells, DNA breakage occurs in a small area, principally on the major BCR (M-BCR), resulting in an 8.5 kb mRNA product and the characteristic 210 kDa BCR-ABL1 protein (p210BCRABL1) (Clark, Mclaughlin, Crist, Champlin, & Witte, 1987)(Groffen et al., 1984). The Ph chromosome is usually seen in B-cell acute lymphoblastic leukemia (B-ALL), however it has a different constitutional breakpoint than CML (Hermans et al., 1987). A smaller mRNA and 185/190 kDa protein (p190BCRABL1) result from DNA breaks within the minor BCR (m-BCR). These protein–disease associations, however, are not always accurate. The generation of multiple BCR-ABL1 fusion transcripts can be influenced by DNA breakage and subsequent splicing.

An N-terminal (oligomerization) domain, a serine/threonine kinase domain (containing a docking site, Y177), a RAS homolog gene family/guanine nucleotide exchange factors (Rho/GEF) kinase domain, and/or a calcium-binding domain (CalB) are among the domains transcribed from the altered BCR [17]. The BCR-ABL1 protein has two lobes in its three-dimensional structure: an N-terminal lobe with five -sheets and a conserved -helix, and a C-terminal lobe with -helices (Panjarian, Iacob, Chen, Engen, & Smithgall, 2013). There are three components that connect the N- and C-terminal lobes: the catalytic segment, the P-loop (phosphate-binding loop), the A-loop (activation loop), and the hinge region, which consists of a 'cleft' between the two lobes. This cleft's ATP-binding and catalytic sites are substantially conserved. When the A-loop on the C-terminal lobe binds to ATP, it changes shape and travels away from the ABL1 kinase's catalytic core, generating an open conformation opposite the inactivated-closed conformation. On the A-loop, tyrosine 393 (Y393) is a crucial residue for activation and substrate binding. The A-loop bends toward the catalytic core and prevents substrate binding when unphosphorylated Y393 forms a hydrogen bond with Asparagine 363 (D363) on the catalytic segment. In addition, at

positions 381–383, the A-loop contains the DFG motif, a highly conserved amino acid residue sequence that is required for kinase activation. It binds to free magnesium ions, which act as catalytic cofactors. In the inactive state, the DFG motif stays away from the catalytic center, but when the A loop is activated, it moves into the catalytic site.

2.6. Signalling pathways in CML

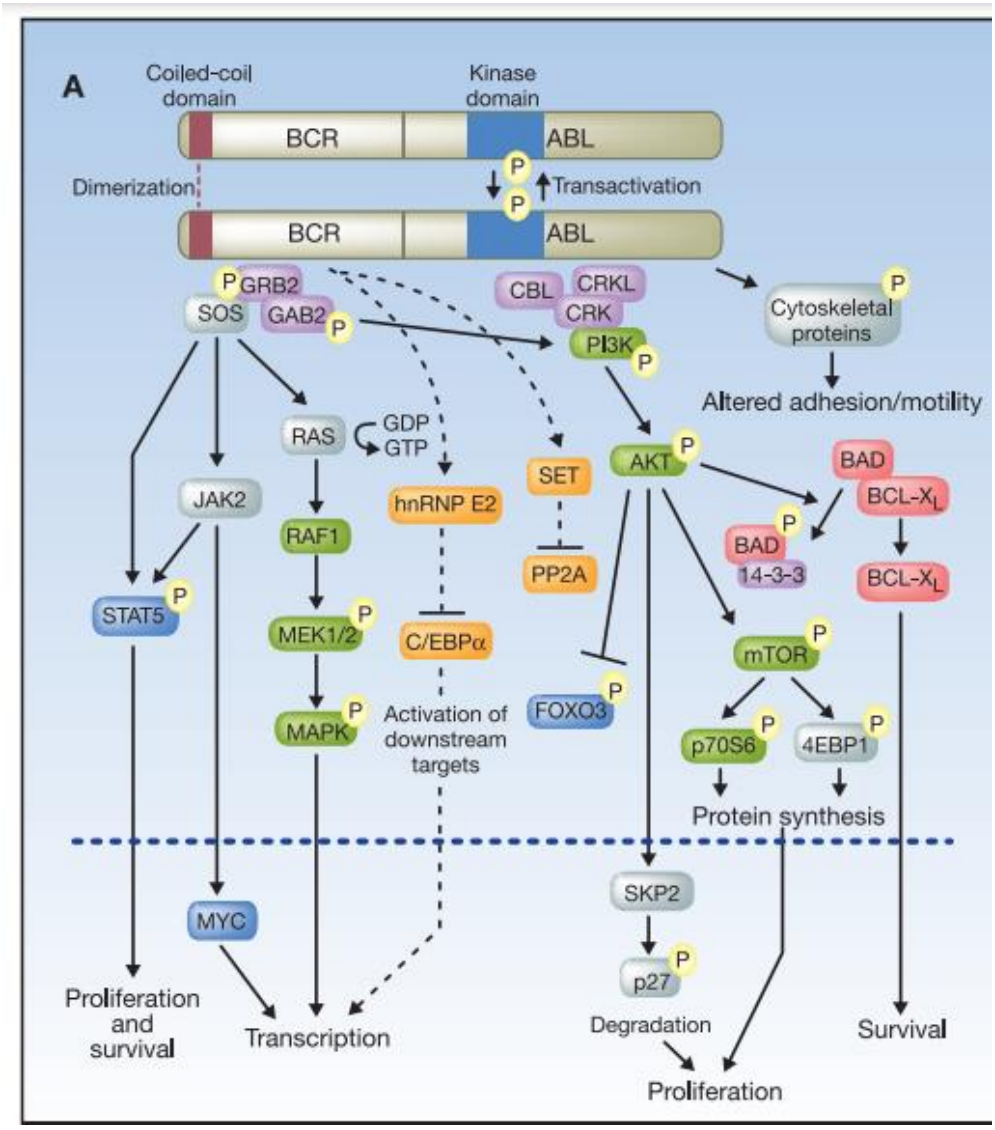


Figure 6. BCR-ABL signaling pathways in CML. Dimerization of BCR-ABL triggers autophosphorylation events that activate the kinase and generate docking sites for intermediary adapter proteins (purple) such as GRB2. BCR-ABL–dependent signaling facilitates activation of multiple downstream pathways that enforce enhanced survival, inhibition of apoptosis, and perturbation of cell adhesion and migration. A subset of these pathways and their constituent transcription factors (blue), serine/threonine-specific kinases (green), and apoptosis-related proteins (red) are shown. A few pathways that were more recently implicated in CML stem cell maintenance and BCR-ABL–mediated disease transformation are shown (orange). *O.Hare et.al.,2010.*

BCR-ABL is a constitutively active tyrosine kinase that promotes cell survival and proliferation via many downstream pathways. BCR-ABL dimerization and trans-autophosphorylation are facilitated by the N-terminal coiled coil domain (McWhirter et al., 1993)(Xun et al., 2002). The autophosphorylation of BCRABL's tyrosine-177 promotes the development of a GRB2 complex with GAB2 and son-of-sevenless (SOS), which activates RAS and recruits phosphatidylinositol 3-kinase (PI3K) and the tyrosine phosphatase SHP2 (Chu, Li, Singh, & Bhatia, 2007) (Sattler et al., 2002). RAS signaling triggers mitogen-activated protein kinase (MAPK), which promotes cell proliferation. PI3K activates the serine-threonine kinase AKT, which is involved in: (1) promoting cell survival by suppressing the activity of forkhead O (FOXO) transcription factors (Naka et al., 2010); (2) enhancing cell proliferation by proteasomal degradation of p27 via upregulation of SKP2, the F-Box recognition protein of the SCFSKP2 E3 ubiquitin ligase(Agarwal et al., 2008); and (3) activation of mTOR, which results in increased protein (Markova et al., 2010)(Ly, Arechiga, Melo, Walsh, & Ong, 2003). STAT5 activation, either directly or indirectly through phosphorylation by HCK or JAK2, is another important BCR-ABL outlet (Ilaria & Van Etten, 1996)(Klejman et al., 2002); STAT5 deficiency prevents both myeloid and lymphoid leukemogenesis (Hoelbl et al., 2010). These pathways work together to control gene transcription (Fig.6).

2.7. Targeting tyrosine kinase in CML management

BCR-ABL1 has been the principal therapeutic target for the last two decades since it is a druggable target uniquely expressed in CML cells and a key driver of leukemogenesis (Andreas Hochhaus et al., 1990)(Kelliher, 1990). Although it is still debatable whether the BCR-ABL1 fusion protein alone is sufficient to launch and maintain CML's leukemogenic process, BCR-suppression ABL1's of tyrosine kinase activity is one of the most successful treatment techniques in cancer medication development history. BCR-ABL1 inhibitors are divided into two kinds based on their method of action: ATP-competitive inhibitors and allosteric inhibitors.

2.7.1. ATP-competitive inhibitors

Inhibitors that compete with ATP for binding to the ABL1 kinase domain through the cleft between the N- and C-terminal lobes are known as ATP-competitive inhibitors. Because inactivated conformations of many kinases are relatively similar, TKIs like imatinib, nilotinib, and ponatinib have been used to target them (Kelliher, 1990).

2.7.1.1. Imatinib

Imatinib mesylate (STI-571), a first-generation TKI that targets BCR-ABL1, was discovered through a screening technique of potential chemical compounds that selectively bonded

to BCR-inactive ABL1's conformational shape(Lee, Basso, & Kim, 2021). Imatinib mesylate is a 2-phenylaminopyrimidine tyrosine kinase inhibitor that targets ABL, platelet-derived growth factor receptor, c-kit, and the Albeson-related gene (Druker, 2003). Imatinib mesylate binds to the amino acids in the ATP binding site of the BCR/ABL tyrosine kinase, stabilizing the inactive, non-ATP-binding form of BCR/ABL and inhibiting tyrosine autophosphorylation and, as a result, phosphorylation of its substrates. The downstream signaling pathways that support leukemogenesis are "switched off" as a result of this process. The Food and Drug Administration (FDA) granted first approval in May 2001 based on better clinical activity in CML patients compared to the standard of therapy at the time (Cohen et al., 2002). Imatinib enhanced progression-free survival (PFS) and overall survival (OS) by 80–90% and 90–95%, respectively, in the IRIS trial, and was linked with greater rates of response than the control arm, cytarabine with interferon-alpha (O'Brien et al., 2003)(Andreas Hochhaus et al., 2017). Fluid retention, gastrointestinal issues, musculoskeletal pain, drug eruption, chronic weariness, and myelosuppression are all possible side effects of using it. Various mutations in the ABL1 kinase region, however, have been reported to give imatinib resistance(Gorre et al., 2001) .

2.7.1.2. Nilotinib

Nilotinib is a second-generation tyrosine kinase inhibitor that was developed to overcome imatinib resistance in a variety of BCR-ABL mutations. It causes faster and deeper molecular responses in a higher percentage of patients than imatinib when administered as a first-line treatment in newly diagnosed chronic myeloid leukemia (CML)(Sacha & Saglio, 2018). Nilotinib is an N-[3-[3-(1H-imidazolyl)propoxy]phenyl] compound. 4-methyl-3-[[4-(3-pyridinyl)-2 pyrimidinyl[amino]benzamide. Nilotinib's binding site is more lipophilic than imatinib's, resulting in greater efficacy and selectivity. Furthermore, nilotinib may bind to the ABL kinase domain's inactive conformation. The H-bond interactions with Glu286 and Asp381 are preserved thanks to alternative binding groups to the Nmethylpiperazine moiety and the retention of an amide pharmacophore. Nilotinib's efficacy is increased by a phenyl group with trifluoromethyl and imidazole substituents, which is linked to a reduction in hydrogen-bond interactions to four(E. Weisberg et al., 2006). Nilotinib has a different structure than imatinib, which results in better affinity and a 20-fold stronger inhibition of wild-type BCR-ABL kinase (Golemovic et al., 2005)(O'Hare et al., 2005)(Ellen Weisberg et al., 2005).

However, nilotinib should be taken with caution in individuals with a history of cerebrovascular accidents, coronary artery disease, or peripheral arterio-occlusive disease (A. Hochhaus et al., 2020), and it should be used with caution in patients with cardiovascular or metabolic comorbidities, such as diabetes mellitus. Nilotinib-associated

cardiovascular events risk has been demonstrated to steadily increase over 5-10 years in long-term follow-up evidence.(Kantarjian et al., 2021)

2.7.1.3. Dasatinib

Dasatinib (BMS-354825, Sprycel®; Bristol-Myers Squibb, New York, NY, USA) is a powerful BCR-ABL multitarget kinase inhibitor of the second generation. In vitro, this compound is more effective than imatinib against unmutated ABL kinase. Dasatinib was designed to block the Src family of kinases, including Fyn, Yes, Src, and Lyk, but it also blocks BCR-ABL, EphA2, platelet-derived growth factor receptor, and c-Kit. It also interacts to other tyrosine and serine/threonine kinases, such as mitogen-activated protein kinases and the discoidin domain receptor 1 receptor tyrosine kinase. (Rix et al., 2007). Except for those with the T315I mutation, dasatinib can reduce the proliferation and kinase activity of wild-type and BCR-ABL mutant cell lines that are resistant to imatinib. (Cortes et al., 2020; O'Hare et al., 2005) Dasatinib is 325-fold more potent than imatinib and 16-fold more potent than nilotinib, against unmutated BCR-ABL, according to in vivo investigations (Müller et al., 2008).

2.7.1.4. Bosutinib

Bosutinib, a 4-[(2,4-dichloro-5-methoxyphenyl)amino]-6-methoxy-7-[3-(4-methylpiperazin-1-yl)propoxy] quinoline-3-carbonitril, is a Src tyrosine kinase inhibitor identified by Boschelli in 2001 (Boschelli et al., 2001). Bosutinib is a dual SRC/ABL kinase inhibitor currently approved in Europe and the United States for the treatment of adult patients with Philadelphia chromosome-positive CML in the chronic phase (CP), accelerated phase (AP), or blast phase (BP) who have previously received one or more TKIs and for whom imatinib, nilotinib, or dasatinib are not regarded as appropriate treatment options. The small molecule inhibitor was developed to treat SRC overexpressing solid tumors. However, its significant activity against BCR-ABL, as seen by a 100-fold increase in potency over IM, as well as a good safety profile with fewer side effects due to low inhibition of PDGFR or c-kit, propelled it into the spotlight as a potential CML treatment.

2.7.1.5. Ponatinib

Ponatinib is a third-generation TKI with the widest target spectrum. Ponatinib, like imatinib, targets the Abl kinase inactive conformation. It has a triple carbon–carbon connection (ethynyl linkage) between the purine and methylphenyl groups, which allows it to accommodate the Isl side chain without steric hindrance. In cellular studies, ponatinib compounds with changed ethynyl linkers show significantly reduced action against T315I,

emphasizing the importance of this area. Importantly, when Thr is changed to Isl, the hydrogen bond is preserved.(Frankfurt & Licht, 2013) Ponatinib is a highly effective treatment for individuals who have developed resistance to a second generation TKI, which is frequently caused by BCR-ABL1 point mutations. To yet, only the T315M mutation in the ATP-binding site has been identified as conferring ponatinib resistance (Deininger et al., 2016) . In vitro results suggest that some compound mutations including T315I (two mutations in the same BCR-ABL1 molecule, hence in the same clone) may affect ponatinib sensitivity and clinical response (Zabriskie et al., 2014) (Müller et al., 2017).

The potential efficacy of ponatinib as a multi-kinase inhibitor against many malignancies, including CML, has been examined. Ponatinib was authorized by the FDA in 2012 based on the PACE trial's remarkable efficacy against CML (Cortes et al., 2012). However, due to the alarming increase in arterial occlusive events (AOEs), which is linked to several targets involving endothelial function and atherosclerosis (Lipton et al., 2016)(Cortes et al., 2012), clinical use should be done with caution.

2.7.2. Allosteric Inhibitors

The activity of the ABL kinase is regulated by intramolecular/intermolecular interactions as well as post-translational changes(Toosi, 2014a). Myristate binding is an important intramolecular interaction for ABL kinase activity. When a myristoylated residue in the N-lobe binds to a hydrophobic pocket in the C-lobe, trapping ABL1/ABL2 into a "closed," or catalytically inactive, conformation, ABL kinases are inhibited. This inhibition echanism is termed as allosteric inhibition. (Jones, Thompson, Tisch, & Tumor, 2021)

Asciminib is an allosteric inhibitor. The myristoylated N-cap of ABL1 is one of the targets, as it modulates ABL1 kinase activity by attaching to a hydrophobic myristate pocket in the C-terminal lobe. This binding causes the kinase domain to shift conformation, which is required for contact with the SH3–SH2 domains, keeping the kinase inactive. The removal of the N-cap myristoyl group happens during BCR-ABL1 gene translocation and transcription, resulting in the kinase's constitutional activation. Compounds that bind to the myristate pocket may help to restore ABL1 kinase activity's normal control. This technique works as follows: is unaffected by the A loop's conformational shift, which is the focus of ATP-competitive inhibitors.

This shows that the two inhibitors could work together to overcome resistance to pre-existing TKIs that bind to the ATP-binding pocket. The only known allosteric inhibitor with

therapeutic efficacy in CML is asciminib (ABL001). It showed promise against CML that had failed three or more ATPcompetitive TKIs and was resistant to them(MICHA, 2017). Asciminib has a very selective on-target impact against the ABL1 kinase with no substantial off-target effects because it binds to the myristate pocket rather than other orthosteric locations(Wylie et al., 2017) . It does not, however, block ABL1-dependent cell growth (Manley, Barys, & Cowan-Jacob, 2020). According to preclinical investigations, asciminib can be coupled with other TKIs to boost efficacy against CML with resistant mutations(Lindström & Friedman, 2020)(Cross, Sarah J. Linker, Kay E. Leslie, 2016). Rash, tiredness, nausea, headache, arthralgia, and pancreatitis were all common side effects of asciminib.

2.8. Resistance in CML

The most prevalent source of acquired resistance in BCR-ABL is point mutations, which typically occur in the Imatinib binding site, the P-loop, the A-loop, and the catalytic domain. (Cang & Liu, 2008). The T315I mutation, which confers resistance to Imatinib by destroying the hydrogen bond between threonine and Imatinib, sterically interfering with drug binding, is the most prevalent mutation in the Imatinib binding site (Patel et al., 2018). Because it limits ATP-competitive inhibitors' access to the active site, T315 is known as the "gatekeeper" residue(Toosi, 2014b) . Mutations in the ATP-binding P-loop, such as G250E, Q252H/R, and E255K, or the activation A-loop, such as H396R, may destabilize the inactive kinase conformation required for Imatinib binding (Reddy & Aggarwal, 2012). In the catalytic domain, replacing F359 with valine may disrupt van der Waals interactions with Imatinib's piperazine ring(Konermann, 2019). The discovery of resistance-causing mutations has driven a quest for medications that can overcome them.

