بسم الله الرحمن الرحيم
Republic of Sudan
Ministry of Higher Education and Scientific research
Shendi University
Faculty of Graduated Studies and Scientific Research

The Role of Association of Tyrosine Kinase Domain Mutation (T315I) with Programmed Death Ligand-1 in Resistant to Imatinib Mesilate in Sudanese Patients with Chronic Myeloid Leukemia

A thesis Submitted Fulfillment of the Requirements for PhD Degree in Medical Laboratory Sciences (Hematology)

Submitted by:
Hassan Babiker Mohamed Lazim

B.Sc. in Medical Laboratory Sciences (University of Sciences & Technology) 2002

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***Supervisor*:**

Prof. Babiker Ahmed Mohamed Ahmed Ismail

Professor of Pathology

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

وَعَلَّمَكَ مَا لَمْ تَكُنْ تَعْلَمُ وَكَانَ فَضْلُ اللَّهِ

عَلَيْكَ عَظِيمًا

النساء (113)

صَدَقَ اللَّهُ الْعَظِيمُ

Dedications

I dedicate this PhD thesis to people I love kind, mother, my wife and my son Mohamed. Also, Special thanks to them for every success I arrive to, because they do help, care and stand with me in every successful step in my live.

I love them very much.

Acknowledgment

A PhD is not an easy degree to obtain. Many complications were happened during the work example COVID19 that affected all of us. I was determined to succeed and to meet all the challenges.

First I would like to thank my supervisor. Professor Babiker Ahmed Mohamed Ahmed for the guidance and help throughout this project.

I am grateful to my family, especially my mother, brothers, sisters, friends for their encouragement, love and support that they have given me.

I am especially thankful to my family.

Abstract

Background: Programmed death receptor ligand-1 (PDL-1) acts an inhibitory molecule for T cells leading to suppressing T-cell and decrease ability of t-cell to kill tumor cells. However, Chronic Myeloid Leukemia cells exploit such molecules to escape immune surveillance.

Objectives: To analyze the association between Tyrosine Kinase Domain Mutation (T315I) and Programmed Death Ligand-1 in resistant to Imatinib Mesilate in Sudanese patients with Chronic Myeloid Leukemia.

Methodology: A cross-sectional case control study, a total of 150 participants, 100 patients with Chronic Myeloid Leukemia in chronic phases of disease, (68 males, 32 females), were conducted from Khartoum Oncology Hospital-Khartoum- Sudan during (2018-2023). All patients treated with Imatinib Mesilate for at least 6 months, with IM dose of 600 mg, while 50 apparently healthy controls (30 males and 20 females). Their mean ages in case group were (54.2±12.4) years, males (54.2±13.9 years) where the females (54.1±11.9 years). their mean ages in control group were (46.8±9.1) years, males (46.8±8.4 years) where the females (46.8±8.49 years). Blood samples were collected from all participants. The analysis of the plasma levels of programmed cell death ligand-1 (PDL1) was done by sandwich ELISA test using commercially available kits, the results was confirmed by repeating two times take the average readings. Also, the BCR-ABLT315I gene mutation was analyzed using molecular techniques (Real Time qPCR).

Result: results of the present study showed that the mean plasma levels of PDL-1 in Sudanese patients with Chronic Myeloid Leukemia resistant to Imatinib Mesilate was significantly increased (0.613ng/ml) when compared to healthy control group (0.336 ng/ml). The prevalence of BCR-ABLT315I mutation in Sudanese patients with Chronic Myeloid Leukemia resistant to Imatinib Mesilate was 11%. A statistical analysis revealed

the correlation between the plasma levels PDL-1 and mutation status in Sudanese patients with Chronic Myeloid Leukemia resistant to Imatinib Mesilate, showed a positive correlation between T315I mutation and plasma levels PDL-1 concentrations, with correlation coefficient (0.3713) indicates moderate correlation. The present study showed cut-off value result, the median plasma level of PDL-1 in CML patients was significant increase were compared to healthy controls (0.595ng/ml vs 0.335ng/ng/ml). Also, the cut-off value result of the median the patients with positive BCR-ABLT315I mutation was a significant increase were compared to those negative BCR-ABLT315I mutation (0.718 ng/ml vs 0.592 ng/ml). In addition, the cut-off values derived from the ROC curve, the plasma levels of PD-L1 was significantly increased in CML patients, showed high accuracy (0.877). At best cut off value (>0.593ng/ml), sensitivity was (100%), specificity was (66.9%) and increased plasma levels of PD-L1 was significantly increase in positive T315I mutation patients showed high accuracy (0.808). At best cut off value (>0.685ng/ml), sensitivity was (63.6%), specificity was (87.6%).

Conclusion: our results indicate that the patients with chronic myeloid leukemia Imatinib Mesilate resistant had higher plasma levels of PDL-1 in their blood compared to the control group and the patients with a positive BCR-ABLT315I mutation were found to had higher levels of plasma levels of PDL-1 than BCR-ABT315I-mutation-negative patients.

Key words: Programmed Death Ligand-1 (PDL-1), Chronic Myeloid Leukemia (CML), BCR-ABLT315I mutation, Imatinib and ELISA.

المستخلص:

خلفية البحث: رابط بروتين موت الخلية المبرمج-1 (PDL-1) يعمل كجزيء مثبط للخلايا التائية مما يؤدي إلى قمعها وتقليل قدرتها على قتل الخلايا السرطانية. ومع ذلك، فإن خلايا ابيضاض الدم الأبيض المزمن تستغل هذه الجزيئات للهروب من المراقبة المناعية.

أهداف البحث: هدفت هذه الدراسة تقييم العلاقة بين مستويات بلازما رابط بروتين موت الخلية المبرمج-1 (PDL-1) ووجود طفرة BCR-ABLT315I لدى المرضى السودانيين المصابين بابيضاض الدم النقوي المزمن ولديهم مقاومة لعلاج الايماتينيب.

طرق البحث: تم استخدام دراسة حالة شاهد مقطعية، شملت 100 مشارك مصاب بابيضاض الدم النقوي المزمن مكونة من (68 ذكور و32 إناث) في مستشفى الخرطوم لعلاج الأورام – السودان و50 شخص سليم كمجموعة مقارنة مكونة من (30ذكور و20 إناث) في الفترة ما بين 2018 الى 2023. تراوحت متوسط أعمار المصابين (54.2±12.4) وكان متوسط اعمار الذكور (54.2±13.9) والإناث (54.1±11.8). ومتوسط أعمار المجموعة المقارنة (46.8±9.1) وكان متوسط اعمار الذكور (46.8±8.4) والإناث (46.8±8.4). تم جمع عينات الدم من المشاركين وإجراء تحليل وقياس بلازما الدم لتحديد نسبة رابط بروتين موت الخلية المبرمج-1 (PDL-1) باستخدام تقنية الاليزا (ELISA) وتم تأكيد التحليل بتكرارها وحساب المتوسط للقراءات. كما تم تحليل الطفرة الجينية BCR-ABLT315I باستخدام التقنيات الجزيئية(Real Time qPCR).

نتائج البحث: أظهرت الدراسة الحالية أن متوسط مستويات رابط بروتين موت الخلية المبرمج-1 (pdl-1) في البلازما لدى المرضى السودانيين المصابين بابيضاض الدم النقوي المزمن ولديهم مقاومة لعلاج الايماتينيب أزيد بفرق إحصائي واضح مقارنة للمجموعة الضابطة متوسط التركيز لدي المرضي 0.613ng/ml مقارنة بمتوسط التركز 0.336ng/ml في المجموعة الضابطة. كما بلغ معدل انتشار نسبة طفرة BCR-ABLT315I في مرضى ابيضاض الدم النقوي المزمن ولديهم مقاومة لعلاج الايماتينيب 11%. كما كشف التحليل الاحصائي عن وجود علاقة ارتباط بين مستويات بلازما رابط بروتين موت الخلية المبرمج-1 (pdl-1) وحالة الطفرة الجينية BCR-ABLT315I لدى المرضى السودانيين المصابين بابيضاض الدم النقوي المزمن ولديهم مقاومة لعلاج الايماتينيب عن وجود علاقة إيجابية بقيمة عامل ارتباط متوسط 0.3713 وبفرق هام من الناحية الإحصائية(P=0.0001).

أظهرت أيضا الدراسة ان القيمة المقطوعة (cut-off value)لمتوسط تركيز مستوى PDL-1 في بلازما الدم لدى مرضى ابيضاض الدم النقوي المزمن زيادة معنوية مقارنة مع المجموعة الضابطة (0.595ng/ml vs 0.335ng/ng/ml) وكانت هناك زيادة ملحوظة لمتوسط قيمة مستوى PDL-1 في بلازما الدم لدى المرضى الذين يعانون من طفرة إيجابية مقارنة بالطفرة السلبية لدى المرضى (0.718 ng/ml vs 0.592ng/ml). بالإضافة إلى ذلك، فإن قيم القطع (cut-off value) المشتقة من منحنى ROC، دلت على زيادة في مستويات PDL-1 في بلازما الدم بشكل كبير لدى مرضى ابيضاض الدم النقوي المزمن، بدقة (0.877) و قيمة قطع (0.593ng/ml>)، كانت الحساسية (100%)، النوعية (66.9%) وجد أيضا زيادة مستويات البلازما الدم لـ PDL-1 بشكل ملحوظ في مرضى ابيضاض الدم النقوي المزمن ولديهم طفرة BCR-ABLT315I الإيجابية بدقة (0.808) وقيمة قطع (0.685ng/ml>)، كانت الحساسية (63.6%)، النوعية (87.6%).

الخلاصة: أظهرت هذه الدراسة أن مرضى ابيضاض الدم النقوي المزمن الذين لديهم مقاومة لعلاج الايماتينيب ولديهم مستويات بلازما أعلى في مستويات رابط بروتين موت الخلية المبرمج-1 (PDL-1) مقارنة بالمجموعة الضابطة. كما وجد أن مرضى سرطان الدم النقوي المزمن ولديهم مقاومة لعلاج الايماتينيب ولديهم طفرة BCR-ABLT315I إيجابية وجدت أن لديهم مستويات أعلى من رابط بروتين موت الخلية المبرمج-1مقارنةً بمرضى سلبية الطفرة لBCR-ABT315I.

الكلمات المفتاحية: بروتين موت الخلية المبرمج-1- ابيضاض الدم النقوي المزمن - طفرة BCR-ABLT315I- إيماتينيب - الاليزا

List of Contents

List of Figures

List of Tables

List of Appendices

Abbreviations

ABL	Abelson murine Leukemia viral oncology homolog 1
ACA	Additional Chromosomal Aberrations
allo-HSCT	allogeneic Hematopoietic Stem Cell Transplantation
AML	Acute Myeloid Leukemia
AP	Accelerated Phase
APC	Antigen Presenting Cells
ATP	Adenosine Triphosphate
B-ALL	Acute B-Lymphoblastic Leukemia
BAP-1	BCR Associated Protein-1
BC	Blast Crisis
BCR	Breakpoint Cluster Region
BM	Bone Marrow
BU	Busulfan
CBC	Complete Blood Count
CCYR	Complete Cytogenetic Remission
CD	Cluster of Differentiation
CHR	Complete Hematologic Remission
CML	Chronic Myeloid Leukemia
CMR	Complete Molecular Response
CP	Chronic Phase
DFS	Disease Free Survival
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ELN	European Leukemia Net
ET	Essential Thrombocythemia
FDA	Food and Drug Administration
FISH	Fluorescent In Situ Hybridization
G-CSF	Granulocyte Colony stimulating factor
GEF	Guanine nucleotide Exchange Factor
GRB2	Growth factor Receptor Bound protein2
HRP	Horseradish Peroxidase
IBMTR	International Blood and Marrow Transplant Registry
IL	Interleukin
ITIM	Immune receptor Tyrosine-based Inhibitory Motif
ITSM	Immune receptor Tyrosine-based Switch Motif
IRIS	International Randomized Study of interferon
LAP	Leukocyte Alkaline Phosphatase
LR	Leukemoid Reaction
LSC	Leukemic Stem Cell
MCYR	Major and Complete cytogenetic Response
MD	Medical Doctorate
MDS	Myelodysplastic Syndromes
MDSCs	Myeloid Derived Suppressor Cells
MDACC	Medical Doctorate Anderson Cancer Center
Me	Methylation