2.9. Diagnosis of CML

The BCR-ABL gene is present in all the cases of Chronic myeloid leukemia (CML). Hence BCR-ABL can be used as a unique marker for diagnosis of Chronic myeloid leukemia. BCR-ABL translocation results in the formation of different types of fusion transcripts depending upon the breaks in BCR region (Irshad, Butt, & Joyia, 2012) These fusion transcripts encode proteins of different sizes. In general three breakpoint clusters have been seen: M-bcr, m-bcr and µ-bcr. M-bcr has two major breakpoints b2a2(e13a2: exon13 of BCR gene and exon 2 of ABL gene) and b3a2(e14a2) resulting in fusion mRNA translated into p210 protein, m-bcr results in e1a2 junction which is translated into p190 protein and µ-bcr has breakpoint between exon 19 and 20 inducing a larger protein p230 (Goh et al., 2006). The variation in transcripts results in the difference in disease outcomes of

patients. Several methods have been used to detect the fusion of BCR-ABL that includes conventional cytogenetics or FISH or RT-PCR(Reverse Transcriptase Polymerase Chain Reaction (Irshad, Butt, & Joyia, n.d.). RT-PCR is the most sensitive of all the methods, however RQ-PCR (Real-time Quantitative Polymerase Chain Reaction) overcoming the quantitative limitations of RT-PCR has been broadly used currently. In this study we have used Real-time Quantitative PCR for identification of the transcripts. However, considering the cost and dependency over this method we tried to design our own conventional primers to diagnose CML using Primer designing.

2.9.1. Primer Designing

A primer is a short synthetic oligonucleotide used in a variety of molecular methods, including PCR and DNA sequencing. These primers are made with a sequence that is the reverse complement of a portion of template or target DNA that we want to anneal to(“Primer Design,” n.d.). The primer designing takes few points into consideration:

1. The target template sequence should first be acquired from genebank and aligned to determine the targeted amplification site. This may be attained via https://www.ncbi.nlm.nih.gov/nuccore and Aliview.
2. The primer should be 18-22bp in length.
3. The melting temperature of primer should be in range of 52 °C to 58 °C (may vary).
4. The GC content of primer should be 40-60%.
5. The primers should not form hairpin loop, self-dimer or cross dimers between one another. In case of unavoidable dimers, ΔG upto -6kcal/mol may be tolerated.
6. Multiple consequetive repeats should be avoided in primers.(“Primer Design Guide for PCR :: Learn Designing Primers for PCR,” n.d.)

   The primer designing may be performed manually or using online tools like https://www.ncbi.nlm.nih.gov/tools/primer-blast/ . Further the quality assessment of primer may be performed using Oligocalc http://biotools.nubic.northwestern.edu/OligoCalc.html or Oligoanalyzer. https://sg.idtdna.com/pages/tools/oligoanalyzer .

Once designed and received, the primers may require to be optimized in wet lab before use. Depending upon the purpose, the primers may be designed accordingly as if it is for diagnosis only or sequencing too. Based on the PCR products’ analysis the frequency of different transcripts can be established and further their correlation with age, sex, lifestyle, caste and geography can also be analysed.

2.10. Virtual Screening

Virtual screening is a technique that is used to understand or predict the interaction between a ligand and a receptor of ligand usually being the substrate or analog or molecule that undergoes some change. The receptor or macromolecule being the protein or enzyme catalyzes or converts the ligand into a different product.

The virtual screening tools help to predict the interaction between the test ligand. The ligands may have the potential to be an inducer or inhibitor for the choice of protein or enzyme.

Virtual screening approaches can be roughly classified into two main types:

1. **Structure-based methods:** Structure-based virtual screening (SBVS) is a computer based method for searching a chemical compound library for novel bioactive compounds against a specific therapeutic target in early-stage drug development. It uses the three-dimensional (3D) structure of the biological target, as determined by X-ray, NMR, or computational modeling, to prioritize the compounds based on their binding affinity to the receptor for further biological evaluation (Sperandio, Villoutreix, & Miteva, 2011).
2. **Ligand-based methods:** Ligand-based virtual screening methods rely on the information contained in known active ligands rather than the structure of a target protein. When no 3D structure of the target protein is available, ligand-based approaches are the sole option. It searches databases for novel bioactive compounds using chemicals known to bind the target of interest as queries. As a result, ligand-based approaches are used to find structural analogs of known substrates that can be used as a competitive inhibitor of the target protein (Bottaro, Larsen, 2008).

2.9.1. Structure-Based Drug Design (Docking):

The basic steps for structure-based drug design are as follows:

1. **Protein Preparation (target preparation):**

The preparation of target sites is at the heart of structure-based drug design. Because SBDD relies on the target's high-quality 3D structure, the 3D structure's availability becomes critical. Many organisms' protein 3D structures are accessible in Protein Data Bank PDB format via protein structure databases such as PDB (http://www.rcsb.org/pdb/home/home.do) and UNIPROThttps://www.uniprot.org/), When the 3D structure is not accessible, homology modeling is used to predict the structure. SWISSMODEL (http://swissmodel.expasy.org/) can be used in predicting the

structure of the protein using similar templates. The GQME and QMEAN are generated to estimate the quality of the models.

The Global Model Quality Estimate (GMQE) is a quality estimate that takes into account attributes such as target-template alignment and template structure. The GMQE is available before we develop a model, and it can help us choose the best templates for the modeling problem we're working on. Once a model is developed, the GMQE is updated, taking into consideration the obtained model's global score to improve quality estimation reliability("Help | SWISS-MODEL," n.d.). Likewise QMEAN is a composite scoring function that can produce both global (i.e. for the entire structure) and local (i.e. per residue) absolute quality assessments.

Both of these scores are in the range [0,1], with one being excellent ("QMEAN," n.d.).

2.4.2. Ligand preparation:

Docking may be performed for the given target molecule using a single ligand or a database of ligands. The ligands for docking may be obtained from Pubchem (https://pubchem.ncbi.nlm.nih.gov/) for known ligands or Zinc database (http://zinc15.docking.org/) is also used for multiple screening. The ligands structure are basically saved in mol2 or sdf formats on download which may or may not be converted to pdb formats for docking ("Ligand Preparation," n.d.).

2.4.3. Docking and Scoring:

Predicting the binding locations and affinity of proteins and ligands based on their three-dimensional (3D) structures is a fundamental task for virtual screening for computer-aided drug discovery. A number of docking tools have been created to help with this task. The majority of them concentrate on docking in user-defined binding sites.

CBDock, (http://clab.labshare.cn/cb-dock/php/), a user-friendly blind docking web service, predicts binding sites of a given protein and calculates the centers and sizes using an unique curvature-based cavity detection approach, and docks with a popular docking application, Autodock Vina (Liu et al., 2020).

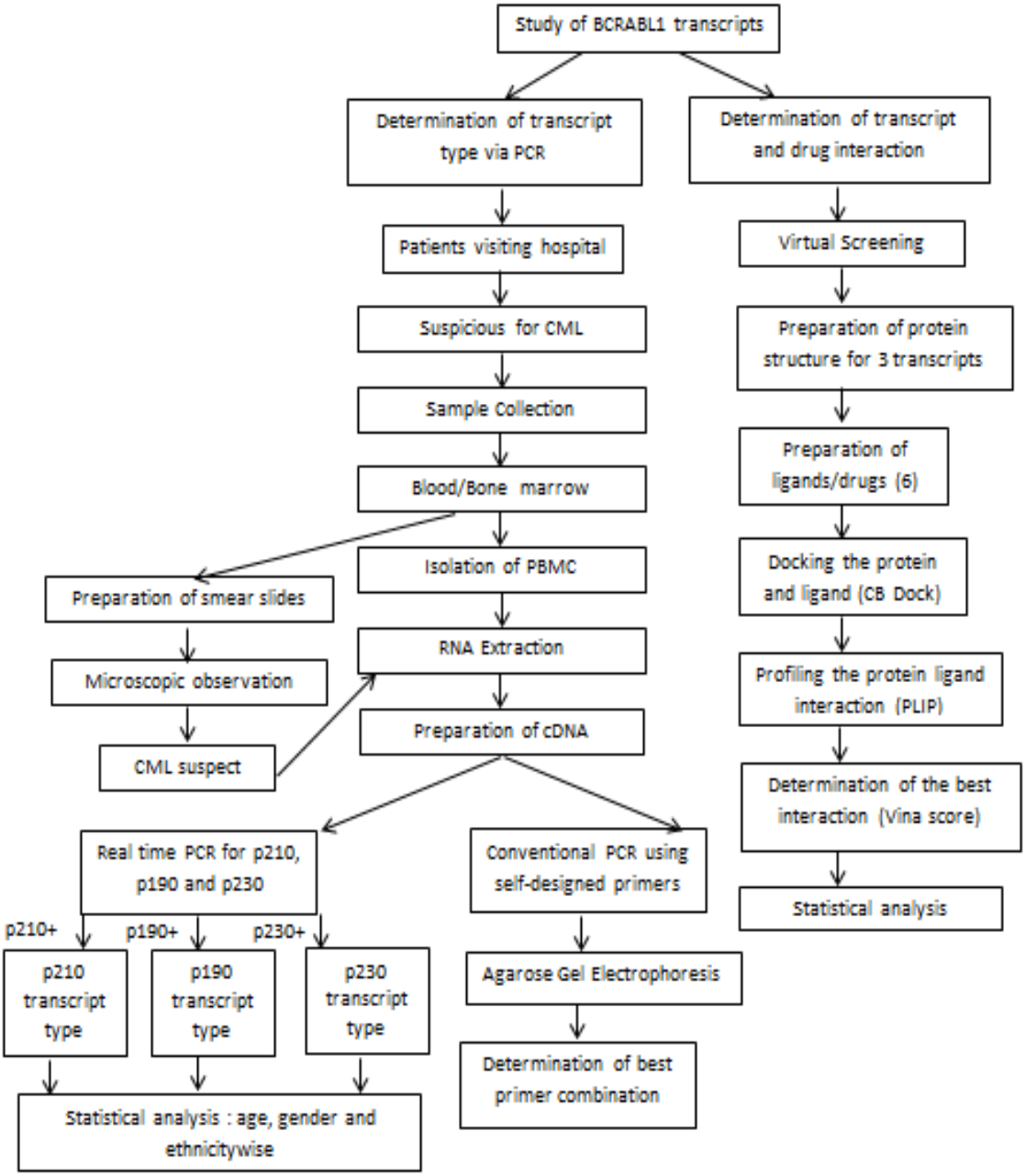
Following the determination of best pose using CB-Dock, the protein ligand Interaction profiler PLIP (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index) can help detect and visualize the interactions, as well as providing data in forms that may be processed further

Chapter 3

3. Materials and Methods

a. Research Design

The research design of the entire study is presented as below:



b. Calculation of sample size

The required sample size for the study was calculated using the formula: $n = \frac{Z^2 P\,(1-P)}{d^2}$

where, P represents the incidence of CML as per literatures ,which is 1 in 100000; (P) = $\frac{1}{100000}$ (Pourhoseingholi, et.al, 2013)

P = 0.00001

Z = 1.96 (at 95% confidence interval)

d = 0.001 (desired precision)

n = $\frac{(1.96)^2 \; x \; 0.00001 \; x \; (1-0.00001)}{(0.001)^2}$

= 38.4

= 39

Minimum of 39 samples were required for the study. As per the convenience and availability we have included 45 samples in our study.

c. Study site

Most part of the study was conducted in Medi Quest Laboratory Clinic Pvt Ltd. Established in 2010, Medi Quest Laboratory Clinic is one of the oldest private diagnostic centers categorized by the government of Nepal as class 'A' Laboratory. It is located in Jawalakhel, Lalitpur and specializes in Blood related disorders tests that includes Haematology, Immunology, Microbiology, Cytology, Biochemistry, Parasitology, Histopathology and many more. The laboratory works in collaboration with Dabur Oncquest, India and has been introducing the new and latest system in cancer diagnostic in Nepal.

The blood samples from different hospitals all over Nepal are referred here for molecular diagnostics.

Some part of the research work was also performed in Central Department of Biotechnology, Tribhuvan University, Kirtipur, Nepal. With the aim to produce high level manpower in the field of biotechnology needed in the country and at the international level, Central Department of Biotechnology was established as one of the youngest institutes of Institute of Science and Technology, Tribhuvan University, Nepal. CDBT has a well facilitated laboratory with advanced equipment for molecular and immunological studies.

d. Collection of Bio-specimen

A total of 45 blood samples collected from the Medi Quest Laboratory Clinic Pvt. Ltd. Suspected with CML were used for the analysis of the BCR-ABL1 transcripts.

The basic inclusion criteria included:

1. The samples had to be brought to Medi Quest Laboratory Clinic Pvt. Ltd. for diagnosis.
2. The samples had to be suspected for CML via microscopic analysis.
3. The samples had to be received within the 6 months of the study.

The exclusion criteria included:

1. The samples with no proper consent from the patients.
2. The samples received beyond the study tenure.
3. The samples not presumed CML from microscopy in Medi Quest Laboratory Clinic Pvt. Ltd.

The median age for the patients was observed to be 43 years (minimum 21 years to maximum 83 years). Among 45 patients, 32 were males and 13 were females. These patients were referred from Civil service hospital and other health centers to Medi Quest Laboratory Clinic for Diagnosis. The study was conducted with the approval from Nepal Health Research Council, informed consent was taken wherever possible and Memorandum of Understanding was signed between Central Department of Biotechnology and Medi Quest Laboratory Clinic Pvt. Ltd. for the overall research.

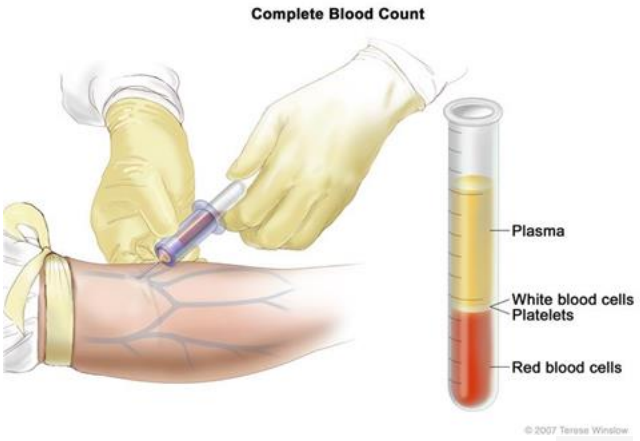


Figure 8. Blood Sample collection for CML test. Blood is collected by inserting a needle into a vein and allowing the blood to flow into a tube. The blood sample is sent to the laboratory and the red blood cells, white blood cells, and platelets are counted.
Source:
https://www.ncbi.nlm.nih.gov/books/NBK65740/

Figure 7. Suspected CML blood sample. The white layer in between the plasma and RBC represents the excessive immature WBC.

e. Microscopy

i. Preparation of stained slides

The suspected blood samples collected by Medi Quest Laboratory Pvt. Ltd. were analysed under microscopy to check for the Total blood count. A drop of blood was taken on a clean slide. Using another slide at 45°C, a think smear was prepared and air dried. The smear was covered with absolute methanol for 5 minutes for fixation and drained off to cover the slide with diluted Giemsa stain(1:10 with Giemsa buffer). The stain was left for 20minutes and washed off with running water and dried for 5 min. The slides were observed under microscope (100X) to observe the stained cells. The samples with unusual WBC counts were then further subjected to molecular diagnosis of CML.

f. Molecular Diagnosis

The molecular diagnosis of CML included three steps: RNA isolation, cDNA preparation and qPCR.

i. RNA isolation

RNA isolation was performed using QIAmp nucleic acid extraction kit. First one volume of blood sample collected in EDTA vials was mixed with 5 volumes of Buffer EL (1ml blood : 5ml EL buffer) in a tube to perform RBC lysis. The tubes were incubated on ice for 10-15 minutes and mixed by vortexing for 2 times during incubation until the cloudy suspension turned translucent indicating complete RBC lysis. Following lysis, the tubes were centrifuged at 400 x g for 10 minutes at 4°C to completely remove and discard the supernatant. The clear pellet obtained were then treated with 600 µl Buffer RLT and vortexed to mix well. If not urgent this resuspended cell lysate were stored at -20°C until extraction. During extraction, the lysate was transferred to QIAshredder spin column consisting 2ml collection tube and centrifuged at maximum speed for 2 minutes to homogenize. The QIAshredder was removed and the homogenized lysate was treated with equal volume (600µl) of 70% ethanol. The lysate was mixed by pipetting and max 700µl of lysate was transferred to new QIAmp spin column. The tubes were centrifuged at 10000rpm for 15s and the flow through was discarded. The QIAmp spin column was transferred to new collection tube and remaining lysate was transferred and centrifuged for 15s at 10000 rpm. Following the binding of nucleic acid to the spin column, washing was performed with 700 µl of Buffer RW1 and 500µl of buffer RPE. Second RPE wash using 500µl of RPE buffer was performed at 14000 rpm for 3 minutes. An empty spin for 1 minute was carried out at maximum speed to remove any residual buffer from the column. Finally, the nucleic acid bound to spin column was eluted using 30µl of RNase free water in a 1.5ml microcentrifuge tube at 10000 rpm for 1 minute.

ii. cDNA preparation

The cDNA preparation was performed using cDNA reagents of TRU-PCR BCR ABL1 kit (Ref:3B1267, 3B BlackBio Biotech India Ltd.) for detection of BCR-ABL1 gene. The 25µl of cDNA mix was prepared with 8 µl RRT1 (RT mix), 1.5 µl RRT2 (Enzyme mix), 5 µl RRT3 (Primer mix), 0.5 µl RRT4 (Enhancer mix) and 10 µl of template RNA. The Reverse transcriptase mix was subjected to following PCR conditions: enzyme activation at 25°C for 10 minutes, reverse transcription at 47°C for 60 minutes and enzyme deactivation at 70°C for 5 minutes.

iii. Real time PCR

Following the synthesis of cDNA, detection of major (M), minor (m) and micro (µ) transcripts of BCR-ABL1 genes were performed using the TRUPCR BCR ABL kit. For each sample, four reaction volumes each of 20µl was prepared using 10µl 2X high master mix, 4µl of RNase free water and 5µl of template cDNA. One microlitre (1µl) of four different primer probe mix targeting major, minor, micro and ABL1 genes were added separately in one of each four reaction. Six different standards STD1($1.08x10^6$) to STD6($1.08x10^1$)were used in place of template with major-BCR-ABL1 primer probe mix for quantification.

iv. Primer design

Following the detection of BCR-ABL1 transcripts using real time PCR, we tried to design our own primers for detection of BCR-ABL1 transcripts using conventional method. We first selected the gene sequences of different BCR-ABL1 transcripts from gene bank and aligned using Aliview software. Following the alignment, we selected the most conserved regions of different transcripts type. We designed the primers to amplify the four different transcript types and checked for the primer efficacy *invitro* using OligoCalc and Oligoanalyzer.

Table 2. Self designed primers for conventional PCR with their Sequences, Tm and expected product sizes

S.N.	Primers	Sequences	Tm
1	M1_M2_u1 F	5'GCAACGGCAAGAGTTACAC3'	57.3°C
2	m1 F	5'AGCCCTACCAGAGCATCTA3'	57.3°C
3	M1_M2_u1_m1 R	5'GGCTCAAAGTCAGATGCTAC3'	58.4°C
4	M1_M2 F	5'GAGTCACTGCTGCTGCTTAT3'	58.4°C
5	ABL F	5'GTAGCATCTGACTTTGAGCC3'	58.4°C
6	M1_M2_ABL R	5'ATAATGGAGCGTGGTGATGAG3’	59.4°C

v. Conventional PCR

For conventional PCR, new lot of RNA extraction was performed using TRIZOL reagent. 200µl of plasma sample was taken and 600µl of TRIZOL (1:3) was added. The tubes were then mixed properly and placed in ice for 5 minutes. Following incubation, 200 µl of Chloroform was added and vortexed for 15 seconds and the tubes were placed at room temperature for 5 minutes. Further, the tubes were centrifuged at 10000 rpm for 5 minutes and the upper aqueous layer was transferred to a new tube and 300µl of Isopropanol was added. Further, the solution was mixed by inversion and incubated in ice for 10 minutes and centrifuged at 10000 rpm for 10 minutes. Finally, the pellet obtained was washed with 1ml of 75% ethanol, centrifuged at 7500 rpm for 10 minutes and the pellet was resuspended in 50µl of NFW. The isolated RNA was then reverse transcribed to cDNA using iScript cDNA synthesis kit (Catalog no.: 18080093, BioRad Laboratories). 10µl of cDNA volume was prepared using 2µl of 5x iScript Reaction mix, 0.5µl iScript Reverse Transcriptase, 4µl of template and final volume adjusted to 10 using 3.5µl using NFW. The cDNA mix was then subjected to the following PCR conditions: priming (25 °C for 5 minutes), Reverse transcriptase (46 °C for 20 minutes) and RT inactivation (95 °C for 1 minute).

A total of 4 forward primers and 2 reverse primers obtained from Macrogen as per the order were used to undergo conventional PCR on the real time PCR positive BCR-ABL1 samples. Initially a 10µl of the PCR volume was prepared using 5µl of 2X master mix with 0.5µl each of forward and reverse primer and the template cDNA of 1 µl was added. The final volume was adjusted to 10µl using NFW. The PCR mix was then subjected to the following PCR conditions: 95°C for 7 minutes, 35 cycles of 95°C for 30s, 55°C for 30s and 72°C for 30 s with final extension at 72°C for 5 minutes and hold at 4°C. The PCR product obtained was observed via electrophoresis using 1.5% gel and 100bp ladder of Solis biodyne.

g. Molecular Docking

The molecular docking study followed three steps:

i. Determination of Target Protein

The determination of target protein was done via literature search. Since the study was subjected to test the variation of transcript type and their interaction with available drugs, we determined basically three transcripts e14a2, e1a2 and e19a2 are prevalent in CML patients. So we extracted the sequences for all the transcripts from NCBI genebank. We selected the sequences AJ131466.1 (e14a2), AF113911.1 (e1a2) and AM491363.1 (e19a2) as the reference sequences. The FASTA files of the respective sequences were downloaded and translated to protein sequences using ExPasy. The translated sequences were modeled using SWISS MODEL to obtain the best possible structure using homology modeling.

ii. Determination of ligands

Using literature search, we determined five different drugs: Imatinib, Nilotinib, Dasatinib, Bosutinib, Ponatinib and Asciminib being used for treatment of CML. The chemical structure of these drugs were obtained from PubChem and converted to pdb files using Open Babel GUI.

iii. Docking

Following the preparation of protein and ligand, protein-ligand docking was performed for the three transcripts using 5 different drugs in CB-Dock. The best pose obtained using CB-dock were further analysed via Protein ligand Interaction Profiler to understand the interacting aminoacids.

Chapter 4

4. Results

a. Enrolled Cases and Clinical manifestations

The patients visiting the lab with typical symptoms of weakness, bone pain, fatigue, weight loss and enlarged spleen were taken in special consideration as suspected CML cases and tested for Total Blood Count using Microscopy. During the duration of 6 months, a total of 47 CML samples were received BCR-ABL1_1 to BCR-ABL1_47. Out of which Sample BCR-ABL1_40 and BCR-ABL1_9 were from same individual and BCR-ABL1_36 and BCR-ABL1_12 were from same individual follow up cases. Hence these four samples were considered as two and the total study size was 45.

b. Microscopic study

The microscopic analysis of all the 45 suspected samples showed presence of large no. of immature granulocytes as shown in the figure 9. The TLC showed that the number of WBC were ranging from $34x10^9$/Lto $35x10^9$ /L which were 3X fold higher than the normal count 4-11X10^9/L.

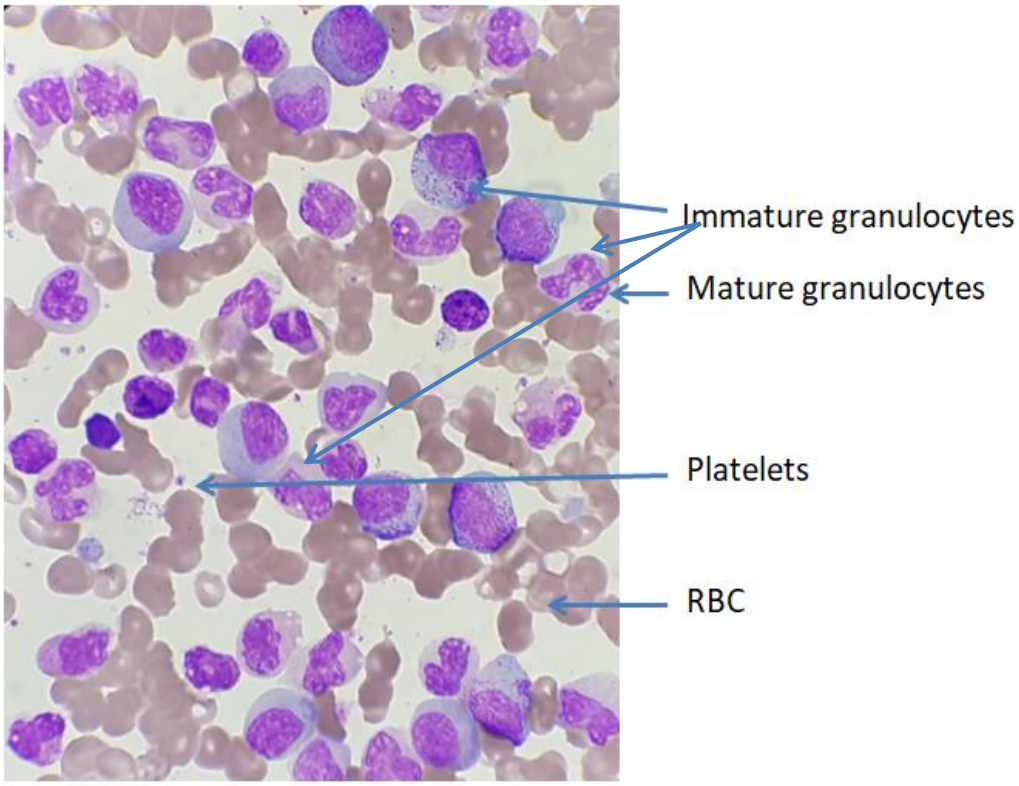
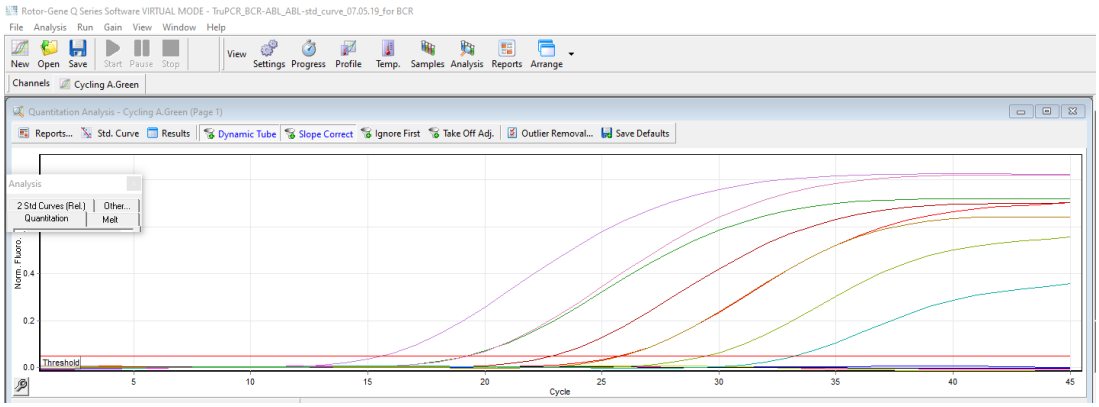


Figure 9. Microscopic observation of blood smear from patient with suspected Chronic Myeloid Leukemia, after Giemsa staining observed at objective 100X. The image demonstrates excessive no. of immature granulocytes with round or oval nucleus in comparision to the mature granulocytes.

c. Amplification of Chimeric BCR/ABL transcripts



a

b

Quant. Results - Cycling A.Green (Page 1)

No.	C	Name	Type	Ct	Ct Comment	Given Conc (cop	Calc Conc (copie	% Var	Rep. Ct	Rep. Ct Stc
1		1M	Unknown	25.79			1,261		25.79	
2		1m	Unknown							
3		1u	Unknown							
9		2M	Unknown	19.24			97,083		19.24	
10		2m	Unknown							
11		2u	Unknown							
18		S1	Standard	15.65		1,000,000	1,051,477	5.1%	15.65	
19		S2	Standard	19.30		100,000	93,395	6.6%	19.30	
20		S3	Standard	22.85		10,000	8,898	11.0%	22.85	
21		S4	Standard	25.93		1,000	1,154	15.4%	25.93	
22		S5	Standard	29.49		100	109	8.9%	29.49	
23		S6	Standard	33.23		10	9	9.0%	33.23	

Figure 10: Real time PCR results of two suspected BCRABL1 positive samples using 6 standards S1 to S6. a.The qPCR amplification curves with sample 2M curve raised near S2 and sample 1M raised near S4. b. M, m and u represents the reaction for major, minor and micro transcripts respectively. 1 and 2 represents two different samples. Both samples 1 and 2 shows amplification of only major transcript.

The Real Time PCR was performed on the cDNA prepared from the extracted RNA using TRU-PCR RT-PCR kit. Standard 1 to 6 (probes for major transcript) along with the samples in 4 replicates each for major, minor, micro and abl gene gave the varied nature of amplification. In every sample the curve for ABL gene was positive indicating the success of RNA extraction. The negatives without template were all clean and all the standard showed significant sigmoidal curves. All the positive samples showed amplification with probes for major transcript and were negative for minor and micro transcript.

d. Distribution of CML transcript types

Among 45 samples, almost all the samples(44) were positive for p210(97.7%). The kit couldn't differentiate between the b2a2 or b3a2 but did show positive results only if either of them were positive for major transcripts. Hence, all the transcripts observed were the major transcripts (except for one case where a rare variant(2.3%), e14a3 was observed. None of the samples showed positive results for p190 or p230.

Table 3. Distribution profile of CML transcripts. 44 out of 45 samples showed positive for major transcript and 1 sample was positive for rare variant with no samples for minor or micro transcripts.

S.N.	Transcript type	No. of cases	Percentage
1	p210 (Major)	44	97.70%
2	Rare variant	1	2.30%
3	p190 (Minor)	0	0%
4	p230 (Micro)	0	0%
	Total	**45**	**100%**

e. Gender based BCR-ABL1 positive case

Genderwise analysis showed that the disease prevalence was significantly high (p= .011) among males 32 (71%) than females 13 (29%). The ANOVA results showed significant impact of gender in CML occurrence as compared to age and gender (Appendix VII)

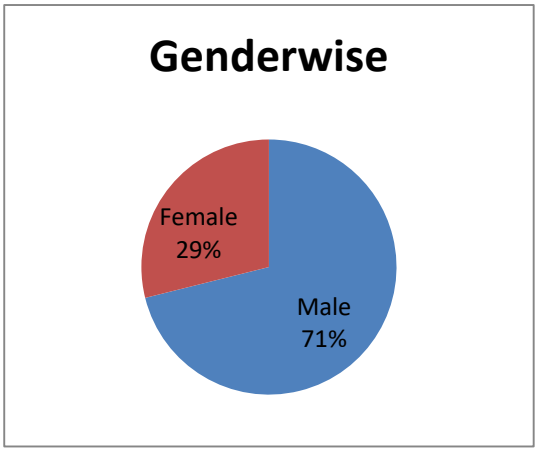


Figure 11. Genderwise CML distribution. Males showed predominance over female by 71% to 29% in CML prevalence.

f. Age based BCR-ABL1 positive cases

Out of the enrolled cases of the age group of 0-15 yrs to 75+ yrs, the disease was not detected in age group 0-15 as the minimum age of patient included in the study was 21 years and found prevalent even at the very old age of 83 years old with 2 samples above 75yrs. However, the most vulnerable age was among 40-50 years old people which gradually decreased to aged group ahead. The most affected median age group fall at 43 years. The ANOVA calculated between two genders for various age groups showed no significant impact of age-groups on occurrence of CML (p=0.051). (Appendix VII) at 5% level of significance.

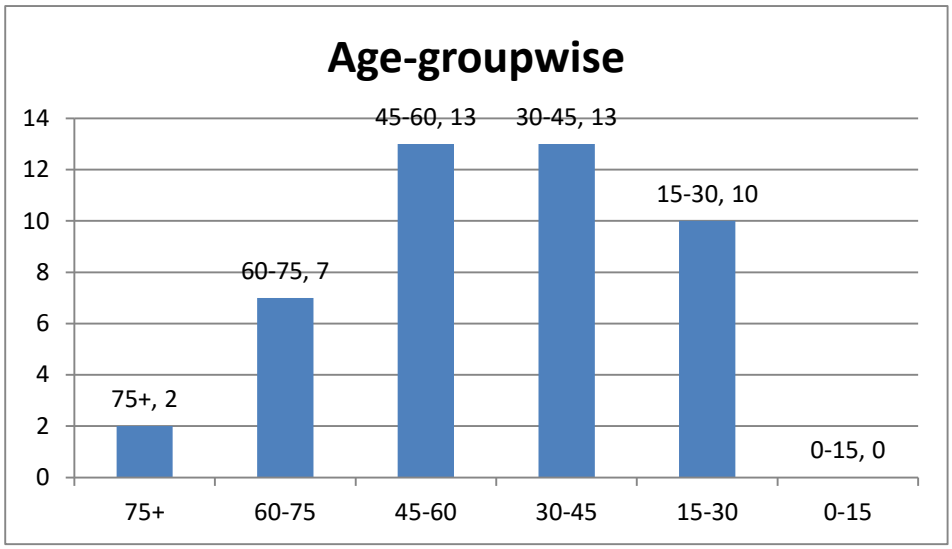


Figure 12. Agewise distribution BCRABL1 positive patients. All the 45 samples (y-axis:no. of individuals) were distributed in six different age groups from 0 to 75 years and 75+ (x-axis)at 15yrs age intervals. No individuals belonged below 15 years and maximum individuals(13) were from 30-45 and 45-60yrs.

Table 4. ANOVA analysis on Agewise distribution of CML Prevalence

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between age group	76.75	5	15.35	4.978378	0.051394	5.050329
Between gender	30.08333	1	30.08333	9.756757	0.026146	6.607891
Error	15.41667	5	3.083333			
Total	122.25	11				

g. Ethnicity based BCR-ABL1 positive cases

On the ethnic grounds, more than 9 ethnic groups of people were identified to be CML patient. The disease was more prevalent in the Kshetri followed by Brahmin community among all other ethnic groups while Magar, Tamang, Muslim and others were found least victimised. However, ANOVA analysis showed no significant impact of ethnicity on CML occurrence (p=0.113) (Appendix VII) at 5% level of significance.

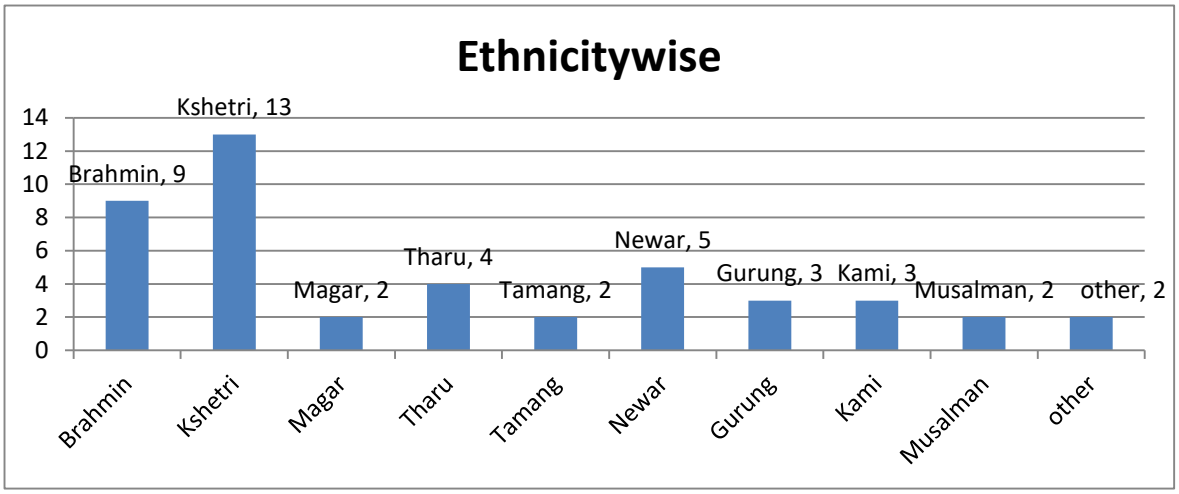


Figure 13.Ethnicity base BCR-ABL1 positive cases distribution. The total samples were distributed into 10 different ethnic groups. Ethnic groups in x-axis and individual no. on y-axis. The maximum distribution occurs in Kshetri community (13), followed by Brahmin(9) and Newar(5).