MCH	Major Histocompatibility complex
miRNA	micro RNA
MMR	Major Molecular Response
MRD	Minimal Residual Disease
NCI	National Cancer Institute
NGS	Next-Generation Sequencing
NIL	Nilotinib
NK cell	Natural killer cell
PB	Peripheral Blood
PDGF	Platelet-Derived growth factor
Ph	Philadelphia chromosome
PD-1	Programmed Death receptor-1
PDL-1	Programmed Death Ligand-1
PMF	Primary Myelofibrosis
PTC	Papillary Thyroid Cancer
PV	Polycythemia Vera
QC	Quality Control
qPCR	quantitative Polymerase Chain Reaction
RICK	Radiation and Isotopes Center Khartoum
RLFP	Restriction Fragment Length Polymorphism
ROC	Receiver-Operating Characteristic
RPM	Revolutions Per Minute
RT-PCR	Real Time Polymerase Chain Reaction
RT	Room Temperature
SH-3	SRC Homology 3 domain
SHP-1	src Homology region 2 domain-containing Phosphatase-1
sPD-L1	soluble serum Programmed Death Ligand- 1
ssDNA	single-stranded DNA
TCR	T Cell Receptor
TKI	Tyrosine Kinase Inhibitor
T315I	Threonine at 315 substituted by Isoleucine
US	United States
WHO	world Health organization

Chapter One: Introduction, Problem, and Objectives

1.1 Introduction:

Chronic Myeloid Leukemia (CML) is a clonal myeloproliferative disorder of a pluripotent stem cell associated with the presence of an abnormal chromosome, the Philadelphia (Ph) chromosome, which carries the Breakpoint Cluster Region-Abelson(BCR-ABL) oncogene(1,2). This oncoprotein is constitutively active in the non-receptor tyrosine kinase in *ABL* region, which leads to activation of multiple signal transduction pathways and inhibition of cells apoptosis(3,4). The *ABL* gene encodes a tyrosine kinase. Its function in a healthy cell is to transfer a phosphate from adenosine triphosphate (ATP) to a target protein which often has serine, threonine, or tyrosine residues. This phosphorylation typically has an impact on the activity of enzymes or protein interactions (5).

Imatinib Mesilate therapy is highly effective treatment for CML patients, and its response rate is excellent. However, some patients especially those with advanced stages of CML diseases, do not respond to Imatinib Mesilate treatment due to the development of acquired resistance or intolerance. Moreover, resistance to Imatinib can be divided into intrinsic primary (never responded to Imatinib therapy) and acquired secondary types of resistance (achieved a transient response which was lost later)(6). Furthermore, the most common cause of TKI resistance is specific point mutations in the ABL kinase domain. which often happen in the first 24 months of TKI therapy. However, among the patients, the mutation frequently appears in the Accelerated and Blast Crisis periods(7–9).

The T315I mutation, which is a single nucleotide change, results in threonine(T) to isoleucine(I) substitution at position 315, was thought to be one of the most common changes in the BCR-ABL domain in CML disease with presence of 43.4%. However, its function results in reduced Imatinib binding affinity(10–12). In addition, the T315I mutation at the gatekeeper residue, found in the advanced stage of the CML disease, also

associated with reduced overall survival rate. However, the overall survival rate of CML patients, is associated with the disease stage and the time T315I mutation was found(13,14).

Programmed death ligand-1 (PDL-1), a cell surface protein expressed on immunological and non-hematopoietic cells, it is an encoded protein that functions as a co-inhibitory molecule for T cells and is important in inhibiting the immune system. By binding the programmed cell death receptor-1 (PD-1) on T cells and its ligand programmed death ligand-1 (PD-L1) on myeloid cancer cells, the T-cell suppress itis function, migration, proliferation, and secretion of cytotoxic mediators, as well as limit the affinity for killing tumor cells(15,16).

Recently, the use of particular checkpoint treatment antibodies directed against the PDL-1 or PD1 receptor has demonstrated increased effectiveness, and improvements are being seen in a number of cancer types, including CML(17,18). As a result, Dr. Tasuku Honjo and Dr. James P. Allison shared the 2018 Nobel Prize in physiology or medicine field for their work on the development of cancer therapy through the inhibition of negative immune regulation of PD-1 receptor and ligands PDL-1 (19).

Therefore, we are conducting our research in the medical field after finding that the prevalence of kinase domain mutations, particularly the T315I mutation, had increased among Sudanese patients with CML diseases and had a high association with treatment compliance resistance(20).

1.2 Problem Statement

There is a relationship between elevated plasma levels of PDL-1 and the development of various types of mutations in the BCR-ABL kinase domain in the most of CML patients, which result in treatment failure. However, the previous researchers addressed the problem of Imatinib Mesilate treatment resistant in CML patients with the research from one side.

Therefore, this study adds an important new factor for CML patients resistant to Imatinib Mesilate treatment and rouse interest in studies testing these mutations in CML patients.

In present study we measure the plasma levels of PDL-1 in presence of BCR-ABL T315I mutations among Imatinib Mesilate resistant CML patients. The problem of the research is going to answer by the following main question: What is the relationship between increased plasma levels of programmed death ligand-1 and the presence of mutation BRR-ABLT315I in Imatinib Mesilate resistant CML patients?

1.3 Research Questions

According to the problem statement, we formulate the research questions as follow:

1- What is the relationship between plasma levels of programmed death ligand-1 and the presence of mutations in Imatinib Mesilate resistant CML patients?

2- What is the relationship between plasma levels of the programmed death ligand-1 and Imatinib Mesilate resistant CML patients?

3- What is the prevalence of BCR-ABLT315I mutation among Imatinib resist CML patients?

4- Are there any statistically significant for the significance level (0.05) in plasma programmed death ligand-1 and the presence of mutations in Imatinib Mesilate resistant CML patients?

1.4 Hypothesis

1-

H0: There is no difference between the cases and controls in the mean concentration of plasma levels of PDL-1

H1: There is a difference between the cases and controls in the mean concentration of plasma levels of PDL-1

2-

H0: There is no association between the plasma levels of PDL-1 of the negative mutation and positive BCR-ABLT315I mutation groups.

H1: There is association between the plasma levels of PDL-1 of the negative mutation and positive BCR-ABLT315I mutation groups.

1.5 Justification

The plasma levels of programmed death ligand-1 (PDL-1) may contribute to tumor immune evasion which increase the apoptosis of tumor cells by suppressing and affecting the immunotherapeutic efficacies. However, there is a connection between elevated plasma levels of PDL-1 and the develop of Imatinib Mesilate resistance in CML patients. In addition, all patients with a mutation in the ABL kinase domain had therapy failure(21).

Tyrosine kinase inhibitors (TKIs) are standard treatment for CML patients who need great selectivity against the *BCR-ABL1*. However, an acquired resistance due to TKIs through ABL1 kinase domain mutations, a serious clinical issue for CML patients particularly the T315I mutation(22). Moreover, over 30% of all CML patients developed resistant to Imatinib Mesilate and progressed to either AP or BP CML. Additionally, there are several factors may increase the resistant to treatment such as extra mutations in the *BCR-ABL1*kinase domain and genomic instability(23). In CML patients, detecting of these mutations may be clinically important. However, the presence of certain mutations may provide prognostic information. But, the mechanisms by which these mutations develop are still enigma(24).

Therefore, this study aimed to determine the role of the programmed death ligand-1 in TK domain mutation (T315I) and itis relationship to Imatinib Mesilate resistant in Sudanese CML patients. PDL-1 was measured by using the direct method of enzyme linked immunosorbent assay (ELISA) and detection of TK domain mutation (T315I) by highly sensitive real time polymerase chain reaction (quantitative real type PCR). In addition to demonstrating if there is significant association between the high plasma levels of PDL-1 and TK domain mutations T315I in Sudanese Imatinib Mesilate resistant CML patients.

1.6 Objectives

1.6.1 General Objective

To determine the association between Tyrosine Kinase Domain Mutation (T315I) and Programmed Death Ligand-1 in resistant to Imatinib Mesilate in Sudanese patients with Chronic Myeloid Leukemia.

1.6.2 Specific Objectives

1- To measure the plasma level of PDL-1 in resistant Imatinib Mesilate in CML patients and control groups.

2- To assess the prevalence of BCR-ABLT315I mutation among resistant Imatinib Mesilate patients with CML.

3- To find out the correlation between the plasma levels of PDL-1and BCR-ABLT315I mutation resistant Imatinib Mesilate patients with CML.

4- To find out the correlation between the plasma levels of PDL-1and risk factor (age, sex).

5- To determine the cutoff value of the plasma levels of PD-L1 in resistant Imatinib Mesilate in patients with CML by ELISA test.

1.7 Organization of Study

The thesis is divided into five chapters in addition to preliminary sections, references and Appendix. The preliminary section includes title page, dedications, acknowledgment, list of contents, list of figures, list of tables, abbreviations and abstract.

Chapter **1** include introduction, problem statement, research questions, hypothesis, justification, objectives and organization of the study. Chapter **2** is about literature review it contains CML, tyrosine Kinase domain, Immune checkpoints molecules (PDL-1) and Previous Studies.

Chapter **3** is about research methodology, it contains study design, study duration, study area, study population, sample size, inclusion and exclusion criteria, sample collection, procedures and regents using in measuring of PDL-1 test and detection of T315I mutation. Chapter **4** is about the results of study. Chapter **5** is the last chapter, it contains discussion, conclusion and recommendations.

Chapter Two: Scientific Background and Literature Review

2.1 Chronic Myeloid Leukemia(CML)

2.1.1 Definition of CML

Chronic Myeloid Leukemia (CML) is a hematopoietic stem cell malignant clonal disorder associated with the presence of abnormal chromosome, the Philadelphia chromosomes (Ph), which express a specific cytogenetic abnormality, *BCR-ABL1* fusion gene(1,2,25).

2.1.2 History of CML disease.

Based on autopsy findings in hematological malignancy, the three pathologists Drs. John Bennet, David Craigie, and Rudolf Virchow independently identified Chronic Myeloid Leukemia (CML) for the first time in 1845. Then Fuller made the initial diagnosis of leukemia in a live patient one year later, in 1846 (see Table 2-1)(26–29)The research of cytogenetic was established between 1950 and 1956, and Tjio and Levan rapidly discovered the diploid 46-chromosome in a normal human cell (30). David Hungerford and Peter Nowell, two physicists, were first reported in Philadelphia city in the USA, in 1960. The Philadelphia chromosome (Ph), a chromosomal aberration, was found in the bone marrow cells of two male patients with chronic myeloid leukemia(31). Later, in 1973, Janet Rowley was discovered the Philadelphia chromosome(Ph) a result of a reciprocal translocation of genetic material on the long arms of chromosome 9 and the short arm of chromosome 22, t(9;22)(q34;q11). The proto-oncogene *ABL* is encoded by chromosome 9, while the breakpoint cluster region gene *BCR* is encoded by chromosome 22 as a molecular outcome of this translocation forming *BCR-ABL* fusion gene(4).

Table 2.1 Milestones in unravelling the biology of CML(32)

Milestones in unravelling the biology of CML.	
1845	Recognition of leukemia (probably CML) as a disease entity
1846	First diagnosis of leukemia in a live patient
1956	Development of cytogenetic study.
1960	Identification of the Philadelphia chromosome.
1973	Recognition of the reciprocal translocation t(9;22).

2.1.3 Molecular characteristics of CML

At the molecular level, CML was the first hematological cancer to be linked to an abnormal chromosome; this abnormality resulted from a reciprocal translocation of genetic material on chromosomes 9 and 22 at (q34;q11), where chromosome 9 encodes the proto-oncogene Abelson murine leukemia (ABL) and chromosome 22 encodes the breakpoint cluster region gene (BCR), which results in the formation of BCR-ABL fusion proteins. **(**Figure 2.1**)**(31).This particular BCR-ABL fusion gene produces a constitutively active non-receptor tyrosine kinase, BCR-ABL1, due to this translation activation of multiple signal transduction pathways and cause of both CML and Ph+ positive acute B-lymphoblastic leukemia(B-ALL)(3,4).However, this fusion gene is present in all myeloid cell lineages and in some lymphoid cells and endothelial cells(5),

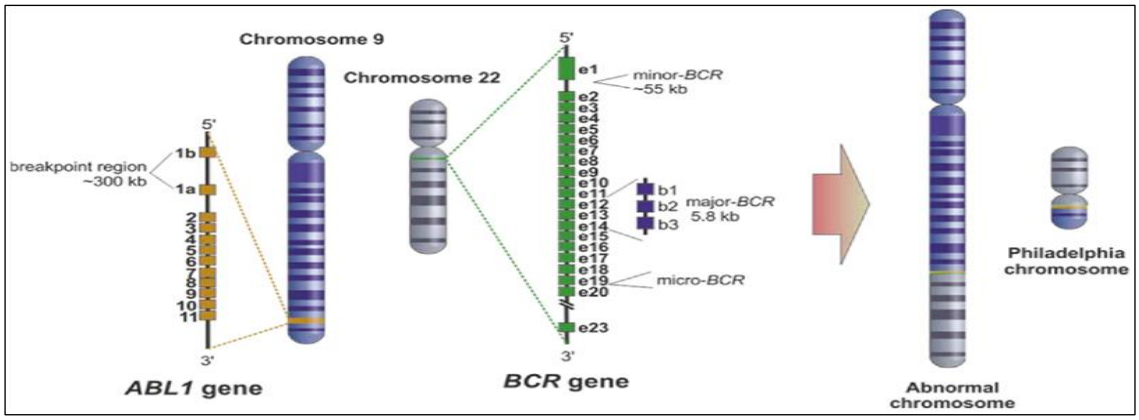

Fig. 2.1 The origin of the Philadelphia chromosome: The Philadelphia chromosome results from the translocation between the Abelson murine leukemia (ABL1) gene on chromosome 9 with the breakpoint cluster region (BCR) gene on chromosome 22(25).