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between ethnicity	61.25	9	6.805556	2.31569	0.113464	3.178893
Between gender	18.05	1	18.05	6.141777	0.035091	5.117355
Error	26.45	9	2.938889			
Total	105.75	19				

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Individuals	32.625[a]	59	.553	1.701	.021
	genderF1M0	.000[b]	59	.000	.000	1.000
Intercept	Individuals	16.875	1	16.875	51.923	<.001
	genderF1M0	30.000	1	30.000	60.000	<.001
Agegroup	Individuals	7.775	5	1.555	4.785	<.001
	genderF1M0	.000	5	.000	.000	1.000
Ethnicity	Individuals	10.208	9	1.134	3.490	.002
	genderF1M0	.000	9	.000	.000	1.000
Agegroup * Ethnicity	Individuals	14.642	45	.325	1.001	.493
	genderF1M0	.000	45	.000	.000	1.000
Error	Individuals	19.500	60	.325		
	genderF1M0	30.000	60	.500		
Total	Individuals	69.000	120			
	genderF1M0	60.000	120			
Corrected Total	Individuals	52.125	119			
	genderF1M0	30.000	119			

a. R Squared = .626 (Adjusted R Squared = .258)
b. R Squared = .000 (Adjusted R Squared = -.983)

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.754	90.557[b]	2.000	59.000	<.001
	Wilks' Lambda	.246	90.557[b]	2.000	59.000	<.001
	Hotelling's Trace	3.070	90.557[b]	2.000	59.000	<.001
	Roy's Largest Root	3.070	90.557[b]	2.000	59.000	<.001
Agegroup	Pillai's Trace	.320	2.289	10.000	120.000	.017
	Wilks' Lambda	.680	2.514[b]	10.000	118.000	.009
	Hotelling's Trace	.471	2.734	10.000	116.000	.005
	Roy's Largest Root	.471	5.657[c]	5.000	60.000	<.001
Ethnicity	Pillai's Trace	.382	1.576	18.000	120.000	.077
	Wilks' Lambda	.618	1.786[b]	18.000	118.000	.035
	Hotelling's Trace	.619	1.995	18.000	116.000	.015
	Roy's Largest Root	.619	4.127[c]	9.000	60.000	<.001
Agegroup * Ethnicity	Pillai's Trace	.470	.410	90.000	120.000	1.000
	Wilks' Lambda	.530	.490[b]	90.000	118.000	1.000
	Hotelling's Trace	.888	.572	90.000	116.000	.997
	Roy's Largest Root	.888	1.184[c]	45.000	60.000	.268

a. Design: Intercept + Agegroup + Ethnicity + Agegroup * Ethnicity

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between ethnicity	20.41667	9	2.268519	3.486056	0.002429	2.095755
Between Agegroup	15.55	5	3.11	4.779169	0.001377	2.422085
Error	29.28333	45	0.650741			
Total	**65.25**	**59**				

The ANOVA did show some significant difference between ethnicity and age-group. However, the multivariate analysis showed no significant difference among ethnicity, age and gender.

h. Conventional PCR of BCR-ABL1 transcripts

Agarose Gel Electrophoresis was performed using five different primer sets. The two primer sets M1M2F/M1M2µ1R and M1M2µ1F/M1M2ABLR seemed to show successful amplification with the positive sample used at around 350bp. The control ABL gene also showed amplification at around 300 bp.

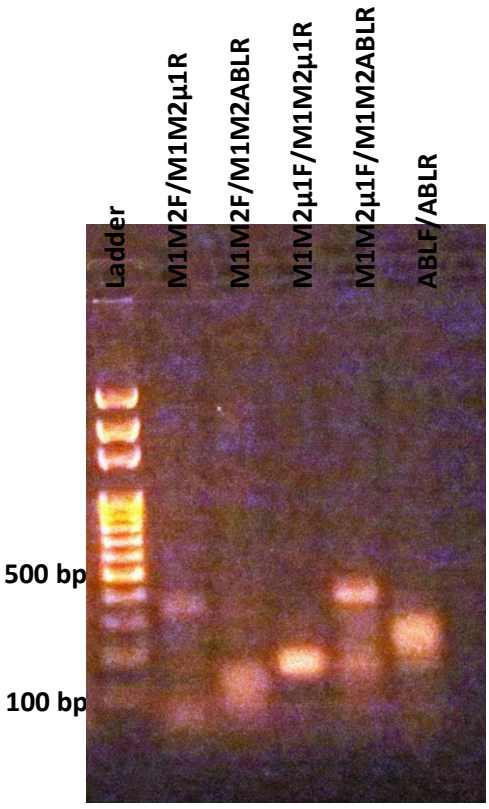


Figure 15.Agarose Gel Electrophoresis of BCRABL1 positive sample using different conventional primers. The agarose gel electrophoresis was carried out at 1.5% gel using 100bp Solis biodyne ladder. For the same sample, 5 different primer combination were tested. Combination 1 (M1M2F/M1M2u1R)) and 4 (M1M2u1F/M1M2ABLR) showed bamds near 300bp to 400bp. ABL bands seem quite dispersed with unintended bands in combination 2 and 3.

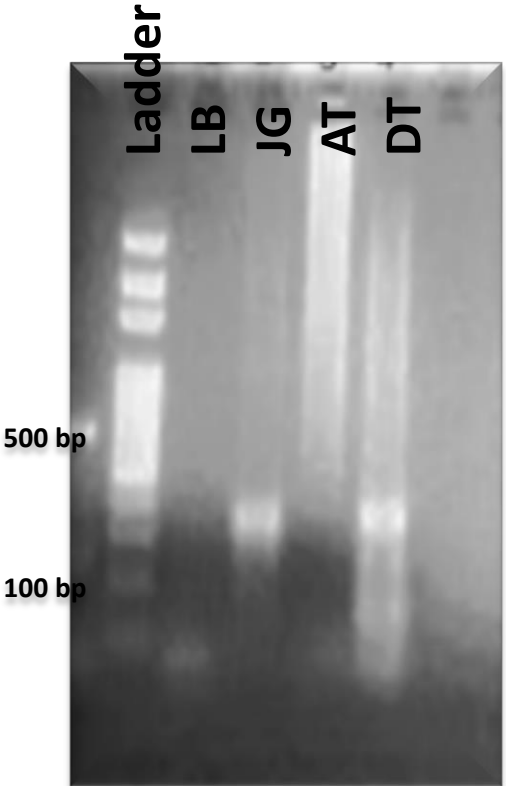


Figure 14. Agarose Gel Electrophoresis of four random samples using M1M2µ1F/M1M2ABLR . Samples JG and DT were positive and sample LB and AT were negative which was later confirmed in real-time PCR.

i. Molecular Docking

i. Determination of Target protein

During this step, the protein sequences obtained were homologously modeled for the templates 1opl.1.A and 5mo4.1.A.with highest GMQE and Identity. The GQME and QMEAN for the three transcripts modeled were as follows:

Table 6. Protein preparation parameters for transcripts of study. The transcripts sequences obtained from genebank were given protein structure using Homology modeling in SWISSMODEL. The template represent the reference protein structure taken for modeling the respective transcripts. The GQME and QMEAN values represent the scores of the finally modeled structures.

S.N.	Transcript	Template	Seq Identity	Description	GQME	QMEAN
1	e14a2	5mo4.1.A	100%	Tyrosine protein kinase ABL1 ABL1 kinase (T334I_D382N)in complex with asciminib and nilotinib	0.56	0.84±0.07
2	e1a2	5mo4.1.A	100%	Tyrosine protein kinase ABL1 ABL1 kinase (T334I_D382N)in complex with asciminib and nilotinib	0.46	0.82±0.07
3	e19a2	1opl.1.A	99.07%	proto-oncogene tyrosine protein kinase Structural basis for the autoinhibition of c-abl kinase	0.34	0.82±0.06

ii. Determination of ligands

The structure of all the ligands were obtained as follows:

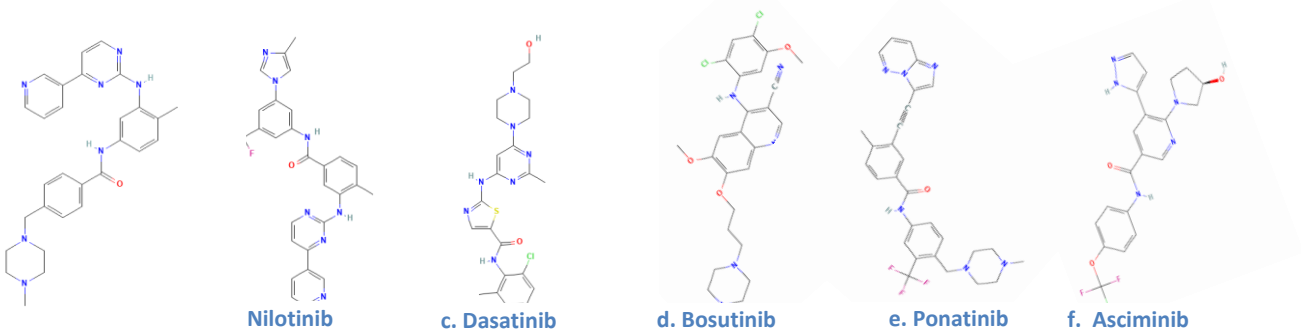


Figure 16.Figure a to f represents the 2D structure of 6 different drugs being used for CML treatment. The structures are all taken from PubChem. The drugs a-e are based on tyrosine kinase inhibition (TKI) mechanism while f.Asciminib is used as allosteric inhibitor for CML treatment.

iii. Docking

Table 7.ANOVA calculation for variation among transcripts and drugs

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Drugs	7.331667	5	1.466333	13.4526	0.000359	3.325835
Between transcript	0.123333	2	0.061667	0.565749	0.585102	4.102821
Error	1.09	10	0.109			
Total	8.545	17				

The results showed no significant difference in the affinity between transcripts (p=0.58) on using various drugs. However, the various drugs (p=0.000359) seem to show significantly different effect among one another at 5% level of significance (Table 7), Appendix (VII).

The results showed Nilotinib as the best drug of choice for transcript e14a2 (-8.9) and e1a2(-8.8) while in case of transcript e19a2, Imatinib(-8.8) and Ponatinib(-8.8) seems to function better. For all three transcripts, Asciminib showed comparatively lesser affinity, except for Bosutinib which showed the least affinity to e19a2 (-6.4). (Table 8).

The transcript e14a2 and e1a2 showed the most active amino acid interacting with most drugs as Tyrosine. However, a variation of amino acids were observed to interact with different drugs in case of e19a2.(Table 8).

The docking of the 3 transcripts with 6 different drugs showed four best different results as:

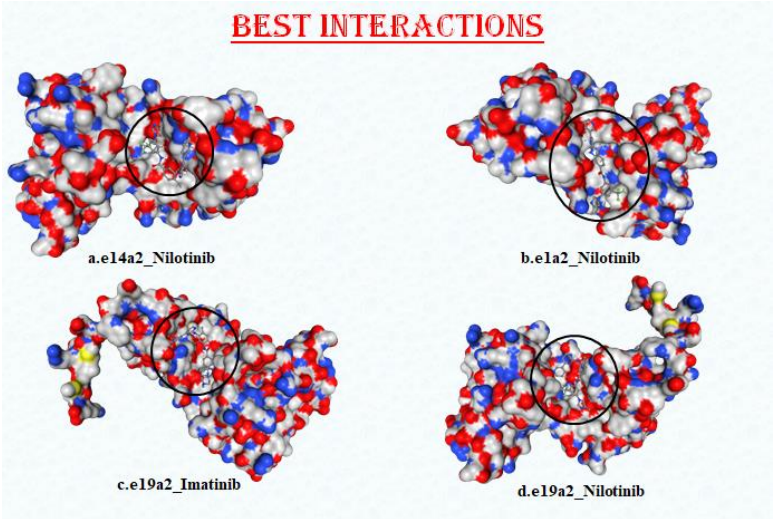


Figure 17. (a-d): Docking poses of three different transcripts with the best drugs of choice. Transcript e14a2 and e1a2 shows best pose with Nilotinib, while e19a2 shows the best pose with Imatinib as well as Nilotinib.

Table 8. Docking results of three transcripts with 6 drugs used. Each transcript was docked against 6 different drugs. The Vina score represents the affinity of the drugs to the transcript. The lowest Vina Score represents the highest affinity. The hydrophobic and hydrogen interaction represents the amino acids of transcript interacting with the drugs.

Transcript	Drugs	Vina Score	Cavity Size	Hydrophobic Interactions	Hydrogen Interaction
e14a2	Imatinib	-8.6	1134	LEU,GLU,HIS,VAL,ALA,TYR,LEU,LEU,LYS	TYR,ILE,THR
	Nilotinib	**-8.9**	**1134**	**LEU,ASN,GLU,TYR,LEU,PRO**	**TYR,THR,ASN**
	Dasatinib	-7.6	1134	LEU,ASN,GLU,LEU,ARG	TYR,TYR,ARG,ASN,ASN
	Bosutinib	-7.7	1134	ASN,PRO	TYR,SER,ASN,THR
	Ponatinib	-8.6	1134	TYR,LEU,PRO	TYR
	Asciminib	-7.4	1134	LEU,LYS,ARG	TYR,TYR,ASN,LYS
e1a2	Imatinib	-8.6	1134	LEU,GLU,HIS,VAL,ALA,TYR,LEU,LEU,LYS	TYR,ILE,THR
	Nilotinib	**-8.8**	**98**	**ASN,GLU,VAL,LEU,LYS**	**TYR,TYR,THR,ARG,ASN**
	Dasatinib	-7.7	1134	TYR,ASN,LYS	TYR,ILE,ASN
	Bosutinib	-7.7	1134	ASN,PRO	TYR,SER,ASN,THR
	Ponatinib	-8.6	1134	TYR,LEU,PRO	ASN,TYR
	Asciminib	-7.5	1134	LEU,LYS,ARG	TYR,TYR,LYS
e19a2	**Imatinib**	**-8.8**	**1371**	**LEU,GLN,ASN,GLU**	**SER,HIS**
	Nilotinib	-8.7	1371	ASN,GLU,	THR,SER
	Dasatinib	-7.6	1371	TYR,ASN,LYS	TYR,ASN,LYS
	Bosutinib	-6.4	1371	LEU,LYS	ASN,HIS,LYS
	Ponatinib	**-8.8**	**1371**	**LEU,TYR,GLU,ASN,GLU**	**TYR,ASN,ASN**
	Asciminib	-7.5	1371	LEU,GLU,LEU	TYR,LYS,ASN

Chapter 5

5. Discussion

Chronic Myeloid leukemia affects 1 in every 100000 people worldwide. The exact fact of its incidence is not available in Nepal. A study (Kulshrestha & Sah, 2009), stated the presence of 35.2% of all the leukemia cases of Nepal while another study (Ghartimagar, et.al., 2012) stated the presence of 16.3% of all the leukemia cases to be CML. Most of the studies are targeted in differentiating the type of leukemia. However we could barely find any literature in context of Nepal that dealt with only CML and the transcripts responsible for CML. This study is therefore a baseline study for CML.

In our study, we have included 45 CML patients to determine the frequency of BCR-ABL1 fusion transcripts. All the 45 samples were clinically suspected for CML as per the WHO criteria of disease classification. Almost all the samples suspected for CML were in the Chronic phase with the height leucocyte and platelets counts. Researches show 80% of the CML cases are in CP CML stage with high WBC count, anemia and high platelets (Ghartimagar et al., 2012).

Using Real Time PCR kit for BCR ABL1 we found the frequency of p210 transcript to be the maximum against p190 and p230. Almost all the cases of suspected CML showed p210 positive.

The results obtained were similar to most of the literatures as can be seen in the Table 8. The major BCR ABL1 transcripts were found in almost 100% cases as seen in the studies by (Baccarani et al., 2019)(Bennour et al., 2013)(Pardanani et al., 2009). Most of the other studies showed the presence of major transcripts above 80 to 90% with co-expression of both transcripts observed. Only a study by Al-Achkar et al., 2016 in Syrian population showed around 72.56% of major transcripts and 2% rare transcripts. Since the kit used in this study was designed to detect both b2a2 and b3a2 transcripts, the co-expression could also have been detected in the process thereby resulting 100% of major transcript. Minor transcripts (e1a2) were found in 1%, 1.17% and 0.75% in Iranian, and Serbian populations respectively as per the studies by Bagheri et.al. 2018, Ayotallahi et.al, 2018 and Zivanovic et.al. 2011 respectively. Bagheri et.al.,2018 showed the presence of micro transcript in 16% of cases in Iranian population and Zivanovic et.al., reported the presence of micro transcripts in 0.75% of Indian population. In our study no micro or mini transcripts were seen. This could possibly be due to the small population size taken. The platelets levels were more elevated in patients with b3a2 transcripts than b2a2 (Bennour et al., 2013). This depicts the essence of transcript type variation associated.

The genderwise classification of CML patients showed male dominance than female with the M:F ratio of 2.44:1. This ratio was higher than the ratios observed in the study by Hamid & Amp; Bokharaei, 2017, where the M:F ratio in Iranian population was 1.4:1 and Kagita,et.al., 2018 , where the M:F ratio in Indian population was 1.78:1. Similar study conducted by Iqbal, 2014, showed higher male dominance with M:F ratio 2.33:1. All these results suggest the occurrence of CML is more in males.

There has not been any actual reasons to explain this but as we look at the transcript type in males and females affected with CML, Khazaal et.al. reported that most males showed b3a2 transcript while females showed b2a2 transcript. In addition b2a2 was reported to show high platelet count while b3a2 showed higher leukocyte (Khazaal, et.al., 2019). Another contradicting results by Osman et.al. in Sudanese population showed higher tendency of males to express b2a2 and females to express b3a2. Yet another study by Al-Achkar et.al showed similar expression pattern of both transcripts irrespective of gender. However the males were always dominant with the expression of either transcripts (Al-Achkar et al., 2016).

Table 9. Distribution of different BCR-ABL1 transcripts around the globe from 2009-2019

S.N.	Country	Author	Total no. of patients	Major (b2a2 Or b3a2)	e14a2 (b3a2)	e13a2 (b2a2)	Both (e14a2 /e13a2)	Minor e1a2	Micro e19a2	others
1	45 countries	(Baccarani, et.al., 2019)	45503	100.00%	62.50%	37.90%	(From 62.25%) 54.45%	(From 666/34561) 0.91%	(From 666/34561) 0.31%	(From 666/34561) 0.7%
2	Iraq	(Khazaal, et.al., 2019)	100	98.00%	59%	39%	1%			1%
3	Iran	(Bagheri, et.al., 2018)	41	77.50%	12.5%.	52.50%	12.50%	35% (atypical)		
4	India	(Kagita, et.al., 2018)	170	99.89%	61.77%	35.29%	2.94%			
5	Iran	(Ayatollahi et al., 2018)	85	91.76%	62.35%	29.41%		1.18%	7.06%(b3a2+e1a2)	
6	Iran	(Hamid & Bokharaei, 2017)	320	86.56%	5% (p210 or p190)			7.81%	0.63%	
7	Brazil	(Vasconcelos et al., 2017)	203	98.00%	64.04%	33.50%	2.46%			
8	Syria	(Al-Achkar, et.al., 2016)	45	72.56%	51.11%	46.67%				2.22%
9	India	(Arun et al., 2017)	1260	94.30%	60%	34.29%	3.89% (e1a2+/)	1.19%	0.32%	0.32%
10	USA	(Jain et al., 2016)	487	83.00%	40.25%	41.07%	17.45%	0.82%		0.41%(b3a3)
11	India	(Mir et al., 2015)	200	100.00%	68%	24%	8% (e14a2 or e13a2)			
12	Pakistan	(Iqbal, 2014)	130	99.99%	63.84%	36.15%				
13	Germany	(Hanfstein et al., 2014)	1119	98.75%	44.32%	40.30%	14.12%	0.27%	0.54%	0.45%
14	Tunisia	(Bennour et al., 2013)	45	97.78%	62%	35.56%				2.22%
15	Pakistan	(Irshad et al., 2012)	23	82.50%	26.09%	56.52%				17.39%(b3a3)
16	Serbia	(Todoric-Zivanovic et al., 2011.)	136	98.50%	73.50%	25%	0.75%	0.75%		
17	Sudan	(Osman et al., 2010)	43	95.40%	41.90%	53.50%	4.6% (e14a2/e13a2 and e14a2/e13a2/e19a2)			
18	Rochester, MN	(Pardanani et al., 2009)	143	97.90%				2.10%		

The impact of age was not not seen significant in CML prevalence however agewise distribution of CML showed the median age group of patients to be 43 years with the minimum age 21 years to maximum 83 years. The median age of 40 years was seen in the study conducted by Kagita et.al. in Indian population in 2018. Similarly, a median age of 35 years was found in Pakistani population as mentioned by Iqbal et.al. 2014. However an increase in median age was seen in China (49.5 yrs; Wang et.al.2010) and Japan (55 yrs; Tauchi et.al. 2015) with the highest of 62years in USA. The CML affected median age group trend in the world suggests that adult people are more vulnerable to the disease. Superficially, the median age group of CML diagnosis seems higher at Western population which seems to have been lower towards the eastern region. This diversity could be a strong indication of the influence of lifestyle and feeding habits which of course is very diverse in the Eastern and Western Population. Intensive research might be essential to study the impact of these essential factors in CML pathophysiology.