2.1.4 Clinical Phases of CML.

Historically, based on the quantity of blasts, CML can be classified into three different phases: an initial stable Chronic Phase (CP), Accelerated Phase(AP) and Blast Crisis Phase (BP). However, the majority of patients are initially identified in the chronic phase(33). Moreover, this classification is based mainly on the number of immature white blood cells (blast), found in the blood or bone marrow, that provided by International Blood and Marrow Transplant Registry (IBMTR), the MD Anderson Cancer Center (MDACC), the World Health Organization (WHO) and the European Leukemia Net (ELN)(34–36) Additionally, In the chronic phase of CML, the patients usually are a symptomatic or have a mild symptom, most of (90-95%) patients are diagnosed during this phase(37).

Chronic phase, in bone marrow and peripheral blood, there are often only a small percentage (10%) of immature leukocytes (myeloblasts), besides that there are an increasing number in the mature functioning white blood cells as well as the number of platelets (31,38). Usually, the CML patient in chronic phase needs a standard treatment to achieve a good response and control the high number of white blood cell count. A small number of patients appear to stop responding to treatment, during the chronic phase, they are progressing to the accelerated phase (33).

Accelerated phase of the disease, the patients may have symptoms such as a fever, low appetite, enlarged spleen, and weight loss. The accelerated phase is characterized by 10-19% blast cells in the peripheral blood or bone marrow, ≥ 20% Promyelocyte, 20% basophils in the peripheral blood and decrease in platelet counts less than (100×10^9 /L). However, Median survival of patients in accelerated phase, without a stem cell transplant (SCT) or tyrosine-kinase inhibitors (TKIs) therapy is only 12-18 months (Table 2-2)(33,36,39)

Blast phase of the diseases, the patients may have symptoms such as; fever, fatigue, weight loss, bleeding from platelets abnormalities, shortness of breath, an enlarged spleen and generally fell unwell (33). However, the blast phase, also characterized by a dramatic increase in the number of blast cells in the bone marrow or peripheral blood (usually 30% or more) and the presence of an extramedullary accumulation of blast cells (infiltration of blast cells regardless of proliferation in the bone marrow involvement other organs), in 7–17% of the patients(40–42).

Table 2.2 Comparison of the criteria established by the ELN and the WHO for definition of the phase of CML(43).

Phase	European Leukemia Net (ELN)	World Health Organization (WHO)
CML-CP	<10% blasts in PB or in BM No criteria fulfilled for CML-AP or CML-BP	• <10% blasts in PB or in BM • No criteria fulfilled for CML-AP or CML-BP
CML-AP	• Persistent thrombocytopenia (<100 × 10^9/L) unrelated to therapy • >20% basophils in the PB • 15–29% blasts in the PB and/or BM • Sum of myeloblasts and promyelocytes >30% in the PB or BM with proportion of blasts <30%	• Persistent or increasing WBC (>10 × 10^9/L), unresponsive to therapy • Persistent or increasing splenomegaly, unresponsive to therapy • Persistent thrombocytosis (>1000 × 10^9/L), unresponsive to therapy • Persistent thrombocytopenia (<100 × 10^9/L) unrelated to therapy • >20% basophils in the PB • 10–19% blasts in the PB and/or BM • Additional clonal chromosomal abnormalities (ACA) in Ph1 cells at diagnosis that include “major route” abnormalities (second Ph1, trisomy 8, is chromosome 17q, trisomy 19), complex karyotype, or abnormalities of 3q26.

Phase	European Leukemia Net (ELN)	World Health Organization (WHO)
CML-BP	• ≥30% blasts in the blood, marrow or both • Extramedullary infiltrates of leukemic cells (with the exception of spleen and liver)	• ≥20% blasts in the blood or BM • the presence of an extramedullary accumulation of blasts (with the exception of spleen and liver) • (As the onset of lymphoid BP may be quite sudden, the detection of any bona fide lymphoblast's in the blood or marrow should raise concern for a possible impending lymphoid BP, and prompt additional laboratory and genetic studies to exclude this possibility).

2.1.5 Epidemiology of CML

2.1.5.1 Prevalence and Incidence

The Chronic Myeloid Leukemia accounts for 15 to 20 % of all leukemia in adults, with an annual incidence rate of approximately 1.5 per 1000,000 persons worldwide (Figure 2-2). CML incidence increases with age and occurs between (50^{th} to 70^{th}) that making mostly an adult disease. However, CML can occur in any age, even in children(44,45). Furthermore, the incidence of CML disease is more in males than females with a ratio varying from 1.2 and 1.7 in different studies(46–48).

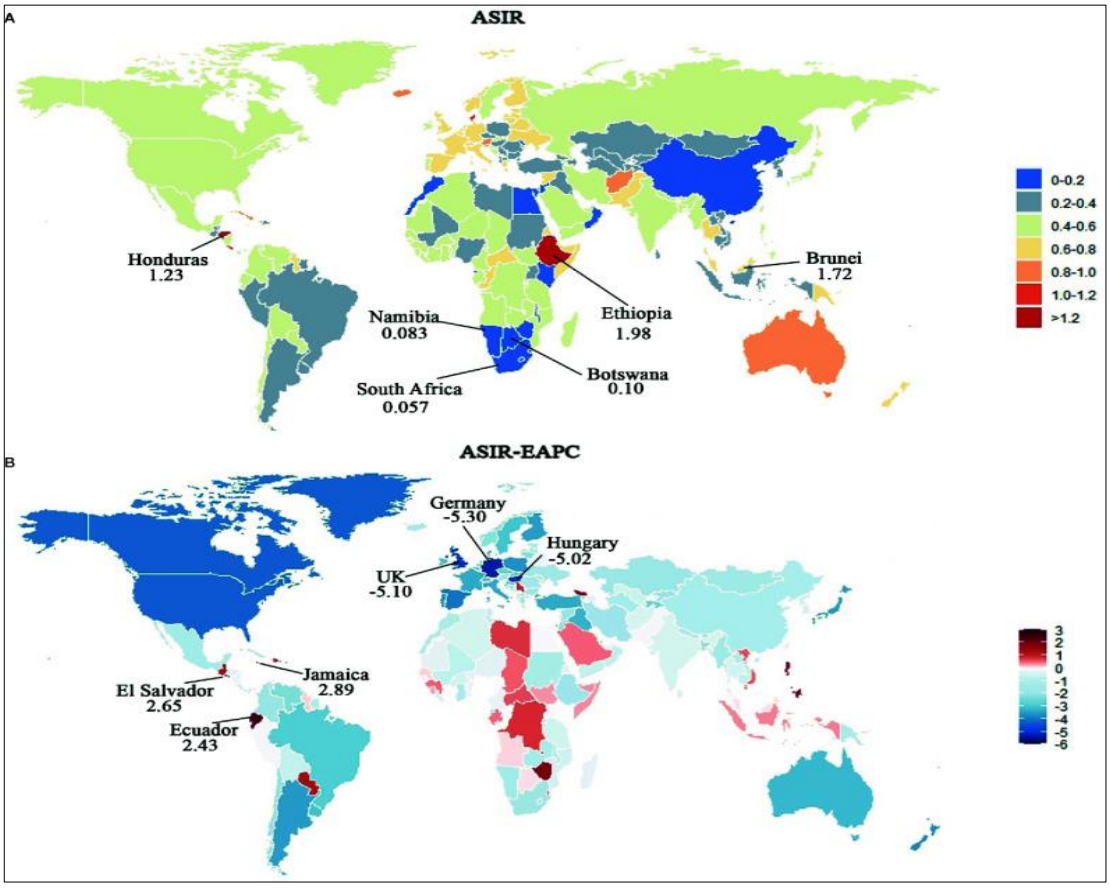

Fig. 2.2 Incidence rates of chronic myeloid leukemia worldwide.(49)

2.1.5.2 Etiology

The majority of cases of CML have no known etiology. Only a few number of known factors linked to exposure to particular chemicals have been linked to the development of CML. One risk factor for CML is prolonged exposure to large amounts of benzene and other organic solvents(50). Although the exact etiology of the disease is unknown, exposure to high doses of ionizing radiation, such as those from the 1945 nuclear disaster in Japan, has been shown to dramatically increase the risk of getting the condition(51).Additionally, the significance of altered myeloid "driver" genes in juvenile CML is coming into greater focus(52).

2.1.6 Pathophysiology of CML.

It is obvious that the BCR-ABL protein is essential for the development of chronic myeloid leukemia. 90% to 95% of CML patients carry the fusion gene for the oncoprotein

BCR-ABL1. Although the etiology of the t(9;22) translocation is unknown, a high dosage of radiation has been linked to an increase in incidence (ionizing radiation)(51).

The Abelson tyrosine kinase (ABL) gene is a non-receptor tyrosine kinase that normally codes for the nuclear ABL protein p145, which is essential for signal transduction, cell growth regulation, and proliferation. The BCR gene, in contrast, codes for a protein called p160 that is constitutively produced in hematopoietic cells(34,53). It is function the Abl in kinase domain catalyzes the transfer of -phosphate from ATP onto tyrosine residues in substrate proteins and peptides. However, the internal regulatory system is disrupted and c-Abl is kept in an inactive form when ABL and BCR combine. Moreover, the t(9;22) translocation creates a *BCR-ABL1* fusion gene that is transcribed *BCR-ABL1* fusion protein, which is constitutively active tyrosine kinase leading to uncontrolled proliferation of myeloid, monocytic, erythroid, megakaryocytic, B-lymphoid, and occasionally T-lymphoid cell lineages(37,54,55).

BCR-ABL1 accomplishes this propagation by inhibiting apoptosis and stimulating cell cycle entry of hematopoietic cell lines even in the absence of growth factors(56). Additionally, depending on where the breakpoint on chromosome 22 is located, several forms of the *BCRABL1* fusion protein have been produced **(**Figure 2-3**)**. The most prevalent variant results from a breakpoint in exon b2 or b3 of the *BCR* linked to exon a2 of the *ABL1* gene, resulting in a 210 kDa protein known as the p210 *BCRABL1* protein. A p230 *BCR-ABL1* protein may result from an alternate *BCR* exon splice at codon e19. Finally, a BCR splice at e1 can result in the production of the p190 *BCR-ABL1* protein, which is more common in Ph-positive acute lymphoblastic leukemia than it is in CML patients. However, the shorter protein (p190) causes more aggressive cell behavior than the longer proteins, indicating that these mutations also have different clinical actions(57).

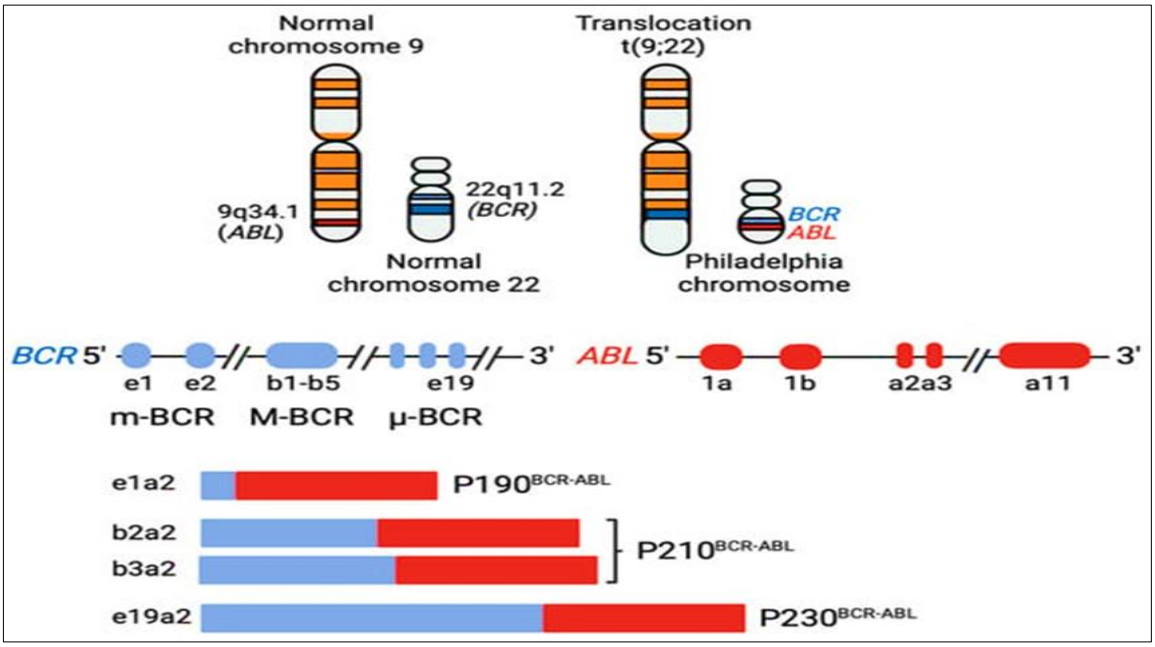

Fig. 2.3 Philadelphia Chromosome isoforms(58).

2.1.7 Diagnosis of CML.

2.1.7.1 Clinical feature of chronic myeloid leukemia

About one-third of CML patients are clinically asymptomatic when they are identified. However, the majority of patients show symptoms may have anemia, including fatigue, weight loss, excessive sweating, especially at night, and symptoms of splenomegaly, including left upper quadrant pain, feeling of a mass, abdominal fullness, and retinal hemorrhage. About 95% of patients have splenomegaly(59).

2.1.7.2 Laboratory diagnosis.

About 85% of patients with chronic myeloid leukemia are diagnosed during the chronic stage of the disease, and 40% of them are asymptomatic. When conducting a routine blood test in a hospital, identify the disease(60). However, a physical examination, hematologic results from tests such the complete blood count (CBC), manual differential blood count, cytogenetic and molecular testing(61). Additionally, the presence of abnormal Philadelphia chromosome abnormality, which is a diagnostic conformation, must be confirmed by using the standard cytogenetics Karyotype method to determine

the number, size, shape, and arrangement of the Philadelphia chromosomes, or by detecting the Ph-related molecular BCR-ABL1 abnormalities by molecular hybridization technique called fluorescent in situ hybridization (FISH) and Real-time Polymerase Chain Reaction (RQ-PCR)(62–65).

2.1.7.2.1 Blood count

Complete Blood Count (CBC) is a common test to count the various blood cell types in peripheral blood is called. However, patients with CML frequently have high white blood cell counts as well as high neutrophil, basophil, and eosinophil absolute counts. Moreover, the platelet count may vary, either high or low, and a slight anemia may also develop as a result of a decreased hemoglobin concentration(66). CML patient also requires a manual differential blood count and morphology study of blood cells in peripheral blood smears and bone marrow aspiration smears to assess the rise in white blood cells and identify any abnormalities(67). Additionally, when examined peripheral blood smear of patient with CML disease under a light microscope show full myeloid spectrum. However, these mature granulocytes have decreased apoptosis, resulting in accumulation of long-lived cells with low or absent enzymatic activity, such as alkaline phosphatase(68).Along with additional anomalies in granulocytes, megakaryocytes, and erythrocyte precursors Large mononuclear forms, numerous small separated nuclei, and microforms of the megakaryocytes; hyper- and hyposegmentation; abnormal lobulation and ring-shaped nuclei of the polymorphonuclear leukocytes; Pelger-like leukocytes; binucleate myelocytes; multinuclearity and karyorrhexis of the erythroblasts, and large mononuclear forms, multiple small separated nuclei and microforms of the megakaryocytes**(**Figure 2.4**)**(69–71).

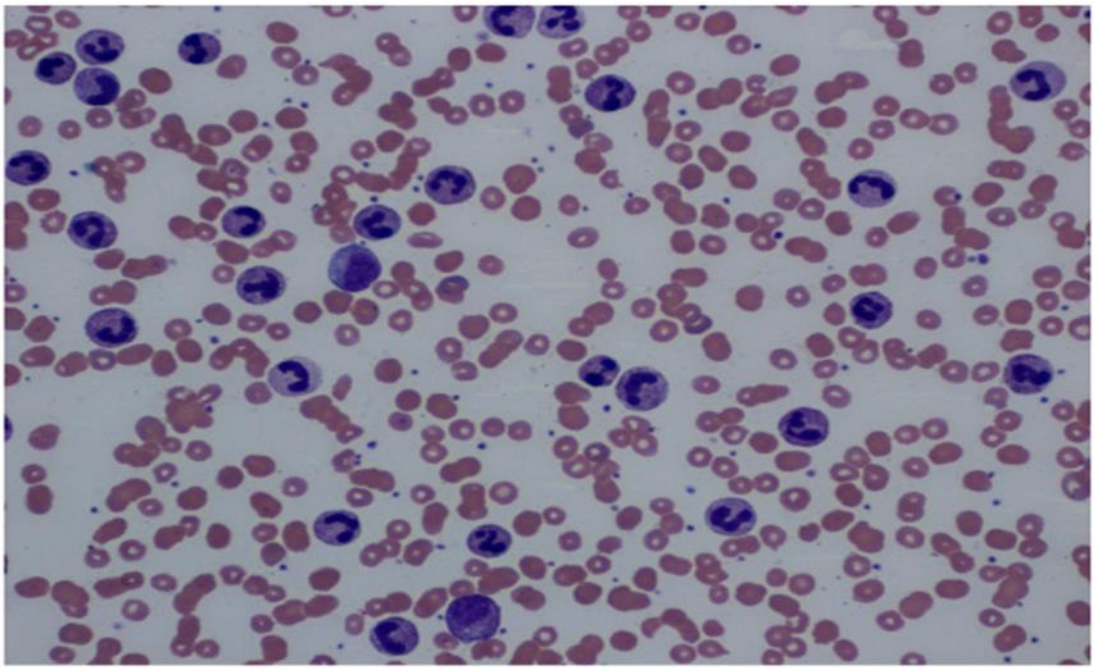

Fig. 2.4 Peripheral blood (PB) fi lm from a patient with chronic myelogenous leukemia with an unusually high white cell count showing an increase of neutrophils, eosinophils, basophils, and granulocyte precursors(72).

abnormalities in the plasma of CML patients, such as elevated uric acid levels that lead to urate stones in the urinary tract, these abnormalities are uncommon. Furthermore, patients with CML often have higher vitamin B12 levels and vitamin B12-binding proteins(73–75).Finally, high basophil counts in peripheral blood when assessed result in elevated histamine levels in the plasma of CML patients. Additionally, increase in serum tryptase levels, a potent indicator for prognosis in CML patients. However, the overall number of mature and immature basophils in CML is reflected by serum tryptase(76).

2.1.7.2.2 Immunophenotyping

Flow cytometry is a technique used for counting, examining and sorting microscopic particles suspended in fluid. Additionally, it enables multiparametric investigation of the chemical and/or physical properties of a single cell as it passes through an optical and/or electronic detecting equipment. In the course of the blast transformation, it plays a crucial role in separating the lymphoid and myeloid lineages. The method used to identify leukemic cells, however, depends on the antigens or markers

that the cells' surfaces contain known as cluster differentiation (CD)(77,78) Moreover, while CD11b, CD11c, and CD15 are primarily found in mature granulocytic cells, the cluster differentiation markers CD34, CD33, and CD13 are present in immature myeloid progenitor cells. But the monocytes and thrombocytes were CD14 and CD61 positive, respectively(79,80).Further, B lymphocyte lineage immature cells exhibit CD34, HLA-DR, and CD10 (high intensity), whereas mature B lymphocyte cells exhibit CD45, CD22, CD19, TdT, and CD10 expression (low-intensity)(81,82).

2.1.7.2.3 Bone marrow aspiration and biopsy

All CML patients must undergo a bone marrow aspiration at the time of diagnosis. BM aspiration helps identify and distinguishing between various CML disease stages and enables the diagnosis to be confirmed. Moreover, a trephine biopsy should be performed to determine the level of fibrosis(67,83) In the lab, bone marrow aspiration is carried out by inserting a specialized needle into one of the bones in iliac crest while the patient is under anesthesia, removing a small sample of bone marrow, and creating a smear to look at the WBCs' morphology. However, assessments of the percentages of myeloblasts, promyelocytes, myelocytes, eosinophils, and basophils are possible with the aspirate (Figure 2.5)(84,85). Additionally, the aspiration of bone marrow from CML patients revealed hypercellularity with granulocytic proliferation, noticeably increased myelocytes, and blast levels that were typically less than 5%. Bone(86).

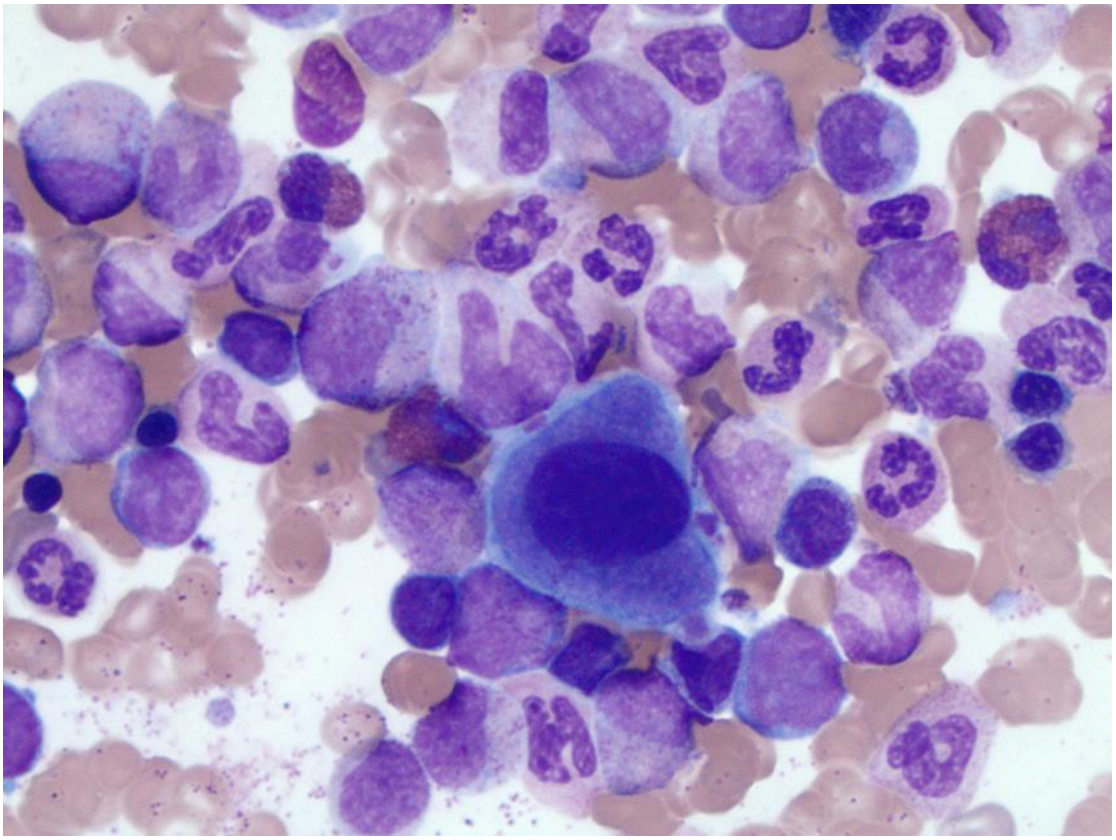

Fig. 2.5 Bone marrow film from a patient with chronic myelogenous leukemia showing increased granulopoiesis(72).

2.1.7.2.4 Biochemical test

Several biochemical tests and functional granulocytic abnormalities both been implicated in CML. The granulocytic abnormalities are often quantitative rather than qualitative and are evident in the adult granulocyte population(87).A screening test called leukocyte alkaline phosphatase (LAP) is used to find the alkaline phosphatase enzyme, which is present in the circulating population of mature granulocytes(88).The LAP test is also used to differentiate between the typical CML diseases and other myeloproliferative disorders(89).