The ethnic distribution of CML in Nepalese population showed the highest expression of BCR-ABL1 in Kshetri community, followed by Brahmin, Newar and Kami community. The ethnic distribution of BCR-ABL1 transcripts in Nepalese population has not been done before to the best of our knowledge. Hence, we are unable to make any literature comparison.

Table 10. Ethnic distribution of Nepalese population as per the Worldatlas source ("Largest Ethnic Groups In Nepal-WorldAtlas.com,"2019)

Rank	Ethnic Groups	Share of Nepalese population
1	Chhetri	16.60%
2	Brahmin	12.20%
3	Magar	7.10%
4	Tharu	6.50%
5	Tamang	5.80%
6	Newar	5.00%
7	Kami	4.70%
8	Madhesi Muslims	4.40%
9	Yadav	4.00%
10	Rai/Other Groups	2.3%/ Less than 2% each

If we look at the ethnic distribution of Nepal as per World atlas as shown in table 2, we may see that the mostly distributed ethnic group in Nepal is Chhetri, this could be a reason why our results showed more CML prevalence in Chhetri community than Brahmin. The prevalence pattern is somehow similar to the ethnic distribution of Nepal. Also at 5% level of significance, the ANOVA analysis of the prevalence data showed no significant

difference. Hence we may not associate the CML prevalence with any ethnic community unless we have sufficient significant molecular evidences.

Following Real time PCR, conventional PCR was performed to check if the primers were able to differentiate the various transcripts. The expected product size was 304bp for major transcript (e14a2), the bands obtained were near 350bp. We consider the primers were successful in amplifying the major transcript. But only combinations: **M1M2F/M1M2µ1m1R and M1M2µ1F/M1M2ABLR** were found to be successful in our test. Since all of our transcripts were major transcript in real time PCR, the results were expectable. Lacking the availability of other transcript, we were not able to test all the primers. Further, the amplification may be performed on multiple samples to ensure the efficacy of the primers.

We further performed docking analysis to check the affinity of the different drugs being used against the three transcript of our interest. Since no proper structure was available for the transcript, we performed homology modeling to determine the protein structure and performed docking with our drugs of interest. For all the three transcripts, Imatinib, Nilotinib and Ponatinib showed better affinity than Dasatinib and Bosutinib. Asciminib, being the allosteric inhibitor showed the least affinity with all the three transcript as expected, except for Bosutinib that showed least affinity to e19a2. All the 6 drugs showed to interact with the three transcripts using various interaction whereby hydrophobic and hydrogen interactions were prevalent among all. 144ATyr appeared as the most essential amino acid in transcript e14a2 to interact with all the 6 drugs while 184Tyr was the common amino acid in e1a2. In case of e19a2, the 320ATyr was found interacting with some drugs but the amino acids varied for different drugs. (Appendix VI).

The results suggest in normal condition, the variation in transcript type makes no drastic changes in drug efficacy. However, while using Asciminib, using it alone may not be as sufficient. So combination therapy may be recommended with other TKIs to treat CML. This could be an option with mutant cases too.

Chapter 6

6. Conclusion

In conclusion, the study could determine the prevalence of major transcripts p210 in almost all the cases of CML under study. There was a male dominance in disease prevalence with median age group of 43 years and Kshetri people seemed more susceptible to CML among all other ethnic groups. However, differences in age and ethnicity were not significant that raises the necessity of larger sample size for study. All other researches showed the prevalence of major transcripts and association of the transcript variants which disease pathophysiology and TKI responses. In our study, however variation of transcript type made no impact over drug affinity in virtual screening. But having proper idea of transcript variants can prove very efficient in effective drug therapy reducing the chances of resistance which has been appearing as a serious problem with CML management. Hence the next approach of the study should be to standardize the diagnostic methods to make the CML treatment more personalized and effective. In addition, the behavioral study among the CML patients of different age groups may provide some insights to understand and control the CML in early ages.

Chapter 7

7. Limitations of the Study

1. The small sample size taken in the study reduced the possibility of occurrence of variable no. of transcripts type in the study.
2. Only molecular parameters were studied which did not give much comparable parameters.
3. No reference gene or protein structure were available so all the simulations are based on predictive model.

Chapter 8

8. Recommendations

1. Larger population size may be selected with incorporation of pathological and behavioural features including detailed questionare could make the study more significant.
2. Conventional PCR may be performed on more samples with positives for all three transcripts to ensure the enability of primers in differentiating transcripts.
3. Simple sanger sequencing may be performed with the PCR products to analyse the variation prevailing between countries.
4. Indepth Docking studies may be performed with wild type and mutant transcripts along with the transcripts obtained in the study to better understand the drug interaction.

Chapter 9

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Appendix I: Composition and reaction conditions

Composition of Giemsa stain

Giemsa stock solution		Giemsa Buffer	
Giemsa powder	1gm	Na_2HPO_4	9.5g/ml
Glycerol	60ml	KH_2PO_4	9.07g/ml
Methanol	66ml		

Giemsa Stain: 10% giemsa stock in giemsa buffer

Preparation of cDNA

Name of the Reagent	Quantity per reaction
RRT1	8 µl
RRT2	1.5 µl
RRT3	5 µl
RRT4	0.5 µl
Sample RNA*	1 µg
Total reaction volume	25

Add upto 10 µl sample RNA (1 µg/mol) and the OD 260/280 of the RNA should be measured spectrophotometrically and should be between 1.7 to 2.0

Program setup

Step	Temperature ˚C	Time	Cycles
1	25	10 min	1
2	47	60 min	1
3	70	05 min	1

i. Quantification of transcripts by Real-time PCR

Reaction preparation for samples

Name of the reagent	**Major BCR ABL1**	**Minor BCR ABL1**	**Micro BCR ABL1**	**ABL1**
2X High Master Mix	10 µl	10 µl	10 µl	10 µl
Major BCR ABL1 Primer Probe Mix	1 µl	-	-	-
Minor BCR ABL1 Primer Probe Mix	-	1 µl	-	-
Micro BCR ABL1 Primer Probe Mix	-	-	1 µl	-
ABL1 Primer Probe Mix	-	-	-	10 µl
Sample cDNA	5 µl	5 µl	5 µl	5 µl
RNAse free water	4 µl	4 µl	4 µl	4 µl
Total Reaction volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**

Reaction preparation for standards

Name of the reagent	**STD1 (10^6 copies)**	**STD2 (10^5 copies)**	**STD3 (10^4 copies)**	**STD4 (10^3 copies)**	**STD5 (10^2 copies)**	**STD6 (10^1 copies)**
Multiples Master Mix	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
Major BCR-ABL1 Primer probe mix	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
Standards	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
Nuclease free water	4 µl	4 µl	4 µl	4 µl	4 µl	4 µl
Total reaction volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**

Prepare three reaction for ABL1 standards

Name of the Reagent	STD 1 (10^6 copies)	STD 1 (10^5 copies)	STD 1 (10^4 copies)
Multiplex Master mix	10 µl	10 µl	10 µl
ABL1 Primer Probe mix	1 µl	1 µl	1 µl
Standards	5 µl	5 µl	5 µl
Nuclease free water	4 µl	4 µl	4 µl
Total reaction volume	20 µl	20 µl	20 µl

Program SetUP

Step	Temperature, C	Time	Dye Acquisition	Cycles
1	94	10 min	-	1
2	94	15 sec	-	45
	60	01 min	Yes	

Detection	Detector channel	Reporter	Quencher	Gain Setup
BCR-ABL1	Green	FAM	None	Auto
ABL1	Green	FAM	None	Auto

Appendix II: Primer design

e14a2:BCR_ABL1:997bp:AJ131466.1

gcgaacaagggcagcaaagctacggagaggctgaagaagaagctgtcggagcaggagtca
ctgctgctgcttatgtctcccagcatggccttcagggtgcacagccgcaacggcaagagt
tacacgttcctgatctcctctgactatgagcgtgcagagtggagggagaacatccgggag
cagcagaagaagtgtttcagaagcttctccctgacatccgtggagctgcagatgctgacc
aactcgtgtgtgaaactccagactgtccacagcattccgctgaccatcaataaggaagat
gatgagtctccggggctctatgggtttctgaatgtcatcgtccactcagccactggattt
aagcagagttcaaaagcccttcagcggccagtagcatctgactttgagcctcagggtctg
agtgaagccgctcgttggaactccaaggaaaaccttctcgctggacccagtgaaaatgac
cccaacctttcgttgcactgtatgattttgtggccagtggagataacactctaagcata
actaaaggtgaaaagctccgggtcttaggctataatcacaatggggaatggtgtgaagcc
caaaccaaaaatggccaaggctgggtcccaagcaactacatcacgccagtcaacagtctg
gagaaacactcctggtaccatgggcctgtgtcccgcaatgccgctgagtatctgctgagc
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Primer list for CML variants

M1_M2_u1 F :	5'GCAACGGCAAGAGTTACAC3'
m1 F :	5'AGCCCTACCAGAGCATCTA3'
M1_M2_u1_m1 R :	5'GGCTCAAAGTCAGATGCTAC3'
M1_M2 F :	5'GAGTCACTGCTGCTGCTTAT3'
ABL F:	5'GTAGCATCTGACTTTGAGCC3'
M1_M2_ABL R :	5'ATAATGGAGCGTGGTGATGAG3’

M1:304bp, M2:229bp, u1:871bp, m1: 487bp

M1:885bp, M2:810 bp, m1: 487bp

e1a2:1079bp:BCR_ABL1:AF113911.1

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Appendix III: Real time PCR details

S.N.	Patient code	Age (in yrs)	Gender	Transcript type
1	BCR-ABL1_1	28	M	P210
2	BCR-ABL1_2	50	F	P210
3	BCR-ABL1_3	48	F	P210
4	BCR-ABL1_4	41	M	P210
5	BCR-ABL1_5	58	M	P210
6	BCR-ABL1_6	21	F	P210
7	BCR-ABL1_7	23	M	P210
8	BCR-ABL1_8	27	F	P210
9	BCR-ABL1_9	28	M	P210
10	BCR-ABL1_10	38	M	P210
11	BCR-ABL1_11	41	F	P210
12	BCR-ABL1_12	38	F	P210
13	BCR-ABL1_13	52	M	P210
14	BCR-ABL1_14	63	M	P210
15	BCR-ABL1_15	80	M	P210
16	BCR-ABL1_16	43	M	P210
17	BCR-ABL1_17	34	M	P210
18	BCR-ABL1_18	28	M	P210
19	BCR-ABL1_19	42	M	
20	BCR-ABL1_20	30	M	P210
21	BCR-ABL1_21	41	F	P210
22	BCR-ABL1_22	60	M	P210
23	BCR-ABL1_23	26	M	P210
24	BCR-ABL1_24	55	M	P210
25	BCR-ABL1_25	49	M	P210
26	BCR-ABL1_26	45	F	P210
27	BCR-ABL1_27	62	F	P210
28	BCR-ABL1_28	42	M	P210
29	BCR-ABL1_29	43	M	P210
30	BCR-ABL1_30	50	M	P210
31	BCR_ABL1_31	61	F	P210
32	BCR_ABL1_32	66	M	P210

33	BCR_ABL1_33	37	M	P210
34	BCR_ABL1_34	46	M	P210
35	BCR_ABL1_35	28	F	P210
36	BCR_ABL1_36	38	F	P210
37	BCR_ABL1_37	47	M	P210
38	BCR_ABL1_38	25	F	P210
39	BCR_ABL1_39	51	M	P210
40	BCR_ABL1_40	28	M	P210
41	BCR_ABL1_41	70	M	P210
42	BCR_ABL1_42	57	M	P210
43	BCR_ABL_43	73	M	P210
44	BCR_ABL_44	43	M	P210
45	BCR_ABL_45	83	M	P210
46	BCR_ABL_46	54	F	P210
47	BCR_ABL_47	24	M	P210

Appendix IV: Protein structure prediction

>AJ131466.1 Homo sapiens partial mRNA for bcr-abl1 e14a2 chimeric protein

GCGAACAAGGGCAGCAAAGCTACGGAGAGGCTGAAGAAGAAGCTGTCGGAGCAGGAGTCACTGCTGCTGC
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Protein seq: 5’3’

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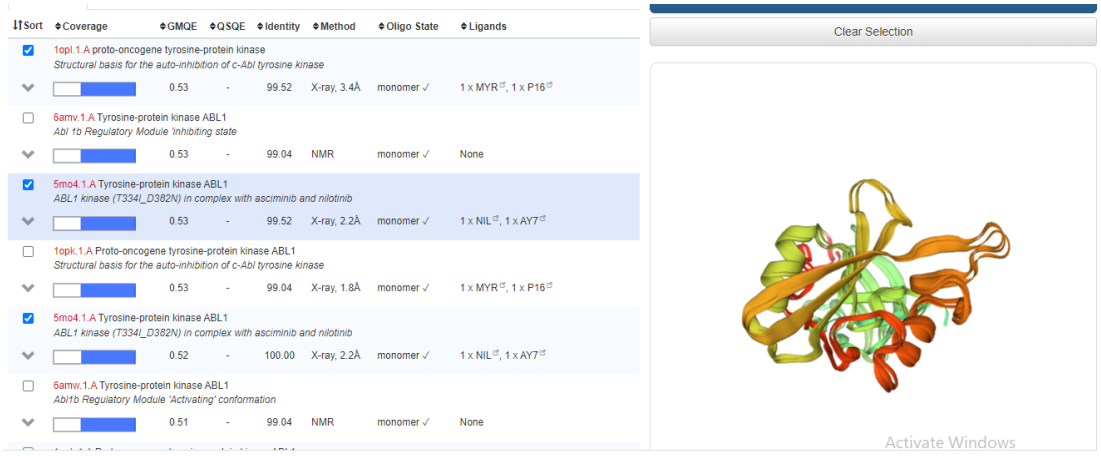

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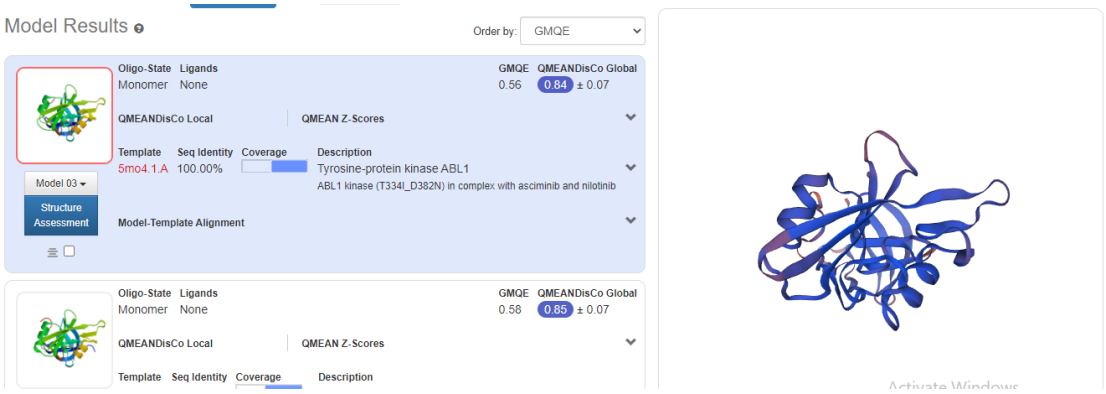

>AF113911.1 Homo sapiens BCR-ABL1 e1a2 chimeric protein (BCR/ABL fusion) mRNA, partial cds

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Protein Seq: 5’-3’

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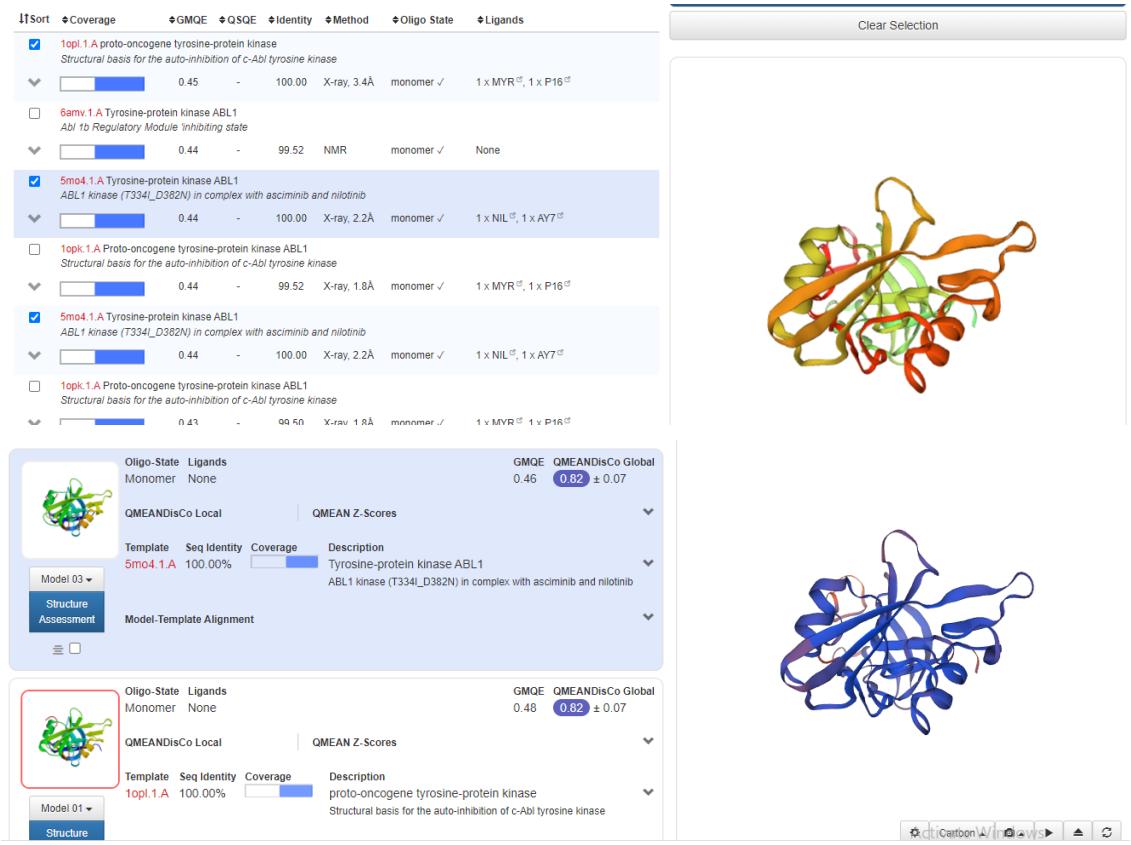

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>AM491363.1 Homo sapiens partial mRNA for bcr-abl1 e19a2 chimeric protein

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Protein seq: 5’-3’

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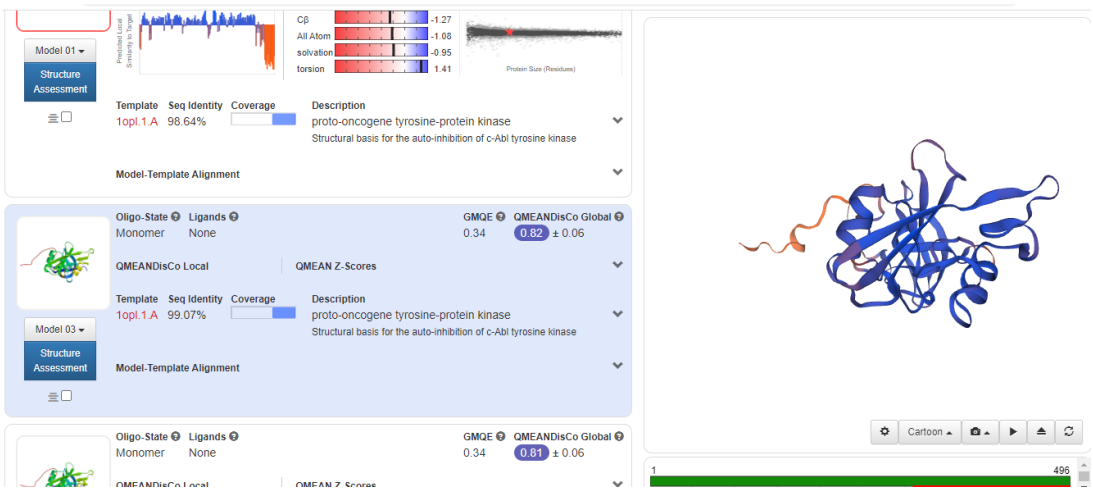

Appendix V: CB-Dock output

Protein	Drug	Vina	Cavity	Center			Size		
e14a2	Imatinib	score	size	x	y	z	x	y	z
		-8.6	1134	-25	9	-7	26	26	26
		-7.9	98	-25	18	-23	26	26	26
		-7.8	53	-15	2	1	26	26	26
		-7.3	77	-20	21	2	26	26	26
		-7.1	74	-29	4	1	26	26	26
		-6.9	96	-29	10	-28	26	26	26
		-6.7	64	-10	17	2	26	26	26
		-6.4	155	-13	0	-24	26	26	26
e1a2	Imatinib	Vina	Cavity	Center			Size		
		score	size	x	y	z	x	y	z
		-8.6	1134	-25	9	-7	26	26	26
		-7.9	98	-25	18	-23	26	26	26
		-7.7	53	-15	2	1	26	26	26
		-7.6	74	-29	4	1	26	26	26
		-7.5	77	-20	21	2	26	26	26
		-6.8	96	-29	10	-28	26	26	26
		-6.8	64	-10	17	2	26	26	26
		-6.7	155	-13	0	-24	26	26	26
e19a2	Imatinib	Vina	Cavity	Center			Size		
		score	size	x	y	z	x	y	z
		-8.8	1371	31	72	40	26	26	26
		-8.1	187	44	78	52	26	26	26
		-7.7	157	11	68	43	26	26	26
		-7.6	569	15	70	51	26	26	26
		-7.6	42	15	72	31	26	26	26
		-7.1	96	14	88	33	26	26	26
		-6.9	126	42	87	48	26	26	26
		-6.8	49	15	83	31	26	26	26

Transcript	Drugs	Vina	Cavity	Center			Size		
e14a2	Nilotinib	score	size	x	y	z	x	y	z
		-8.9	1134	-25	9	-7	24	24	24
		-8.7	98	-25	18	-23	24	24	24
		-7.7	53	-15	2	1	24	24	24

		-7.6	74	-29	4	1	24	24	24
		-7.5	64	-10	17	2	24	24	24
		-7.4	155	-13	0	-24	24	24	24
		-7.3	96	-29	10	-28	24	24	24
		-7.1	77	-20	21	2	24	24	24
e1a2	Nilotinib	**Vina**	**Cavity**	**Center**			**Size**		
		score	**size**	**x**	**y**	**z**	**x**	**y**	**z**
		-8.8	98	-25	18	-23	24	24	24
		-8.7	1134	-25	9	-7	24	24	24
		-7.9	53	-15	2	1	24	24	24
		-7.5	74	-29	4	1	24	24	24
		-7.5	64	-10	17	2	24	24	24
		-7.3	96	-29	10	-28	24	24	24
		-7	77	-20	21	2	24	24	24
		-6.9	155	-13	0	-24	24	24	24
e19a2	Nilotinib	**Vina**	**Cavity**	**Center**			**Size**		
		score	**size**	**x**	**y**	**z**	**x**	**y**	**z**
		-8.7	1371	31	72	40	24	24	24
		-8.2	569	15	70	51	24	24	24
		-8.2	157	11	68	43	24	24	24
		-7.7	187	44	78	52	24	24	24
		-7.7	126	42	87	48	24	24	24
		-7.7	96	14	88	33	24	24	24
		-7.7	49	15	83	31	24	24	24
		-6.7	42	15	72	31	24	24	24

Transcript	**Drugs**	**Vina**	**Cavity**	**Center**			**Size**		
e14a2	bosutinib	**score**	**size**	**x**	**y**	**z**	**x**	**y**	**z**
		-7.7	1134	-25	9	-7	25	25	25
		-6.4	53	-15	2	1	25	25	25
		-6.2	77	-20	21	2	25	25	25

		-6	74	-29	4	1	25	25	25
		-6	64	-10	17	2	25	25	25
		-5.6	98	-25	18	-23	25	25	25
		-5.3	155	-13	0	-24	25	25	25
		-5.2	96	-29	10	-28	25	25	25
e1a2	bosutinib	**Vina**	**Cavity**	**Center**			**Size**		
		score	**size**	**x**	**y**	**z**	**x**	**y**	**z**
		-7.7	1134	-25	9	-7	25	25	25
		-6.4	53	-15	2	1	25	25	25
		-5.9	77	-20	21	2	25	25	25
		-5.9	64	-10	17	2	25	25	25
		-5.7	98	-25	18	-23	25	25	25
		-5.7	96	-29	10	-28	25	25	25
		-5.3	74	-29	4	1	25	25	25
		-4.2	155	-13	0	-24	25	25	25
e19a2	bosutinib	**Vina**	**Cavity**	**Center**			**Size**		
		score	**size**	**x**	**y**	**z**	**x**	**y**	**z**
		-6.4	1371	31	72	40	25	25	25
		-6.4	187	44	78	52	25	25	25
		-6.3	157	11	68	43	25	25	25
		-5.9	42	15	72	31	25	25	25
		-5.6	126	42	87	48	25	25	25
		-5.5	569	15	70	51	25	25	25
		-5.3	49	15	83	31	25	25	25
		-5.2	96	14	88	33	25	25	25

Transcript	**Drugs**	**Vina**	**Cavity**	**Center**			**Size**		
e14a2	Ponatinib	**score**	**size**	**x**	**y**	**z**	**x**	**y**	**z**
		-8.6	1134	-25	9	-7	30	30	30
		-8.6	77	-20	21	2	30	30	30

		-8.6	74	-29	4	1	30	30	30
		-8	98	-25	18	-23	30	30	30
		-7.9	53	-15	2	1	30	30	30
		-7.3	64	-10	17	2	30	30	30
		-7.2	96	-29	10	-28	30	30	30
		-6.7	155	-13	0	-24	30	30	30
e1a2	Ponatinib	**Vina**	**Cavity**	**Center**			**Size**		
		score	**size**	**x**	**y**	**z**	**x**	**y**	**z**
		-8.6	1134	-25	9	-7	30	30	30
		-8.4	77	-20	21	2	30	30	30
		-8.4	74	-29	4	1	30	30	30
		-7.9	53	-15	2	1	30	30	30
		-7.7	98	-25	18	-23	30	30	30
		-7.1	96	-29	10	-28	30	30	30
		-6.9	64	-10	17	2	30	30	30
		-6.3	155	-13	0	-24	30	30	30
e19a2	Ponatinib	**Vina**	**Cavity**	**Center**			**Size**		
		score	**size**	**x**	**y**	**z**	**x**	**y**	**z**
		-8.8	1371	31	72	40	30	30	30
		-8	569	15	70	51	30	30	30
		-7.9	157	11	68	43	30	30	30
		-7.9	42	15	72	31	30	30	30
		-7.6	187	44	78	52	30	30	30
		-7.2	49	15	83	31	30	30	30
		-7.1	96	14	88	33	30	30	30
		-7	126	42	87	48	30	30	30

Transcript	**Drug**	**Vina**	**Cavity**	**Center**			**Size**		
e14a2	Asciminib	**score**	**size**	**x**	**y**	**z**	**x**	**y**	**z**
		-7.4	1134	-25	9	-7	26	26	26

		-6.3	77	-20	21	2	26	26	26
		-6	98	-25	18	-23	26	26	26
		-6	96	-29	10	-28	26	26	26
		-5.2	155	-13	0	-24	26	26	26
e1a2	Asciminib	**Vina**	**Cavity**	**Center**			**Size**		
		score	**size**	**x**	**y**	**z**	**x**	**y**	**z**
		-7.4	1134	-25	9	-7	26	26	26
		-6.3	77	-20	21	2	26	26	26
		-6.1	96	-29	10	-28	26	26	26
		-6	98	-25	18	-23	26	26	26
		-5.9	155	-13	0	-24	26	26	26
e19a2	Asciminib	**Vina**	**Cavity**	**Center**			**Size**		
		score	**size**	**x**	**y**	**z**	**x**	**y**	**z**
		-7.6	1371	31	72	40	26	26	26
		-6.5	569	15	70	51	26	26	26
		-6.4	157	11	68	43	26	26	26
		-6.3	126	42	87	48	26	26	26
		-6.1	187	44	78	52	26	26	26

Appendix VI: Different Interactions observed in docking

Hydrophobic interaction							
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance**	**Ligand Atom**	**Protein Atom**
e14a2	Imatinib	1	143A	LEU	3.42	1340	46
		2	197A	GLU	3.67	1343	467
		3	203A	HIS	3.87	1333	526
		4	206A	VAL	3.74	1360	548
		5	210A	ALA	3.75	1365	579
		6	213A	TYR	3.69	1365	601
		7	214A	LEU	3.75	1365	579
		8	214A	LEU	3.84	1364	612
		9	293A	LYS	3.57	1333	1229
e1a2	Imatinib	1	183A	LEU	3.43	1344	46
		2	237A	GLU	3.66	1341	467
		3	243A	HIS	3.91	1333	526
		4	246A	VAL	3.74	1360	548
		5	250A	ALA	3.74	1365	579
		6	253A	TYR	3.7	1365	601

		7	254A	LUE	3.71	1365	611
		8	254A	LUE	3.81	1364	612
		9	333A	LYS	3.5	1333	1229
		10	333A	LYS	3.96	1332	1230
e19a2	Imatinib	1	319A	LEU	3.77	1479	61
		2	348A	GLU	3.78	1504	284
		3	364A	ASN	3.88	1469	410
		4	373A	GLU	3.68	1478	482
Hydrophobic interaction							
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance**	**Ligand Atom**	**Protein Atom**
e14a2	Nilotinib	1	143A	LEU	3.56	1360	46
		2	188A	ASN	3.78	1364	395
		3	197A	GLU	3.54	1360	467
		4	213A	TYR	3.89	1342	601
		5	214A	LEU	3.52	1342	613
		6	297A	PRO	3.46	1334	1265
e1a2	Nilotinib	1	228A	ASN	3.62	1360	395
		2	237A	GLU	3.83	1334	467

		3	246A	VAL	3.88	1344	548
		4	254A	LEU	3.54	1342	613
		5	333A	LYS	3.83	1333	1230
e19a2	Nilotinib	1	364A	ASN	3.57	1503	410
		2	373A	GLU	3.6	1471	482
Hydrophobic interaction							
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance**	**Ligand Atom**	**Protein Atom**
e14a2	Bosutinib	1	188A	ASN	3.94	1333	395
		2	297A	PRO	3.94	1335	1266
e1a2	Bosutinib	1	228A	ASN	3.93	1333	395
		2	337A	PRO	3.94	1335	1266
e19a2	Bosutinib	1	319A	LEU	3.79	1495	61
		2	469A	LYS	3.58	1472	1242
Hydrophobic interaction							

Transcript	Drugs	Index	Residue	AA	Distance	Ligand Atom	Protein Atom
e14a2	Dasatinib	1	143A	LEU	3.63	1359	46
		2	188A	ASN	3.99	1362	395
		3	197A	GLU	3.87	1359	467
		4	214A	LEU	3.85	1336	613
		5	294A	ARG	3.68	1334	1237
e1a2	Dasatinib	1	207A	TYR	3.93	1334	230
		2	228A	ASN	3.78	1336	395
		3	333A	LYS	3.6	1359	1229
e19a2	Dasatinib	1	343A	TYR	3.71	1473	245
		2	364A	ASN	3.56	1475	410
		3	469A	LYS	3.72	1498	1243
Hydrophobic interaction							
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance**	**Ligand Atom**	**Protein Atom**
e14a2	Ponatinib	1	213A	TYR	3.84	1357	601
		2	214A	LEU	3.58	1354	613
		3	297A	PRO	3.86	1336	1266
e1a2	Ponatinib	1	253A	TYR	3.87	1357	601
		2	254A	LEU	3.58	1354	613
		3	337A	PRO	3.82	1336	1266

e19a2	Ponatinib	1	319A	LEU	3.46	1476	61
		2	320A	TYR	3.95	1475	71
		3	348A	GLU	3.58	1506	284
		4	364A	ASN	3.82	1492	410
		5	373A	GLU	3.59	1472	482
Hydrophobic interaction							
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance**	**Ligand Atom**	**Protein Atom**
e14a2	Asciminib	1	214A	LEU	3.57	1342	613
		2	293A	LYS	3.81	1331	1229
		3	294A	ARG	3.46	1342	1238
e1a2	Asciminib	1	254A	LEU	3.56	1342	613
		2	333A	LYS	3.77	1331	1229
		3	334A	ARG	3.48	1342	1238
e19a2	Asciminib	1	319A	LEU	3.28	1482	61
		2	373A	GLU	3.61	1478	482
		3	390A	LEU	3.66	1497	625

Hydrogen Interaction

Transcript	Drugs	Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein Donor?	Side Chain	Donor Atom	Acceptor Atom
e14a2	Imatinib	1	144A	TYR	2.17	3.08	165.51	**Yes**	**Yes**	59[O3]	1337[O2]
		2	190A	ILE	2.96	3.82	141.64	No	No	1346[N3]	414[O2]
		3	191A	THR	3.15	4.05	155.88	Yes	Yes	424[O3}	1346[N3]
e1a2	Imatinib	1	184A	TYR	2.15	3.06	163.65	Yes	Yes	59[O3]	1337[O2]
		2	230A	ILE	2.96	3.8	140.03	No	No	1346[N3]	414[O2]
		3	231A	THR	3.16	4.06	155.66	Yes	Yes	424[O3]	1346[N3]
e19a2	Imatinib	1	363A	SER	2.31	3.08	135.38	NO	No	1494[Npl]	403[O2]
		2	379A	HIS				NO	No	1485[N3]	540[O2]

Hydrogen Interaction

Transcript	Drugs	Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein Donor?	Side Chain	Donor Atom	Acceptor Atom
e14a2	Nilotinib	1	144A	TYR	2.07	2.99	166.53	YES	YES	59[O3]	1362[Nar]
		2	191A	THR	2.45	3.19	133.26	YES	YES	424[O3]	1367[Nar]
		3	295A	ASN	3.47	4.05	119.89	YES	NO	1244[Nam]	1348[N2]
e1a2	Nilotinib	1	184A	TYR	2.89	3.74	151.96	YES	YES	59[O3]	1355[NpL]
		2	184A	TYR	3.2	3.74	116.64	N	YES	1355[Npl]	59[O3]
		3	231A	THR	2.03	2.89	147.39	YES	YES	424[O3]	1367[Nar]
		4	334A	ARG	3.36	3.97	121.97	YES	NO	1233[Nam]	1348[N2]
		5	335A	ASN	2.57	3.33	134.27	YES	NO	1244[Nam]	1348[N2]

e19a2	Nilotinib	1	367A	THR	2.68	3.52	143.01	NO	YES	1494[Npl]	439[O3]
		2	474A	SER	3.07	3.94	147.6	YES	NO	1281[Nam]	1487[N2]
Hydrogen Interaction											
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance H-A**	**Distance D-A**	**Donor Angle**	**Protein Donor?**	**Side Chain**	**Donor Atom**	**Acceptor Atom**
e14a2	Bosutinib	1	144A	TYR	2.13	3.03	160.33	YES	yes	59[O3]	1352[O3]
		2	187A	SER	2.16	3.05	145.13	No	no	1361[N3]	388[O2]
		3	188A	ASN	3.19	3.97	138.34	YES	yes	398[Nam]	1328[Nar]
		4	298A	THR	2.46	3.4	159.1	YES	yes	1268[Nam]	1329[N1]
e1a2	Bosutinib	1	184A	TYR	2.12	3.02	160.98	YES	yes	59[O3]	1352[O3]
		2	227A	SER	2.15	3.05	145.96	No	no	1361[N3]	388[O2]
		3	228A	ASN	3.18	3.97	138.68	YES	yes	398[Nam]	1328[Nar]
		4	338A	THR	2.48	3.41	158.62	YES	yes	1268[Nam]	1329[N1]
e19a2	Bosutinib	1	364A	ASN	3.54	4.01	112.12	YES	yes	413[Nam]	1500[N3]
		2	379A	HIS	2.45	3.08	121.3	YES	yes	543[Nar]	1467[Nar]
		3	469A	LYS	3.14	3.81	124.7	YES	yes	1245[N3]	1493[O3]
Hydrogen Interaction											
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance H-A**	**Distance D-A**	**Donor Angle**	**Protein Donor?**	**Side Chain**	**Donor Atom**	**Acceptor Atom**
e14a2	Dasatinib	1	144A	TYR	2.16	2.9	135.27	YES	YES	59[O3]	1355[Nam]
		2	144A	TYR	1.93	2.9	168.08	NO	YES	1355[Nam]	59[O3]