In CML, the LAP activity usually decreased or absent in patients at diagnosis. However, increasing during infections or when the high leukocyte count is reduced by chemotherapy(90,91).Although the scientist found some biochemical may have a higher percentage of CD34-positive hematopoietic stem and progenitor cells than healthy

individuals. But this rise suggests that the disease is moving from a chronic to an advanced stage in CML patients(92–94).

2.1.7.2.5 Cytogenetics (chromosome analysis)

The study of chromosome structure, function, and variation is known as cytogenetics, which derives its name from the combination of the terms cytology and genetics. However, cytogenetics is separated into two categories: traditional cytogenetics karyotype and molecular cytogenetics(95).

2.1.7.2.5.1 Conventional Cytogenetics(karyotype)

A conventional cytogenetic karyotype, which was initially created in the field of cytogenetics, is still the most important method for helping clinical hematologists in the detection of chromosome abnormalities in hematological malignancies. It is an easy technique to find out the chromosome abnormalities (Figure 2.6)(96,97). A successful detection of the Ph chromosome done by cytogenetic analysis, by use sample collected either by bone marrow or peripheral blood. In most cases, 2 to 3 mL of high-quality, heparinized peripheral blood samples or at least 5 mL of heparinized bone marrow samples are sufficient (98). Each culture requires 1–10 million cells to produce 20–25 cells in the metaphase. However, the cytogenetic analysis is appropriate with at least 80 metaphases(99,100).

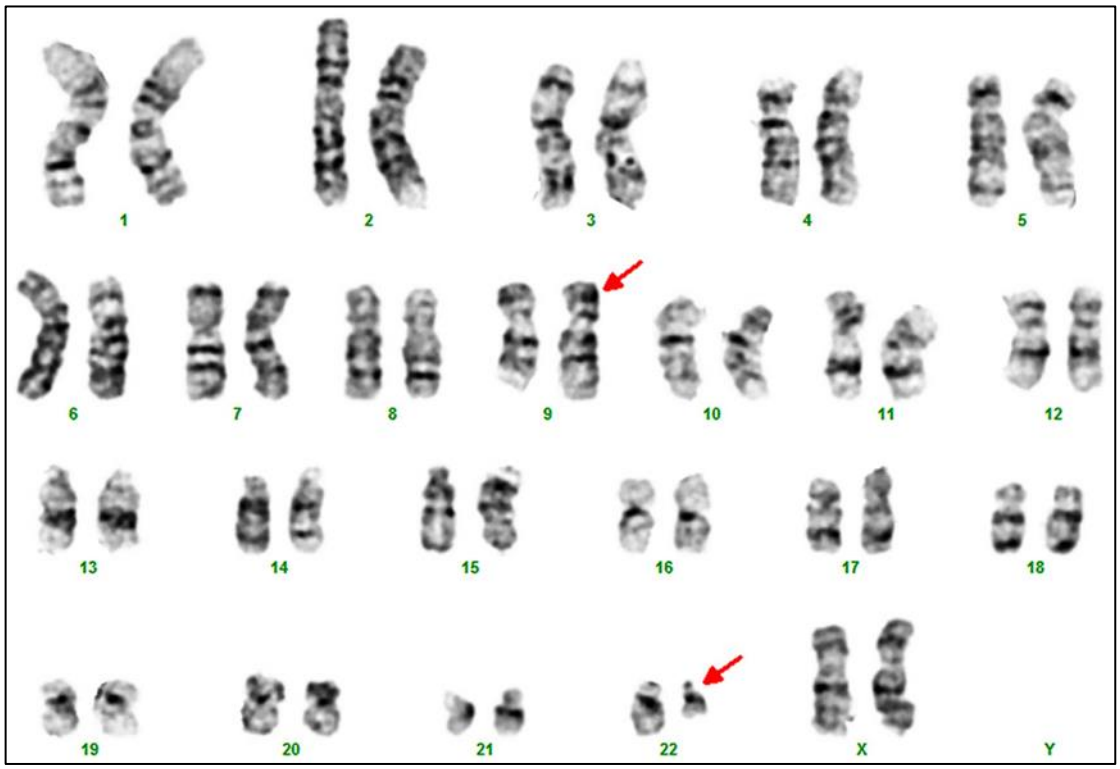

Fig. 2.6 Conventional cytogenetics of a patient with chronic phase CML who progressed into accelerated phase after first-line treatment of Imatinib which showed positive Philadelphia chromosome. G-banding with trypsin/Giemsa(101).

When using the traditional cytogenetic karyotype approach, the sample must first be properly prepared in culture media with a base medium supplemented with L-Glutamine, fetal bovine serum, and antibiotics before the chromosomes are examined under a microscope (Gentamicin).However, use careful sterile reagents and tools to prevent contamination before starting to culture the sample in media(102).Then, Colchicine, a mitotic inhibitor, was given to the culture after 48–72 hours to stop mitosis at the metaphase stage. A phase-contrast microscope can be used to examine stained slides of the chromosomes in order to identify and detect any abnormalities(103).Additionally, chromosome-specific bands are created using stains and dyes; one of the most popular techniques is the G-banding technique, where the chromosome is first treated with the enzyme trypsin before being stained with the gemsa dye. Positive bands are those that exhibit strongly stained regions, whereas negative bands are those that exhibit weakly stained regions(104,105).

2.1.7.2.5.2 Molecular Cytogenetics Fluorescent in situ Hybridization (FISH)

Fluorescent in situ hybridization (FISH) is a molecular cryptogenic technique. which is rapid, sensitive, and capable of detecting specific DNA sequences on chromosomes in the metaphase or the interphase. FISH technique converts the double strands of DNA into two separate single strands by denaturing the chromosome by treating it with a formamide solution in a high temperature medium. Moreover, The DNA probe was previously used in direct probe labeling techniques with fluorescent dyes like Spectrum Green such that the fluorescence microscopy analysis, washing to remove the unbound probe, and visualization of the probe target complexes immediately following their hybridization(63,106–109). FISH probes that are utilized for the detection process can be labeled with fluorescent compounds that have various excitation and emission properties. However, it is possible to create probes for the detection of complete chromosomes, fragments of chromosomes, centromeres, telomeres, genes, and fragments of genes(110).Besides that, FISH analysis is divided into three categories based on the chromosomes that are detected: rapid prenatal (detect sex chromosome constitution in newborns at high risk of chromosome abnormality), interphase (detect numerical abnormalities of the chromosomes, such as trisomies 13, 18, and 21), and metaphase (detect chromosome imbalances, Marker chromosome and Suspected gain or loss of a chromosome segment)(111,112). Although, FISH was performed to determine the presence of BCR/ABL fusion gene in CML diseases and monitoring the response of his therapy(113).

Additionally, the most accurate method for diagnosing CML is the FISH technique, which can identify the BCR/ABL fusion gene in around 95% of patients, as well as about 5% of cases with "masked" translocations that cytogenetics miss(114–116).

2.1.7.2.6 Molecular testing.

2.1.7.2.6.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR), developed by American scientist Kerry in 1985, is a molecular diagnostic method for amplifying one or more copies of a fragment of DNA. But in 1993, he received the chemistry Nobel Prize for creating PCR(117,118).

The principle of PCR operation is based on three main steps: thermal denaturation of double-stranded DNA into single-stranded DNA (ssDNA), hybridization (annealing) of oligonucleotide primers to both ends of a target sequence, and synthesis completed by addition of four nucleotide bases and a Taq polymerase(78). In addition, it is a rapid, affordable, and easy approach that has since grown in popularity in molecular biology. Even though the source DNA material is of very low quality, it is precise, sensitive, and specific for amplifying particular DNA fragments from small amounts of source DNA material. Additionally, thermostable DNA polymerase can tolerate repeated heating to 94°C and is growing in a hot spring over 110°C. The extension catalyst is already there each time the liquid is cooled to allow the oligonucleotide primers to bind(78). Practically, PCR process is carried out in a tube using a series of 30–40 cycles, each cycle consisting of three stages to produce amplified DNA fragments as the end result. But in order to identify the PCR product, a separation method like agarose gel electrophoresis is needed. After that, the DNA was dyed with ethidium bromide and seen under ultraviolet light(119). Finally, The PCR technology sector has recently seen greater advancements than the traditional one. Numerous additional PCR variants have been developed recently, including multiplex-PCR, RT-PCR, Nested PCR, Inverse PCR, Colony PCR, Asymmetric PCR Helicase PCR, and Allele-Specific PCR. However, each of these methods was able to obtain a particular outcome for the amplification of DNA or RNA fragments(120).

2.1.7.2.6.2 Real-time Polymerase Chain Reaction (RT-PCR)

Real-time PCR, is The most accurate and sensitive quantitative method for detecting the presence of aberrant DNA or RNA transcripts is real-time PCR. Due to its ability to produce quantitative data, RT-PCR has grown in significance in clinical diagnostics and research laboratories. Unlike traditional PCR, which only shows the qualitative results, this technology enables accompanying the reaction and presenting of data in a faster and more precise manner(121,122).

2.1.8 Differential diagnosis of CML.

Differentiating CML from other myeloproliferative or myelodysplastic diseases can be challenging (Table 2.3). However, Polycythemia Vera can manifested with leukocytosis and thrombocytosis(123). Additionally, Leukemoid Reaction (LR) is the primary differential diagnosis for CML in patients who have a leukocyte count between 50,000 and 100,000 cells/L and a notable rise in mature neutrophils with a clear leftward shift(65,124–129).

Table 2.3 Differentiates chronic myeloid leukemia from other myeloproliferative neoplasms(130).

Myeloproliferative neoplasms (MPN)	Clinical manifestations		Diagnosis			
	symptoms	**Physical examination**	**leukocytes**	**blast**	**Left shift**	**Basophils**
chronic myeloid leukemia BCR-ABL1 +	Asymptomatic Bleeding Infection	Splenomegaly Purpura Anemia related Priapism	↑	< 2%	+	↑
Chronic neutrophilic leukemia	Asymptomatic Bleeding Infection	Splenomegaly Hepatomegaly Purpura Anemia related	↑	Minimal	+	NL
Polycythemia Vera (PV)	Thromboembolism Bleeding Pruritus after a worm bath. PUD related	Facial ruddiness Splenomegaly Renal bruit	NL or↑	None	–	↑or ↓
Primary myelofibrosis (PMF)	Anemia related Bleeding Infection Abdominal pain	Hepatosplenomegaly Petechiae & ecchymoses Abdominal distension Lymphadenopathy	↓	Erythroblasts	_	Absent
Essential thrombocythemia (ET)	Headache Dizziness Visual disturbances Priapism Acute chest pain	Splenomegaly Skin bruises	NLor↑	None	–	↓
Chronic Eosinophilic leukemia	Rash Rhinitis Gastric	Hypertension Eczema Ulcers	↑			↑

	Thromboembolism related	Angioedema Anemia Lymphadenopathy		Present	+	
Myelodysplastic syndromes (MDS)	Constitutional Anemia related Bleeding Infection	Pallor Petechiae Organomegaly	↓	Variable	_	↓
Acute myeloid leukemia (AML)	Constitutional Anemia related Bleeding Bone pain Joint pain Infections	Infection related Pallor Leukemia cutis Bruising & Petechiae Lymphadenopathy Hepatosplenomegaly	NL or ↑	↑	N/A	↑ or ↓

2.1.9 Treatment of CML.

2.1.9.1 Arsenic therapy

he first CML therapy that was shown to be effective was Fowler solution, a chemical salt of the metal arsenic. It was created in the middle of the eighteenth century by Thomas Fowler. Later in the 1800s, the first brand-new therapy for CML diseases was started(131,132).

In addition, Heinrich Lissauer described the use of arsenic as an active therapy in 1865 for the treatment of two patients with leukemia(133).

2.1.9.2 Radiotherapy

he clinical practice began introducing as a new therapeutic step for CML patients in the 1920s. Radiation therapy was, however, the main form of treatment for CML patients for more than 50 years(134,135). Moreover, radiation that was given to the human body or to the spleen caused a decrease in the leukocyte count and a reduction in the size of the spleen, but it might be employed in some circumstances(32).

2.1.9.3 Conventional Cytotoxic Chemotherapy

Beginning in the early 1990s, conventional cytotoxic chemotherapy introduced a new group of drugs, such as interferon-alpha, Hydroxyurea, and busulfan(136).