		3	294A	ARG	2.75	3.41	125.18	YES	NO	1233[Nam]	1331[Nar]
		4	295A	ASN	2.02	2.99	167.87	YES	NO	1244[Nam]	1331[Nar]
		5	295A	ASN	2.57	3.37	138.62	NO	NO	1328[Npl]	1247[O2]
e1a2	Dasatinib	1	184A	TYR	2.41	3.27	153.44	YES	YES	59[O3]	1354[O2]
		2	230A	ILE	2.89	3.28	104.1	NO	NO	1328[Npl]	414[O2]
		1	364A	ASN	2.26	2.93	124.22	YES	YES	413[Nam]	1485[O3]
e19a2	Dasatinib										
Hydrogen Interaction											
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance H-A**	**Distance D-A**	**Donor Angle**	**Protein Donor?**	**Side Chain**	**Donor Atom**	**Acceptor Atom**
e14a2	Ponatinib	1	213A	TYR	3.01	3.56	119.33	YES	YES	605[O3]	1363[N2]
e1a2	Ponatinib										
		1	228A	ASN	3.42	3.8	104.35	No	no	1343[N3]	394[O2]
		2	253A	TYR	3	3.55	119.3	yes	yes	605[O3]	1363[N2]
e19a2	Ponatinib	1	320A	TYR	2.11	3.09	160.51	No	YES	1482[N3]	74[O3]
		2	364A	ASN	3.1	3.65	116.8	YES	YES	413[Nam]	1502[Nar]
		3	364A	ASN	3.47	3.91	108.92	NO	NO	1468[Nam]	409[O2]
Hydrogen Interaction											
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance H-A**	**Distance D-A**	**Donor Angle**	**Protein Donor?**	**Side Chain**	**Donor Atom**	**Acceptor Atom**
e14a2	Ascicimib	1	144A	TYR	2.28	3.1	145.01	YES	YES	59[O3]	1350[N2]
		2	144A	TYR	2.19	3.1	151.83	NO	YES	1350[N2]	59[O3]

		3	188A	ASN	3.44	3.97	116.81	NO	NO	1360[O3]	394[O2]
		4	213A	TYR	3.3	4.03	135.87	YES	YES	605[O3]	1344[O3]
		5	293A	LYS	2.11	3.07	154.99	YES	YES	1232[N3]	1351[N2]
e1a2	Ascicimib	1	184A	TYR	2.3	3.12	145.39	YES	YES	59[O3]	1350[N2]
		2	184A	TYR	2.23	3.12	149.45	NO	YES	1350[N2]	59[O3]
		3	253A	TYR	3.22	3.96	136.41	YES	YES	605[O3]	1344[O3]
		4	333A	LYS	2.14	3.09	153.48	YES	YES	1232[N3]	1351[N2]
e19a2	Ascicimib	1	320A	TYR	2.74	3.57	142.74	NO	YES	1489[N2]	74[O3]
		2	469A	LYS	2.65	3.59	153.81	YES	YES	1245[N3]	1474[O2]
		3	471A	ASN	2.65	3.05	105.36	NO	YES	1499[O3]	1263[O2]

Halogen Interaction									
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance**	**Donor Angle**	**Acceptor Angle**	**Donor Atom**	**Acceptor Atom**
e1a2	Nilotinib	1	250A	ALA	3.66	145.71	94.61	1354[F]	578[O2]
Halogen Interaction									
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance**	**Donor Angle**	**Acceptor Angle**	**Donor Atom**	**Acceptor Atom**
e1a2	Bosutinib	1	336A	LYS	3.4	136.17	113.43	1349[CL]	1255[O2]
Halogen Interaction									
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance**	**Donor Angle**	**Acceptor Angle**	**Donor Atom**	**Acceptor Atom**
e14a2	Ponatinib	1	202A	TYR	3.27	137.01	127.6	1340[F]	513[O2]
e19a2	Ponatinib	1	378A	TYR	3.89	161.92	112.98	1478[F]	528[O2]
Halogen Interaction									
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance**	**Donor Angle**	**Acceptor Angle**	**Donor Atom**	**Acceptor Atom**
e14a2	Asciminib	1	295A	asn	3.12	147.49	90.13	1348[F]	1250[O2]
e1a2	Asciminib	1	335A	ASN	3.09	149.67	90.37	1348[F]	1250[O2]

Salt Interaction								
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance**	**Protein Positive?**	**Ligand Group**	**Ligand Atom**
e19a2	Dasatinib	1	348A	GLU	5.37	NO	Tertamine	1479

pi interaction									
Transcript	**Drugs**	**Index**	**Residue**	**AA**	**Distance**	**OFFSET**	**Protein Charged**	**Ligand Group**	**Ligand Atom**
e14a2	Ponatinib	1	294A	ARG	5.07	1.65	YES	Aromatic	1362, 1363, 1364, 1365, 1366, 1367
e1a2	Ponatinib	1	334A	ARG	5.08	1.68	YES	Aromatic	1362, 1363, 1364, 1365, 1366, 1367

Appendix VII: Statistical Analysis

Genderwise Calculation of ANOVA

SUMMARY	*Count*	*Sum*	*Average*	*Variance*		
<15	2	0	0	0		
15-30	2	10	5	2		
30-45	2	13	6.5	24.5		
45-60	2	13	6.5	12.5		
60-75	2	7	3.5	4.5		
75+	2	2	1	2		
Female	6	13	2.166667	3.366667		
Male	6	32	5.333333	15.06667		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between age group	76.75	5	15.35	4.978378	0.051394	5.050329
Between gender	30.08333	1	30.08333	9.756757	0.026146	6.607891
Error	15.41667	5	3.083333			
Total	122.25	11				

Ethnicitywise Calculation of ANOVA

SUMMARY	*Count*	*Sum*	*Average*	*Variance*		
Brahman	2	9	4.5	24.5		
Kshetri	2	13	6.5	12.5		
Magar	2	2	1	0		
Tharu	2	4	2	0		
Tamang	2	2	1	0		
Newar	2	5	2.5	0.5		
Gurung	2	3	1.5	0.5		
Kami	2	3	1.5	4.5		
Musalman	2	2	1	2		
Other	2	2	1	0		
Female	10	13	1.3	1.344444		
Male	10	32	3.2	8.4		

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between ethnicity	61.25	9	6.805556	2.31569	0.113464	3.178893
Between gender	18.05	1	18.05	6.141777	0.035091	5.117355
Error	26.45	9	2.938889			
Total	105.75	19				

Summary table for variation among drugs and transcript

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Imatinib	3	26	8.666667	0.013333
Nilotinib	3	26.4	8.8	0.01
Dasatinib	3	22.9	7.633333	0.003333
Bosutinib	3	21.8	7.266667	0.563333
Ponatinib	3	26	8.666667	0.013333
Asciminib	3	22.4	7.466667	0.003333
e14a2	6	48.8	8.133333	0.406667
e1a2	6	48.9	8.15	0.331
e19a2	6	47.8	7.966667	0.946667

ANOVA calculation for variation among transcripts and drugs

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Drugs	7.331667	5	1.466333	13.4526	0.000359	3.325835
Between transcript	0.123333	2	0.061667	0.565749	0.585102	4.102821
Error	1.09	10	0.109			
Total	8.545	17				

Comparsion among different drugs

LSD : 1.11						
	Imatinib	Nilotinib	Dasatinib	Bosutinib	Ponatinib	Asciminib
Imatinib		-0.13	1.03	1.40	0.00	1.20
Nilotinib			1.17	1.53	0.13	1.33
Dasatinib				0.37	-1.03	0.17
Bosutinib					-1.40	-0.20
Ponatinib						1.20
Asciminib						

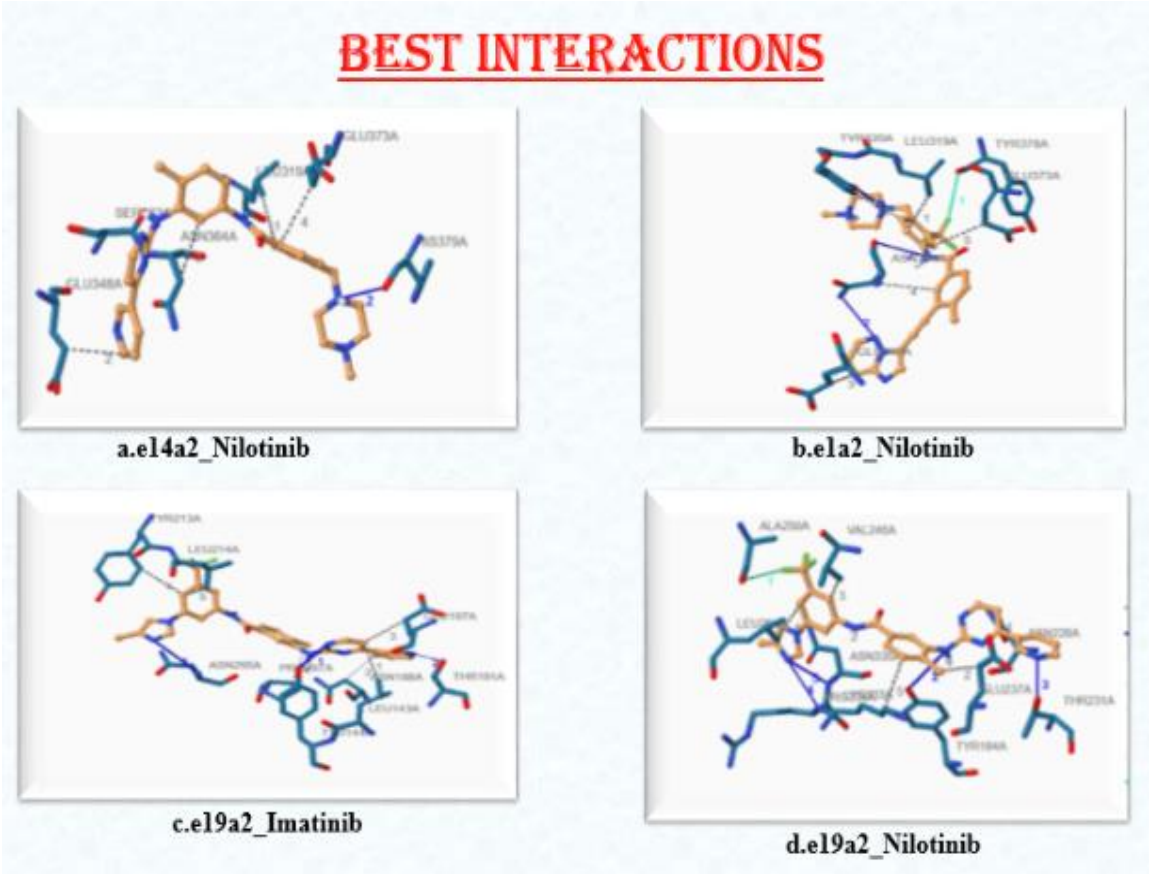

Figure 18. 2D structure of best interactions observed in molecular docking of different transcripts and different drugs used.

Appendix VIII: Documents

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 2638

25 March 2019

Ms. Roji Raut
Principal Investigator
Central Department of Biotechnology, TU
Kirtipur, Kathmandu

Ref: **Approval of thesis proposal** entitled **Frequency of BCR-ABL1 transcripts in Nepalese patients with Chronic Myeloid Leukemia from Civil Service Hospital and Medi Quest Laboratory Clinic Pvt. Ltd.**

Dear Ms. Raut,

It is my pleasure to inform you that the above-mentioned proposal submitted on **20 February 2019** (**Reg. no. 129/2019**) has been approved by Nepal Health Research Council (NHRC) National Ethical Guidelines for Health Research in Nepal, Standard Operating Procedures Section 'C' point no. 6.3 through Expedited Review Procedures.

As per NHRC rules and regulations, the investigator has to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made so and implemented after prior approval from this council. Thus, it is compulsory to submit the detail of such changes intended or desired with justification prior to actual change in the protocol. Expiration date of this proposal is **August 2019.**

If the researcher requires transfer of the bio samples to other countries, the investigator should apply to the NHRC for the permission. The researchers will not be allowed to ship any raw/crude human biomaterial outside the country; only extracted and amplified samples can be taken to labs outside of Nepal for further study, as per the protocol submitted and approved by the NHRC. The remaining samples of the lab should be destroyed as per standard operating procedure, the process documented, and the NHRC informed.

Further, the researchers are directed to strictly abide by the National Ethical Guidelines published by NHRC during the implementation of their research proposal and **submit progress report in between and full or summary report upon completion.**

As per your thesis proposal, the total research budget is **Self-Funded** and accordingly the processing fee amounts to **Rs 1,000.** It is acknowledged that the above-mentioned processing fee has been received at NHRC.

If you have any questions, please contact the Ethical Review M & E Section at NHRC.

Thanking you,

Nirbhay Kumar Sharma
Deputy Chief Administrative Officer

Tel: +977 1 4254220, Fax: +977 1 4262469, Ramshah Path, PO Box: 7626, Kathmandu, Nepal
Website: http://www.nhrc.org.np, E-mail: nhrc@nhrc.org.np

संकेत नं :

FREQUENCY OF BCR-ABL1 TRANSCRIPTS IN NEPALESE POPULATION WITH CHRONIC MYELOID LEUKEMIA FROM CIVIL SERVICE HOSPITAL AND MEDI QUEST LABORATORY CLINIC PVT. LTD.

सम्बन्धि अध्ययनमा सहभागीको मन्जूरीनामा फाराम

Chronic Myeloid Leukemia (CML) नेपालीहरुमा अत्यधिक रुपमा देखापरेको एक प्रमुख प्रकारको रगतको क्यानसर हो । यस रोगको पुष्टिका लागि मुख्यत : Philadelphia क्रोमोजोम अथवा BCR-ABL1 फ्यूजन जीनको परीक्षण गरिन्छ । तपाईलाई यस अध्ययनमा सहभागी हुन आह्वाहन गर्नुको मूख्य उद्देश्य तपाईको शरीरबाट लिइएको रगत वा Bone marrow को नमूनामा गरिने नियमित BCR-ABL1 फ्यूजन जीन परीक्षणका परिणामलाई हाम्रो अध्ययनमा समावेश गरेर Chronic Myeloid Leukemia (CML) रोगका कारक विविध transcripts हरुको आवृत्ति पत्ता लगाउनु हो । यस अध्ययनमा तपाईका नियमित परीक्षणका परिणामलाई मात्र समावेश गरिनेछ । यसका लागि तपाईले अतिरिक्त रगत वा Bone marrow का नमूना प्रदान गर्नुपर्ने अथवा अन्य परीक्षण गर्नुपर्ने छैन । बेलैमा थाहा हुंदा CML रोगको सजिलै निवारण हुनसक्छ तर यस रोगसम्बन्धि पर्याप्त अनुसन्धानको अभावमा नेपालीहरुमा यस रोगको प्रमुख कारक transcript अझै अन्योलमै छ । यसकारण यो रोगको निदान गर्दा कुन कुन transcript हरुको जाँच गर्नु आवश्यक छ भन्ने पत्ता लगाउनु नै यस अध्ययनको मूल उद्देश्य हो । यस अध्ययनको प्रमुख कारण भन्नुनै Chronic Myeloid Leukemia (CML) रोगको सहज र सरल निदानका विधिहरुको विकास गर्नु हो । यस मञ्जुरीनामाको प्रमुख उद्देश्य तपाईलाई यस अध्ययनसम्बन्धि पर्याप्त जानकारी दिनु हो जसमार्फत तपाई यस अध्ययनमा सहभागि हुने नहुने कुराको सही निर्णय लिन सक्नुहुनेछ । तपाईलाई प्रष्ट नभएका कुनै पनि कुराहरुसम्बन्धि खुलेर प्रश्न गर्न सक्नुहुनेछ । तपाईबाट प्राप्त रगत र Bone marrow का नमूनाहरु Medi Quest Laboratory Clinic Pvt. Ltd. एवम् त्रिभुवन विश्वविद्यालय केन्द्रिय विभागमा विश्लेशण गरिने छ । संकलित नमुनाहरुलाई संकेत नं दिइनेछ जसले गर्दा तपाईको व्यक्तिगत परिचय गोप्य रहनेछ ।

फाइदा : यस अनुसन्धानमा सहभागी भएर तपाई वा तपाईको परिवारलाई प्रत्यक्ष रुपमा फाइदा हुन वा नहुन पनि सक्छ । यस अध्ययनबाट प्राप्त जानकारी मार्फत भविष्यमा तपाई वा CML रोगको शंका भएका कुनै पनि बिरामीहरुलाई सरल तथा सहज रुपमा यस रोगकोनिदानका निम्ति सहयोग पुग्नेछ ।

गोपनीयता : यस अनुसन्धान र अध्ययनको नतिजा प्रकाशित गर्न सकिनेछ तर त्यसमा तपाईको नाम तथा परिचय उल्लेख हुने छैन ।

स्वेच्छिक सहभागिताको बयान : यस अनुसन्धानमा मेरो सहभागिता स्वेच्छिक हो । मैले आफ्नो इच्छाले बिना जरिवाना , बिना डरत्रास, अनुसन्धानकर्तासमक्ष पूर्व सूचना बिनानै कुनै पनि समय यस अनुसन्धानबाट सहभागिता परित्याग गर्न सक्नेछु । मैले माथि लेखिएका कुराहरु पढेको छु अथवा मलाई माथि लेखिएका कुराहरु पढेर सुनाइएको छ । मेरो प्रश्नहरुको जवाफ दिइएको छ र आफ्नो इच्छाले यस फाराममा सही गरेको छु ।

सहभागीको हस्ताक्षर :
अथवा सहभागीको हकमा अनुमती प्रदान गर्ने व्यक्तिको हस्ताक्षर)

अन्य व्यक्तिको हस्ताक्षर भए नाम र सम्बन्ध खुलाउनु होस् :

सहभागिको नाम :

ठेगाना :

सम्पर्क नं :

अनुसन्धानकर्ताको नाम :

अनुसन्धानकर्ताको सम्पर्क नं :

अनुसन्धानकर्ताको हस्ताक्षर : **मिति :**

संकेत नं :

FREQUENCY OF BCR-ABL1 TRANSCRIPTS IN NEPALESE POPULATION WITH CHRONIC MYELOID LEUKEMIA FROM CIVIL SERVICE HOSPITAL AND MEDI QUEST LABORATORY CLINIC PVT. LTD.