2.1.9.3.1 Busulfan (Bu)

In the past 30 years, there has been a significant advancement in the treatment of CML disorders. Busulfan, introduced in the 1950s as an alkylating agent used to treat

CML patients, has swiftly gained popularity as a novel form of chemotherapy(137).Busulfan, is accessible as tablets that are taken orally, initially for hematopoietic stem cell transplant recipients and thereafter for patients with chronic myeloid leukemia Later, in 1960, radiation was completely supplanted by oral busulfan chemotherapy for the treatment of CML disorders(138–140). Later, in 1996, the intravenous form of Busulfan was discovered and is now used in combination with the oral form. However, compared to oral form, intravenous Busulfan chemotherapy typically causes less bone marrow toxicity in humans and is prepared to be safe(141–143). Additionally, oral busulfan is easily absorbed from the digestive system, immediately binds to plasma proteins like albumin and red blood cells, and then swiftly leaves the circulation. The median survival durations of busulfan therapy, however, for CML illness patients, varied from 35 to 47 months(144–146). Although, in patients receiving busulfan, sister chromatid exchange and chromosomal abnormalities are more common in the peripheral blood. Also, busulfan can cause amenorrhea, fibrosis, hypotension, nausea, vomiting, amenorrhea, and testicular shrinkage(147–150).Lastly, in patients receiving the busulfan agent, it causes haematotoxicity, immunosuppression, and bone marrow depression. However, a reduction in apoptotic cell death was seen when a sample from a BM biopsy belonged to a patient who had received busulfan treatment(148,151).

2.1.9.3.2 Hydroxyurea (Hydroxycarbamide)

A new medication for CML patients known as Hydroxyurea is a standard chemotherapeutic agent that was introduced in the United States of America and used to lower the white blood cell proliferation in CML disorders. It was first used to treat CML patients in the United States of America in 1972(152). However, by quickly reducing the high levels of White Blood Cells in the Chronic Phase in CML patients and blocking the

enzyme ribonucleotide reductase, which prevents DNA synthesis, Hydroxyurea can induce a hematological response. It can also eliminate cells in the S phase of the cell cycle and synchronize cells in the G1 or pre-DNA synthesis phase(153).As a result, the CML patients began treatment with an initial dose of 40 to 50 mg/kg/day in divided doses to lower WBC counts near to normal levels. Once the diagnosis of Philadelphia chromosome was confirmed, the patients could quit treatment and begin treatment with a TKI such Imatinib(154).

Moreover, more investigations revealed that patients receiving Hydroxyurea chemotherapy outlived those receiving busulfan chemotherapy. However, in CML patients, the median survival durations following Hydroxyurea therapy range from 44 to 58 months(155,156). Additionally, even at high doses, Hydroxyurea is quite successful at controlling the hematological abnormalities associated with this illness but has no helpful cytogenetic effects(157).

2.1.9.3.3 Interferon-alpha

Interferon (IFN) was originally introduced as a therapeutic treatment for CML patients in the chronic phase of the illness in the 1980s. It was intended to be the first medication to stimulate both hematological and cytogenetic responses in CML patients during the chronic phase(158).

Prior to the development of Imatinib, chemotherapy was preferred over interferon-alpha therapy as a single treatment for CML patients because to its efficacy(159,160). Further, interferon-alpha directly inhibits the proliferation of CML progenitor cells, where it exerts its antileukemic impact. Additionally, since the 1990s, a small number of CML patients receiving interferon-alpha therapy have seen complete cytogenetic responses, which have persisted for several years after the interferon Alfa treatment was discontinued(161–163).

The second generation of TKIs, Dasatinib and nilotinib, are used in conjunction with interferon alpha(164,165). However, a significant incidence of adverse events was linked to the combination of the nilotinib medication with an interferon alpha dose of 90 g per week, reduced to 45 g per week after one month of treatment(166).

2.1.9.4 Allogeneic hematopoietic stem cell transplantation (AlloSCT)

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) evolved into the preferred treatment for CML patients in the chronic phase later in the 1980s. But for patients who were still relatively young and had acceptable stem cell donors, allogeneic stem cell transplantation was the advised initial treatment(167). Additionally, autologous stem cell transplantation was performed in the 1990s on patients who were ineligible for allo-HSCT. Allogeneic stem cell transplantation is additionally regarded as a curative therapy in late stages of CML patients, however it is linked to high rates of morbidity and mortality(168,169).

2.1.9.5 Leukapheresis

The term "apheresis" is generally used to describe the removal of a single blood component and the safe return of the remaining blood components to the patient. However, hyperleukocytosis, which is an increase in white blood cell counts above 100 x 10^9 cells/l, can cause leukocytosis, a condition that can be fatal in CML patients(170). In CML, When WBCs reach more than 300x10^9cell/l, there is a clear risk of leukostasis in chronic myeloid leukemia illness. Additionally, experienced, well-trained staff members working in specialist facilities should perform the therapeutic leukocytophersis. However, a single leukopheresis procedure might cause a 10- to 70% decrease in WBC count(171,172).

2.1.9.6 Tyrosine kinase inhibitors(TKIs)therapy

Since the 1960s, when the Philadelphia (Ph) Chromosome was found to be aberrant, and up till the late 1990s, when the BCR-ABL fusion gene was found to be the primary cause of CML illness. All patients with CML disorders express a suitable target for CML therapy Tyrosine kinase inhibitors, a new class of medications that debuted later in 1998, completely altered the way CML patients were treated(173,174).

First-generation TKI therapy is Imatinib Mesilate, and second-generation TKIs Dasatinib, Nilotinib, and Bosutinib are recommended for newly diagnosed CML patients in the chronic phase of the disease and for patients with relapsed or refractory disease. The third-generation TKIs (Ponatinib) is recommended for patients who have previously become resistant to or intolerable to TKIs because of mutations in the ABL kinase domain(175). These TKIs are more effective against many of the kinase domain mutants that have developed resistance to Imatinib because they have a higher binding affinity to the BCR-ABL kinase domain than Imatinib does. Omacetaxine, a protein synthesis inhibitor, has just received US approval for the treatment of CML patients who have developed resistance to or intolerance to two or more BCR-ABL1TKIs.Finally, although CML treatment outcomes are much better than those for the majority of other malignancies, the cure-rate for CML is still not thought to be high(176).

2.1.9.6.1 Imatinib-First generation TKIs

Imatinib Mesilate, often known as Gleevec, is a 2-phenylaminopyrimidine substance that was the first selective inhibitor of *BCR-ABL* tyrosine kinase and is now the go-to first-line therapy for CML patients who have just been diagnosed. But in 2001, Sweden accepted and registered it. Moreover, Imatinib Mesilate inhibits the activity of numerous protein kinases, including the platelet-derived growth factor receptor (PDGFR), Kit, TEL-ABL, and ABL kinase activity, as well as the growth and viability

of cells transformed by any of these ABL oncogenes, but has no effect on the Src-family kinases(177). Since then, it has developed into the gold standard medication and the most popular first-line therapy for CML patients in the chronic phase, administered at a dose of 400 mg daily(178,179).

Imatinib inhibits the Breakpoint Cluster Region *BCR-ABL* tyrosine kinase activity, which is a key factor in determining the genomic instability of leukemic cells, by blocking the adenosine triphosphate-binding site of the *bcr-Abl* tyrosine kinase and specifically preventing the transfer of terminal phosphate from ATP to tyrosine residue(180–182).A comprehensive evaluation of Imatinib has been made possible by the findings of a 5-year follow-up study from the International Randomized Study of Interferon Versus ST1571 (IRIS) experiment. In 97% of patients, a full hematopoietic response was seen, and in 82% of patients, a full cytogenetic response. Estimated progression-free survival over five years was 84%. The anticipated 5-year survival rate, however, was 93% without transition to the accelerated phase or blastic transformation. If fatalities unrelated to CML were taken out of the equation, the overall survival on first-line Imatinib increased to 95.4% from 89.4% on an intention-to-treat analysis(173). Moreover, regardless of when the response was obtained, it was evident that individuals who experienced a large molecular response had a very low probability of progressing to the accelerated or blastic phases of CML. This finding was corroborated by several trials, which revealed that, contrary with earlier therapy, there was no rise in the incidence of progression to the accelerated phase or blastic transformation with time(183). Furthermore, the majority of patients who were randomly assigned to receive Imatinib exhibited detectable leukemia as determined by reverse transcriptase-polymerase chain reaction (RT-PCR) for BCR-ABL, and most of these patients had a complete cytogenetic response(184).

The majority of patients experience gastrointestinal symptoms (nausea, abdominal pain, and diarrhea), fluid retention, weight gain, bone aches, exhaustion, rash, and abnormalities in liver function as a result of Imatinib side effects, which are caused by hematological toxicity. Additionally, it results in pancytopenia; however, patients with anemia can administer erythropoietin, and those with neutropenia may be able to tolerate Imatinib at its full dosage if Granulocyte Colony-Stimulating Factor (G-CSF) is given to them(185,186). Similarly, numerous analyses have demonstrated that patients who do not experience adequate cytogenetic or molecular responses to Imatinib at predetermined time points experience a worse result, which is characterized by an elevated risk of relapse, of progression, and of mortality(187,188).Imatinib therapy aims to generate hematologic remission in nearly 100% of patients with early chronic phase CML and a substantial cytogenetic response in 87% of patients; however, only 8% and 16% of individuals treated for blast crisis experience these types of response(178)(189).

According to the European Leukemia Net 2010, complete hematologic remission (CHR) would demonstrate a return to normal bone marrow morphology, peripheral blood cell counts, and total white blood cell counts with platelet levels under $450x10^9$/L. The lack of peripheral blast, immature granulocytes such promyelocytes and myelocytes, and complete hematologic remission were also criteria. The categories of cytogenetic remission were complete, major, partial, minor, and non-responder. Complete cytogenetic response (CCyR) was defined as the absolute elimination of the Ph+ chromosome in cytogenetic analysis, while partial cytogenetic response was defined as the presence of fewer than 35% Ph+ cells in bone marrow (PCgR). Patients with a modest cytogenetic response had 36–65% Ph+ cells in their bone marrow, whereas patients with a minimal cytogenetic response had 66–95% Ph+ chromosomal positivity. Imatinib non-responders were defined as patients with above 95% Ph+ chromosomes in their bone marrow(190,191).

Imatinib clinical trials started in 1998 with patients with chronic-phase CML who had become intolerant to or resistant to IFN- therapy. 53 out of 54 patients in a Phase I study had a complete hematologic response (CHR) after just 4 weeks of treatment, and 96% of these patients kept their CHR for more than a year. Additionally, hematological responses were obtained in 55% of the patients in the expedited phase or blast crisis (21/38)(174).However, some individuals developed Imatinib resistance because to acquired mutations in the kinase domain of BCR-ABL, making Imatinib ineffective at blocking the binding of ATP, especially in the latter stages of CML with positive Philadelphia Chromosome(173).Although, majority of Imatinib-treated patients with chronic phase CML have well-controlled illness, however some patients have relapsed or advanced to an accelerated phase or blast crisis(14).

Point mutations in the BCR-ABL kinase domain, mutations outside the KD, patient adherence to therapy, the drug's bioavailability, pharmacodynamics, and genetic changes are thought to combine to cause the development of this resistance to Imatinib(192,193). However, the P-loop (codons 244–256), Imatinib-binding area (codons 315 and F317), catalytic domain (codons 350–363), and activation loop (codons 315–317) are the four locations where the mutations in the BCR-ABL kinase domain are concentrated (codons 381–407)(184).

This is particularly intriguing because it has been demonstrated that patients still have BCR-ABL positive cells when detected using accurate DNA-based tests. because the TKI therapy was stopped. Last but not least, according to some scientific studies, about half of CML patients on Imatinib who have achieved a lasting full molecular response are able to quit taking it without relapsing(194,195).

Switching to second generation TKI Nilotinib or Dasatinib can be more effective. However, some studies comparing second-line Nilotinib or Dasatinib with high-dose Imatinib (400 mg BID) have shown that the newer TKIs have much greater rates of CHR,

CCyR, and MMR(193,196,197). There are currently a number of second-line CML therapeutic options available. Patients who are resistant to conventional doses (400 mg/day) of Imatinib might be treated by increasing the dose (600-800 mg/day). According to studies, 35 to 40 percent of CML patients who did not respond to standard-dose Imatinib later experienced a Major and Complete Cytogenetic Response(MCyR) after switching to high-dose Imatinib(198).

2.1.9.6.2 Dasatinib-Second generation TKIs

Based on its effectiveness and tolerance in clinical trials, Dasatinib (Sprycel, Bristol-Myers Squibb) is a thiazolecarboximide drug that is an orally strong inhibitor of the BCR-ABL1 fusion gene. In order to treat CML patients in the chronic phase and the accelerated or blast phase who are resistant to or intolerant to the first-line medication Imatinib therapy, was approved by the United States Food and Drug Administration (FDA) in 2006(199). However, Dasatinib blocks the activity of a number of oncogenic kinases, such as the SRC family of kinases (SRC, LCK, YES, FYN), c-KIT, ephrin A receptor, and platelet-derived growth factor (PDGFR) receptor kinases(200,201).

Dasatinib is also 350 times more potent than Imatinib in vitro, which is another factor favoring its superiority. It, with the exception of the T315I mutation, is active against the majority of BCR-ABL1 mutations(202,203). Furthermore, Dasatinib interacts to the ATP-binding site, which is supported by the crystal structure, which shows that Dasatinib inhibits Bcr-Abl tyrosine kinase activity. The Abl kinase domain can bind to both the active and inactive conformations(204).

The CML patients whom Imatinib-resistant or intolerant in chronic phase of the disease we recommended starting dose of Dasatinib 100 mg once daily or 70 mg twice daily. However, the rate of hematologic and cytogenetic responses to Dasatinib 70 mg

twice daily was considerably higher than that of high-dose Imatinib (800 mg/day) in patients with Imatinib-resistant CP CML(205).

Dasatinib can cause gastrointestinal bleeding, rashes, lethargy, headaches, diarrhea, vomiting, and nausea in patients with CP CML. As a result, more specific adverse effects caused by Dasatinib included pleural effusions (27%; grades 3-4 6%), peripheral edema (18%), pericardial effusion (4%), CHF (5%), neutropenia (grade 3-4 48%), thrombocytopenia (grade 3-4 48%), and anemia (grades 3-4 18%)(206,207).

Dasatinib treatment resulted in greater MMR rates than Imatinib treatment (64% vs 46% after 24 months) and a reduced advancement rate to AP or BC (2.3% vs 5.0%) in newly diagnosed CP patients. However, Dasatinib has a 12-month survival rate of between 22 and 49% when used alone to treat BC(208).

2.1.9.6.3 Ponatinib-Third generation TKIs

The only third-generation TKI that is regarded as a multi-targeting BCR-ABL/SRC kinase that has action against every BCR-ABL1 mutation that has been evaluated, including mutant T315I, is Ponatinib. However, because of its side effects, which include peripheral vascular, cardiovascular, and cerebrovascular events as well as arterial and venous thromboembolism(60)(209). However, the typical dose is not known for sure, however studies are being done on doses of once-daily 15–45 mg. Recent clinical trial data, however, indicate that Ponatinib might potentially be helpful for patients without T315I mutations if dose is improved(210).

2.1.9.7.7 Omacetaxine

Omacetaxine is an organic alkaloid that comes from evergreen trees. This has been approved for the treatment of CML patients who have resistance to or intolerance to their medication in the US. However, this medication causes leukemic cells, including

those from CML patients who have the T315I mutation, to undergo apoptosis. Directly attaches to the ribosome in order to prevent the first stage of protein(209,211,212).

2.2 Tyrosine Kinase domain

2.2.1 The BCR gene of kinase domain

The BCR gene contains approximately 21 exons and is 130 kb in size. However, this gene was discovered and identified as a result of the discovery that the breakpoints on chromosome 22 in Ph-positive CML were grouped within a small region known as the Major BCR (M-bcr)(213). The BCR protein has a multi-domain, intricate structure. The first exon is composed of two SH2 domains, a putative serine/threonine kinase domain, a growth factor receptor bound protein 2 (Grb2) binding site, a BCR associated protein-1 (BAP-1) integration site, and several other elements. A core guanine nucleotide exchange factor (gef) domain is present in exons 3–8, followed by exons 19–23, which also contain a RacGap domain(214).

2.2.2 The ABL gene of kinase domain

The ABL gene is part of the Abelson murine leukemia virus (9q34.12). Based on alternatively spliced starting exons, ABL encodes a nonreceptor tyrosine kinase with a molecular weight of 145 kDa. A 6–7 kb Mrna transcript is the outcome. The N-terminal portion of the ABL protein is thought to be localized in the nucleus and is encoded by exon 1a (ABLA), whereas exon 1b (ABL1B) encodes the N-terminal glycine, which is mesilated and is thought to drive the protein to the plasma membrane(215). Additionally, cell motility, adhesion, and apoptosis, as well as cytoskeleton remodelling in response to environmental stimuli(216).

2.2.3 BCR/ABL fusions in CML

ABL1 gene fusion within the BCR gene creates a fusion protein with a constitutively active ABL1 tyrosine kinases, which are believe to be causative in CML.

The majority of BCR-ABL1 proteins contain juxtaposed SH3-SH2 domains, which are located upstream to the tyrosine kinase domain and regulate kinase activity through their interactions with other proteins(217,218). It has been shown that the interaction between BCR-ABL1 SH3 and RAD51 proline-rich regions caused RAD51 phosphorylation, resulting in secondary chromosomal aberrations that contribute to disease progression and relapse in CML in contrast, small protein apoptin, a tumor-selective killer, is a negative regulator of BCR-ABL1 kinase via its ability to interact with BCR-ABL1 SH3 domain. (Figure 2.7) (219,220).

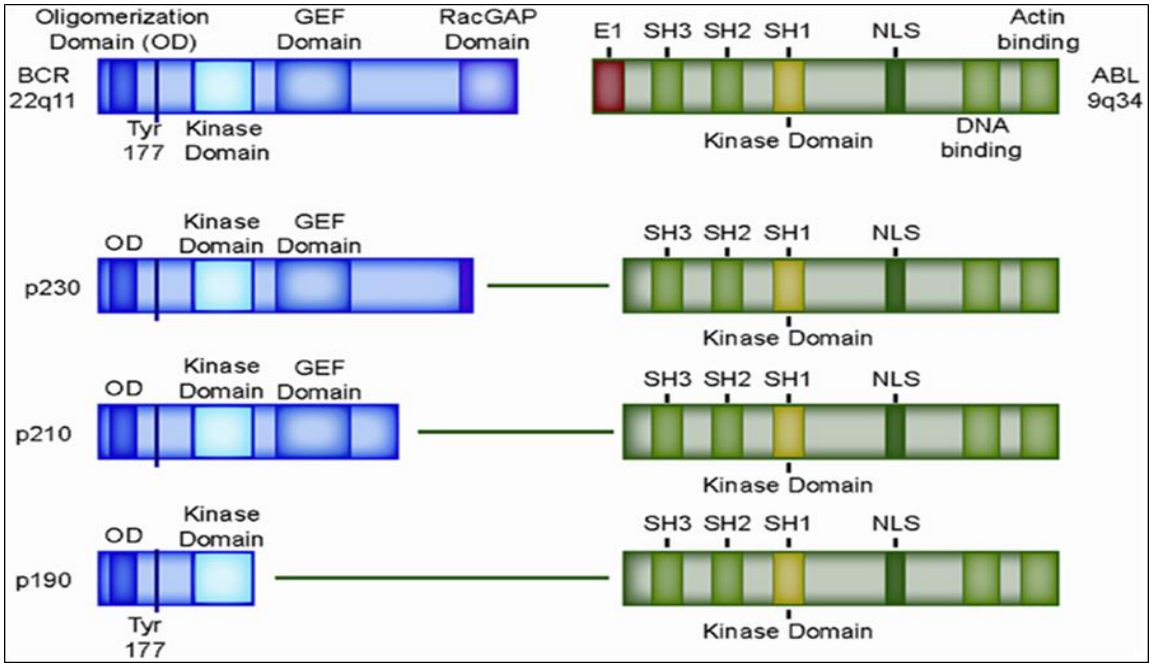

Fig. 2.7 Structure of the most common BCR–ABL1 fusion genes. Domain structure of wild type BCR and wild type ABL1 protein(221).

2.2.4 Types of Oncogene BCR-ABL

Three are three main types of BCR-ABL gene can be found, most patients with CML have breakpoints in introns 1 and 2 of the ABL gene and in the major breakpoint cluster (M-bcr), of the BCR gene, either between exons 13 and 14 (b2), or 14 and 15 (b3). These breakpoints produce BCR-ABL fusion genes that transcribe either b2a2 or b3a2 mRNA; and The final product of this genetic rearrangement is a 210 kDa cytoplasmic

fusion protein, $p210^{BCR-ABL}$, which is crucial and necessary for the malignant transformation of CML, and responsible for the phenotypic abnormalities of chronic phase CML. The ensuing e1a2 mRNA is translated into a 190 kDa protein, $p190^{BCR-ABL}$. A third breakpoint cluster region (μ-bcr) has been identified downstream of exon 19, and it gives rise to a 230 kDa protein, $p230^{BCR-ABL}$, found in some cases of the rare Ph-positive neutrophilic leukemia (Figure 2.8)(134,222).

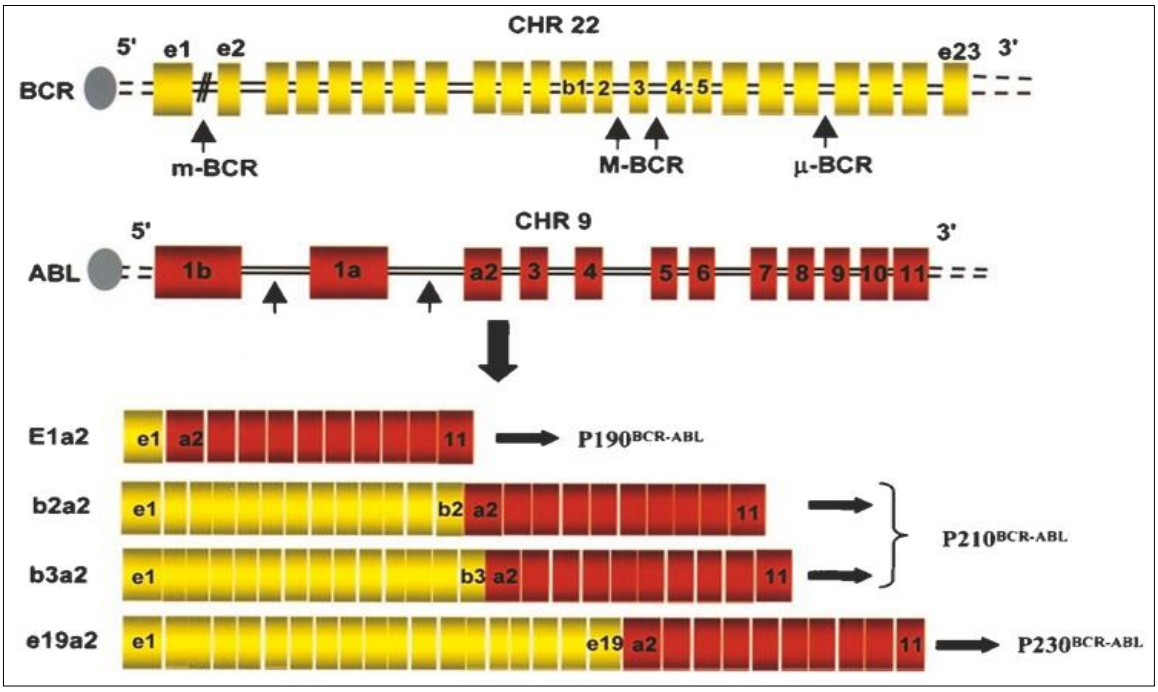

Fig. 2.8 Locations of the breakpoints in the ABL and BCR genes and structure of the chimeric BCR/ABL mRNA transcripts derived from the various breaks(223)**.**

The increased tyrosine kinase activity of $p210^{BCR/ABL}$ results in phosphorylation of several cellular substrates and in auto phosphorylation of $p210B^{CR/ABL}$, which in turn induces recruitment and binding of a number of adaptor molecules and proteins. Activation of a number of signal pathways by $p^{210BCR/ABL}$ leads to malignant transformation by interfering with basic cellular processes, such as control of cell proliferation and differentiation , adhesion and cell survival(224,225).

$p210^{BCR/ABL}$ activates signal transduction pathways such as RAS/MAPK, PI-3 kinase, c-CBL and CRKL pathways, JAK-STAT and the Src pathway. Of these, the ras, Jun-kinase, and PI-3 kinase pathways have been demonstrated to play a major role in transformation and proliferation. However, Inhibition of apoptosis is thought to result from activation of the PI-3 kinase and RAS pathways, with induction through AKT of c-myc and BCL-2(226,227).