अभिभावकको लागि सहमति पत्र

(१८ वर्ष मुनिका बिरामीका लागि मात्र)

Chronic Myeloid Leukemia (CML) नेपालीहरुमा अत्यधिक रुपमा देखापरेको एक प्रमुख प्रकारको रगतको क्यानसर हो । यस रोगको पुष्टिका लागि मुख्यत : Philadelphia क्रोमोजोम अथवा BCR-ABL1 फ्यूजन जीनको परीक्षण गरिन्छ । तपाईको बच्चालाई यस अध्ययनमा सहभागी हुन आह्वाहन गर्नुको मूख्य उद्देश्य तपाईको बच्चाको शरीरबाट लिइएको रगत वा Bone marrow को नमूनामा गरिने नियमित BCR-ABL1 फ्यूजन जीन परीक्षणका परिणामलाई हाम्रो अध्ययनमा समावेश गरेर Chronic Myeloid Leukemia (CML) रोगका कारक विविध transcripts हरुको आवृत्ति पत्ता लगाउनु हो । यस अध्ययनमा तपाईको बच्चाको नियमित परीक्षणका परिणामलाई मात्र समावेश गरिनेछ । यसका लागि तपाईको बच्चाले अतिरिक्त रगत वा Bone marrow का नमूना प्रदान गर्नुपर्ने अथवा अन्य परीक्षण गर्नुपर्ने छैन । बेलैमा थाहा हुंदा CML रोगको सजिलै निवारण हुनसक्छ तर यस रोगसम्बन्धि पर्याप्त अनुसन्धानको अभावमा नेपालीहरुमा यस रोगको प्रमुख कारक transcript अझै अन्योलमै छ । यसकारण यो रोगको निदान गर्दा कुन कुन transcript हरुको जाँच गर्नु आवश्यक छ भन्ने पत्ता लगाउनु नै यस अध्ययनको मूल उद्देश्य हो । यस अध्ययनको प्रमुख कारण भन्नुनै Chronic Myeloid Leukemia (CML) रोगको सहज र सरल निदानका विधिहरुको विकास गर्नु हो । यस मञ्जुरीनामाको प्रमुख उद्देश्य तपाईलाई यस अध्ययनसम्बन्धि पर्याप्त जानकारी दिनु हो जसमार्फत तपाई यस अध्ययनमा सहभागि हुने नहुने कुराको सही निर्णय लिन सक्नुहुनेछ । तपाईलाई प्रष्ट नभएका कुनै पनि कुराहरुसम्बन्धि खुलेर प्रश्न गर्न सक्नुहुनेछ । तपाईको बच्चाबाट प्राप्त रगत र Bone marrow का नमूनाहरु Medi Quest Laboratory Clinic Pvt. Ltd. एवम् त्रिभुवन विश्वविद्यालय केन्द्रिय विभागमा विश्लेशण गरिने छ । संकलित नमुनाहरुलाई संकेत नं दिइनेछ जसले गर्दा तपाईको व्यक्तिगत परिचय गोप्य रहनेछ ।

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गोपनीयता : यस अनुसन्धान र अध्ययनको नतिजा प्रकाशित गर्न सकिनेछ तर त्यसमा तपाईको बच्चाको वा तपाईको नाम तथा परिचय उल्लेख हुने छैन ।

स्वेच्छिक सहभागिताको बयान : म ....., ... वर्षीय..... को अभिभावक, यस अनुसन्धानमा आफ्नो बच्चाको रगतको नमुना दिनको लागि सहमत भएको छु । यस अनुसन्धानमा मेरो सहभागिता स्वेच्छिक हो । मैले आफ्नो इच्छाले बिना जरिवाना , बिना डरत्रास, अनुसन्धानकर्तासमक्ष पूर्व सूचना बिनानै कुनै पनि समय यस अनुसन्धानबाट सहभागिता परित्याग गर्न सक्नेछु । मैले माथि लेखिएका कुराहरु पढेको छु अथवा मलाई माथि लेखिएका कुराहरु पढेर सुनाइएको छ । यसरी लिइएको रगतको नमुनालाई यही अनसन्धानका अतिरिक्त अन्य काममा दुरुपयोग हुने छैन भन्ने कुरा मलाई जानकारी गराइएको छ । मेरो प्रश्नहरुको जवाफ दिइएको छ र आफ्नो इच्छाले यस फाराममा सही गरेको छु ।

बच्चाको पुरा नाम :

बच्चाको हस्ताक्षर :

ठेगाना :

अनुसन्धानकर्ताको नाम :

अनुसन्धानकर्ताको सम्पर्क नं :

अनुसन्धानकर्ताको हस्ताक्षर :

अभिभावकको नाम :

अभिभावकको सम्पर्क नं :

अभिभावकको हस्ताक्षर :

मिति :

FREQUENCY OF BCR-ABL TRANSCRIPTS IN NEPALESE POPULATION WITH CHRONIC MYELOID LEUKEMIA FROM CIVIL SERVICE HOSPITAL AND MEDI QUEST LABORATORY CLINIC PVT. LTD.

INFORMED CONSENT FORM:
Questionare Format

ID No.: .....

NAME OF PARTICIPANT: .....

SAMPLE GIVEN : .....

AGE: .....

GENDER: ☐ Male ☐ Female ☐ Others

HEIGHT: .....

WEIGHT: .....

FAMILY HISTORY OF CANCER OR CML: ☐ Yes ☐ No ☐ Don't know

If YES, specify your relationship: .....

Any additional information (optional) .....

.....

Appendix IX: Photo gallery

Figure 19. MOU between Central Department of Biotechnology (Head of Department, Prof. Dr. Krishna Das Manandhar on left) and Medi Quest Laboratory Clinic Pvt. Ltd (Director and Chairman, Mr. Sunil Shrestha, on right)

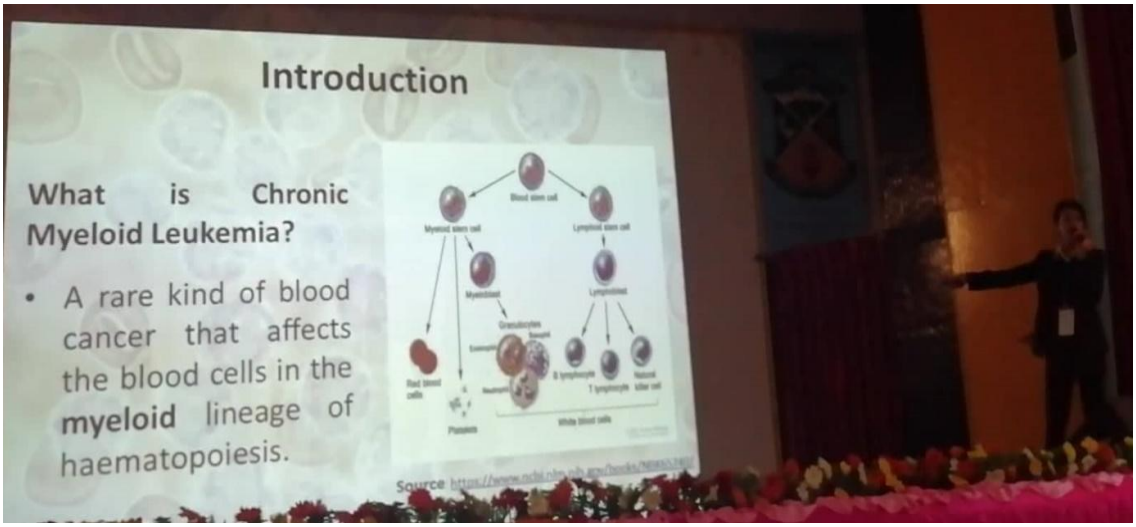

Figure 20. Figure: Oral presentation in One day Conference on Unravelling Life Sciences- A quest for sustainability held on 29th November 2019 at St. Xaviers College, Kathmandu

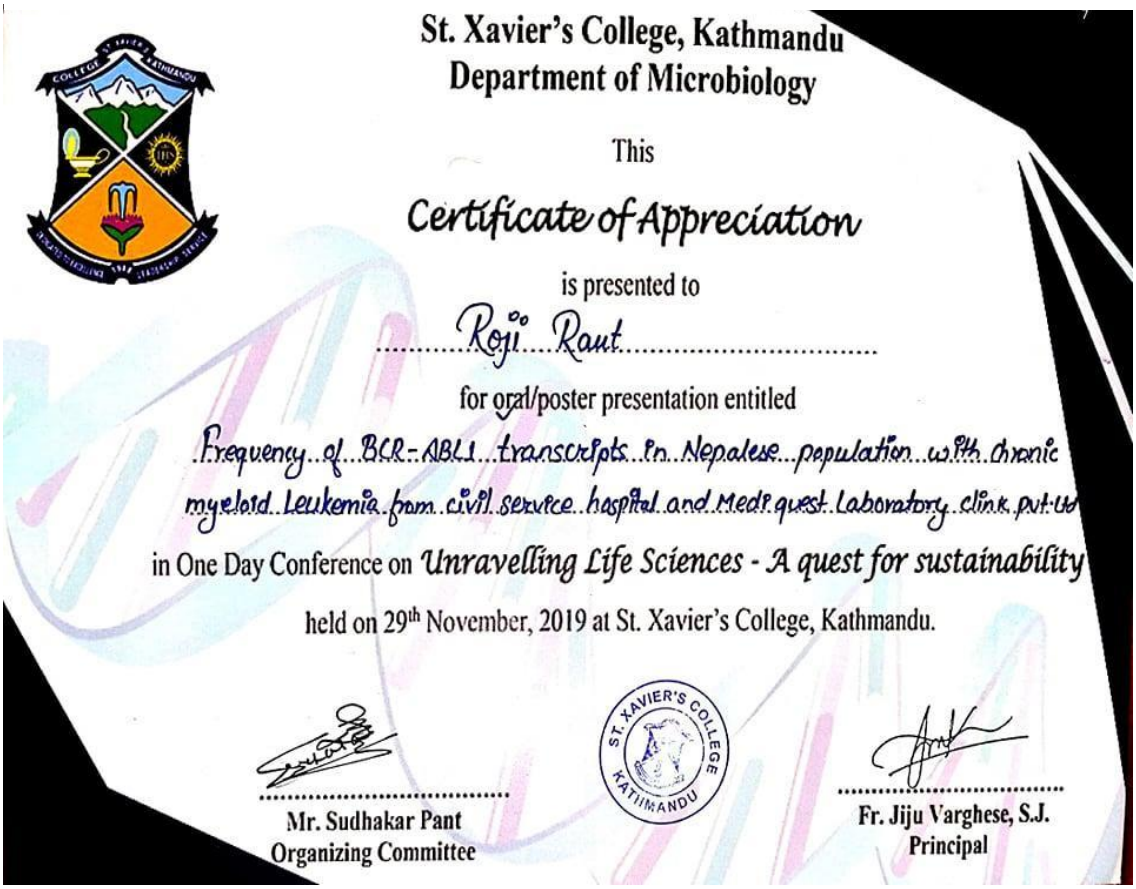

Figure 21. Certification of Participation in One Day Conference on Unravelling Life Sciences – A quest for sustainability held on 29th November 2019, St. Xaviers College, Kathmandu

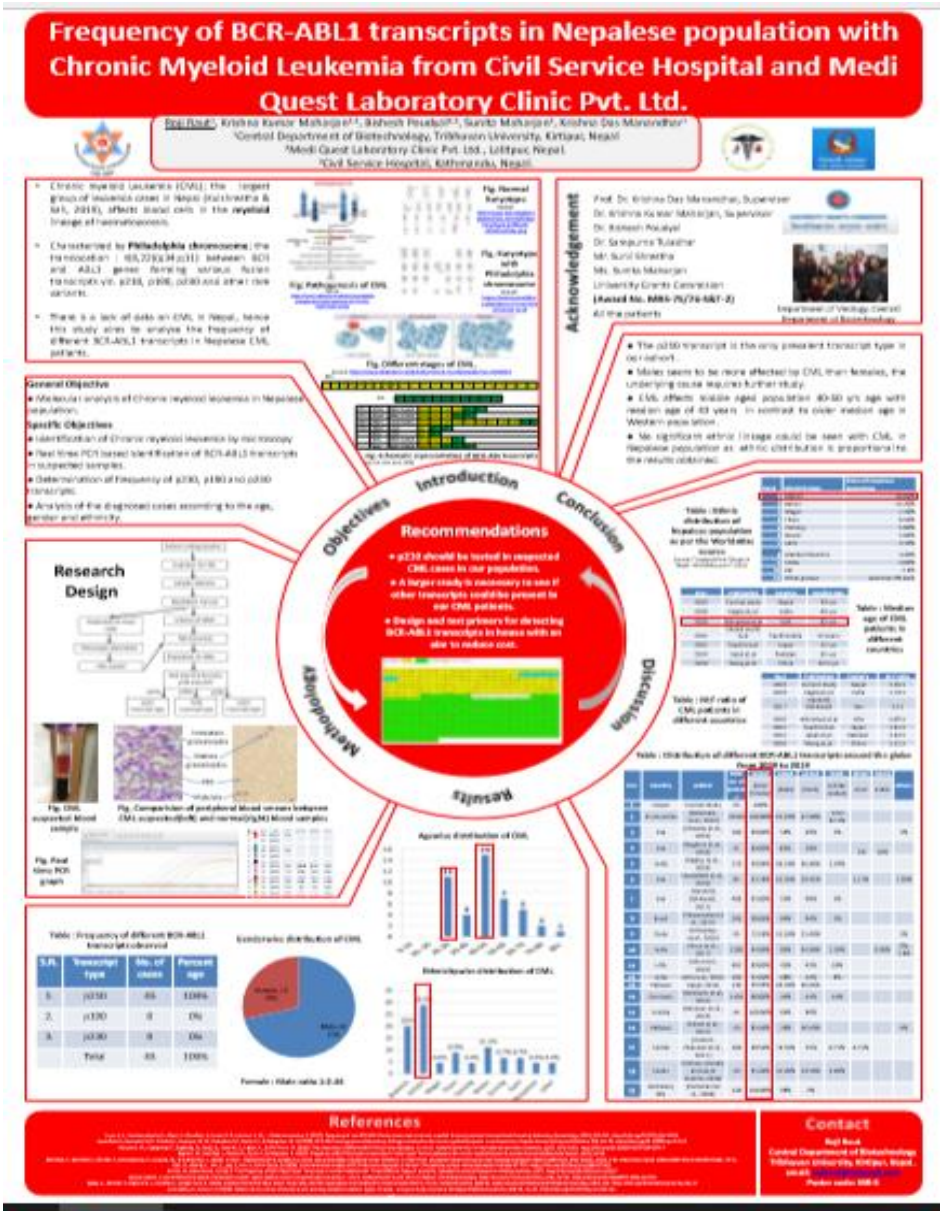

Figure 22. Poster presentation in Southeast Asian Regional Symposium on Microbial Ecology (SARSME-2020)

Figure 23. Certificate of participation in Southeast Asia Regional Symposium on Microbial Ecology (SARSME-2020)

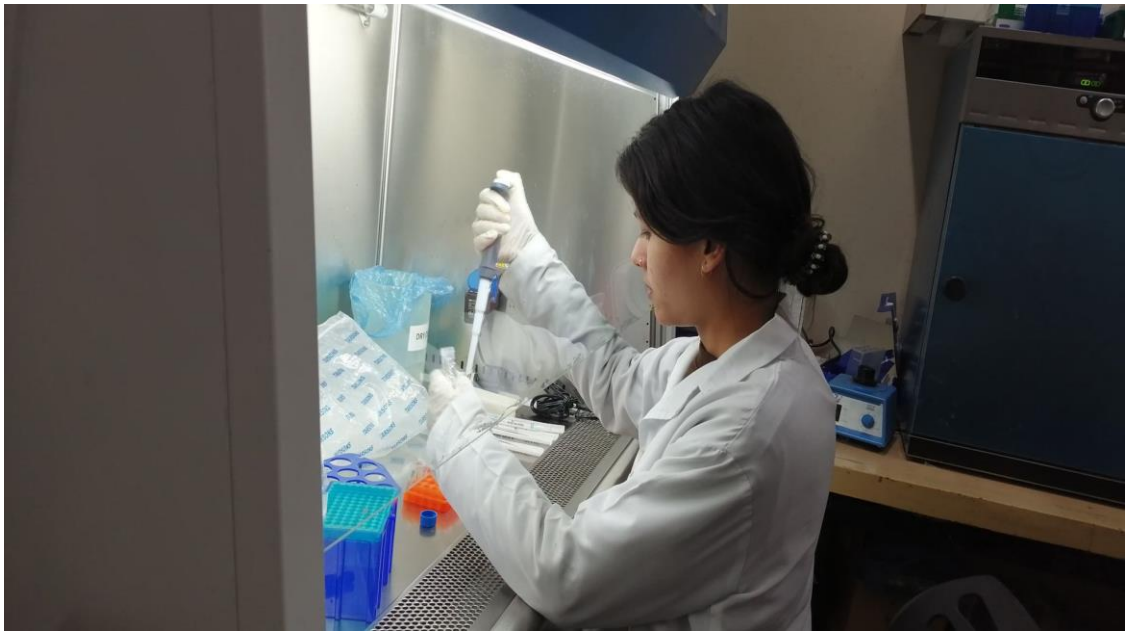

Figure 24. Manual RNA extraction from CML Suspected samples at Central Department of Biotechnology, TU, Kirtipur

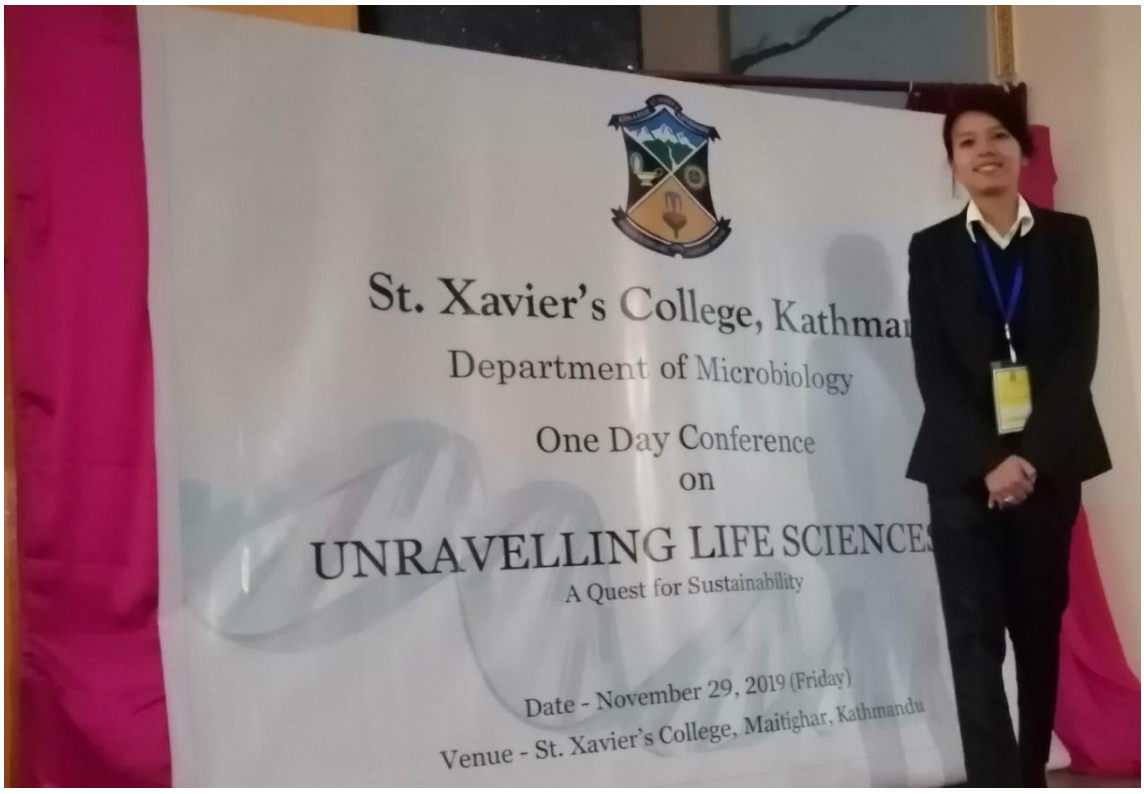

Figure 25. After oral presentation in One Day conference on Unravelling Life Sciences , A quest for sustainability at St. Xavier's College

Figure 26. Team Central Department of Biotechnology after attending the SARSME conference in Pokhara

Figure 27. Team Virolab