In CML the cytoplasmic protein tyrosine kinase will elevated and dysregulated enzymatic activity that plays a vital role in the pathogenesis and progression. However, this fusion protein $p210^{BCR/ABL}$ is found in 95% of patients with CML and 30% of adult patients with ALL(213,228).

2.2.5 Mutations in BCR-ABL domain

Point mutations in the Bcr-abl kinase domain decrease and/or inhibit the interaction of TKI and the oncogenic Bcr-abl protein depending on the location of the mutation (Figure 2-9). Alterations in critical contact points due to amino acid substitutions increase the failure of agent binding to the target site. In addition, drug treatment can induce mutations leading to the development of drug resistance and, thus, drug efficacy decreases during treatment of CML. Point mutations are found more frequently in advanced phase CML as compared to the chronic phase of the disease. Mutations in the genome can lead to dysfunction(229,230).

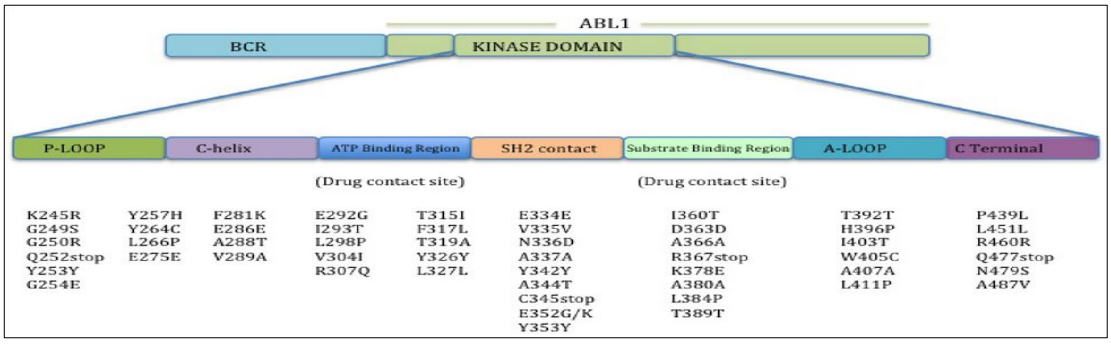

Fig. 2.9 Distribution of the mutations with respect to the main regions of the Bcr-abl kinase domain(231).

Moreover, point mutations, which often occur in the Imatinib binding site, the P-loop, the A-loop, and the catalytic domain, are the most common cause of acquired resistance in *BCR-ABL*(232).

T315I is a missense mutation that results from a change in a single nucleotide; in contrast, a new amino acid, commonly referred to as a gatekeeper residue, is changed from a threonine residue to an isoleucine residue at position 315. Its frequency in Sudan was (5/50) 10%, and it accounts for 15% of all cases. Additionally, the T315I missense mutation in the BCR-ABL kinase domain makes the affected Imatinib binding site drug-resistant because it causes a hydrogen bond to form with Imatinib that prevents Imatinib from binding to the ATP site(23,233).Further, a number of hydrophobic contacts are stabilized by the large hydrophobic functional group in isoleucine, which helps to support the enzyme's active conformation(234). Furthermore, the original structure of BCR-ABL1, particularly the residues crucial in inhibitor binding, is altered as a result of the mutation at position 315, which leads in a weak interaction with inhibitors. When there are inhibitors present, the mutant protein is kept active thanks to these conformational changes(235).Lastly, there are also mutations in the ATP-binding P-loop and the activation A-loop, such as G250E, Q252H/R, and E255K(Table1.4).

Table 2.4 Description of substitution of amino acids in mutation. (236)

Mutation	Description of substitution
T315I	Threonine at 315 substituted by Isoleucine
G250E	Glycine at 250 substituted by Glutamic acid
M244V	Methionine at 244 substituted by Valine
M351T	Methionine at 351 substituted by Tyrosine
Q252H	Glutamine at 252 substituted by Histidine
E255K	Glutamic acid at 255 substituted by Lysine
Y253F	Tyrosine at 253 substituted by Phenylalanine
H296P	Histidine at 296 substituted by Proline
H396R	Histidine at 296 substituted by Arginine
F359V	Phenylalanine at 359 substituted by Valine
F311L	Phenylalanine at 311 substituted by Leucine
E255V	Glutamic acid at 255 substituted by Valine
F317L	Phenylalanine at 317 substituted by Leucine

2.2.6 Detection of mutations in BCR-ABL domain

BCR-ABL1 mutations may be a biological indicator of the development of the disease in CML patients. Therefore, it is essential to do a cytogenetics test and a qualitative PCR to determine the BCR-ABL1 fusion transcript type at the time of diagnosis to identify the Philadelphia chromosome(20).

During treatment, cytogenetic tests are only advised in cases with additional chromosomal aberrations (ACA) in the Philadelphia-positive clone, whereas quantitative PCR for *BCR-ABL1* has to be performed after the achievement of a complete cytogenetic response (no metaphases positive for the Ph chromosome out of a minimum of 20 metaphases examined) performed on a regular basis to monitor transcript levels (minimal residual disease (MRD)). Only quantitative PCR for the BCR-ABL1 transcript is required for follow-up. It is advised to use Sanger sequencing or next-generation sequencing to look for *BCR-ABL1* alterations in situations with an inadequate response(20).

2.3 Immune checkpoints molecules

Immune checkpoints are molecules in the immune system, which are regulators of immune activation by regulating the antigen recognition of T cell receptor (TCR) during the process of immune response. However, immune checkpoints molecules are divided into two types: co-stimulatory immune checkpoints; turning up a signal in the immune response. and co-inhibitory immune checkpoints; turning down a signal in the immune response(Figure 2.10)(237,238).

The co-inhibitory and costimulatory refers to how these antigens independent second signals modify the first signal, provided by interaction of antigenic peptide-MHC complex with the TCR, which confers specificity to the response(239). However, in order for lymphocytes to recognize a particular antigen (signal 1) and receive additional signals (signal 2, also known as costimulatory signals), the antigen-specific lymphocytes must

first recognize that antigen.(240)The co-stimulatory immune checkpoints are further divided into two groups: those that can stimulate immunological development, such as CD27, CD40, OX40, GITR, and CD137, which are members of the tumor necrosis factor (TNF) receptor superfamily; and those that do not. and stimulatory checkpoint molecules, including as CD28 and ICOS, are members of the B7-CD28 superfamily(241–247).

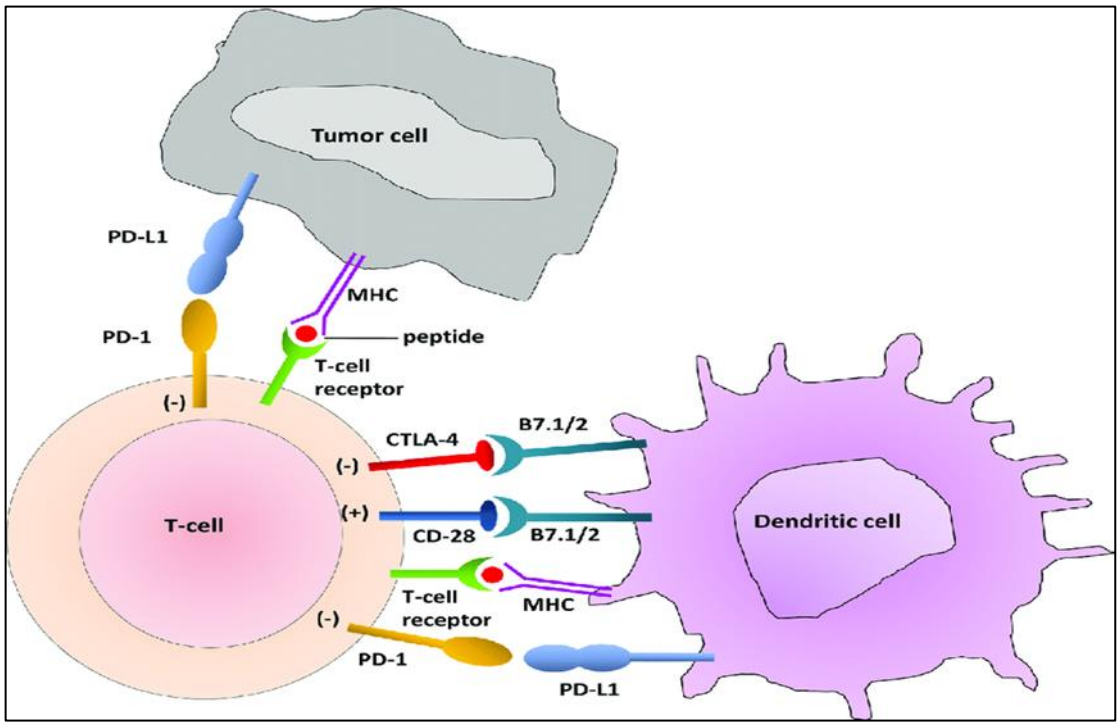

Fig. 2.10 Immune checkpoint molecules and their ligands. The immune checkpoints CTLA-4 and PD-1/PD-L1 are highlighted in the interactions among T-cells, dendritic cells and tumor cells. (248)

2.3.1 PD-1 receptor and It is ligands

2.3.1.1 Structure and expression of PD-1 receptor

PD-1, a monomeric type I transmembrane glycoprotein that belongs to the CD28/B7 immunoglobulin superfamily, was first discovered via subtractive hybridization utilizing a T-cell hybridoma that was undergoing programmed cell death(249). However, PD-1 receptor is also 288 amino acids (50–55kDa), comprising an extracellular N-terminal IgV-like domain, a transmembrane domain, and a cytoplasmic tail(250).

The intracellular cytoplasmic domain of PD-1 contains of two tyrosine; an immune receptor tyrosine-based switch motif (ITSM) and an immune receptor tyrosine-based inhibitory motif (ITIM). However, the ITSMs are essential for the delivery of inhibitory signals(251). Moreover, PD-1 receptor protein is normally expressed on the cell surface of an activated T cells, B cells, natural killer T cells, monocytes and myeloid. However, it is function can be induced following T-cell receptor (TCR)-mediated activation and stimulation by cytokines such as interleukin (IL)-2, IL-7, IL-15, and IL-21(252,253).

2.3.1.2 PD-1 receptor ligands

PD-1recepter has two ligands which are PD-L1 (B7-H1(homolog 1), CD274) and PD-L2 (B7-DC, CD273). However, PD-L1 is expressed on the cells of the hematopoietic lineage. Is also expressed on activated vascular endothelial cells, cultured bone marrow–derived mast cells and mesenchymal stem cells(254,255). While PDL-2 expressed normally on macrophages and dendritic cells. However, it is demonstrating that can be expressed on many tumor cell types(256,257).

2.3.2 Types of PDL-1

There are three different types of PD-L1: soluble PD-L1(sPDL-1), exosomal PD-L1(exoPDL-1), and membrane-bound (mPDL-1). The sPD-L1 is generated from mRNA expression Four alternatively spliced PD-1 mRNA transcripts. However, sPDL-1 can be produced and released by both tumor cells and activated mature DCs(258,259). In healthy human serum, sPD-L1 was expressed, and its levels rose with advancing age. The expression of sPD-L1 was lowest in children aged 3 to 10 years and highest in adults aged 51 to 70 years(260). Additionally, sPD-L1 still exhibits biological activity, suggesting that it may be able to precisely bind to the PD-1 receptor on active T cells before they

reach the tumor site, squelching T cell activity and impeding an anticancer response on a systemic level(261).

2.3.3 PD-1/PDL-1 interaction

When PD-1 and its ligands are bound, the intracellular domain of PDL-1, which contains the immune receptor tyrosine-based switch motif (ITSM) and the immune receptor tyrosine-based inhibitory motif (ITIM), is both phosphorylated. However, this recruits the phosphatases Src homology region 2 domain-containing phosphatase-1(SHP-1) and Src homology region 2 domain-containing phosphatase-2(SHP-2) to the intracellular domain of PD-1, SHP-1 and SHP-2 dephosphorylate the immune-receptor tyrosine-based activation motifs of the T- cell Receptor (TCR) resulting in the dampening of T-cell receptor (TCR) signaling(262). By blocking TCR signaling, PD-1 blocks the activation of the PI3K/Akt and c-Myc pathways, which in turn restricts CD8+ T cells from surviving, proliferating, and producing cytokines. T-cell fatigue is largely caused by the interaction between the programmed cell death-1 receptor and its ligand(263,264). However, when T cells become exhausted, they lose their ability to operate as effector cells and exhibit phenotypic characteristics such the expression of immune-inhibitory receptors on their surface, likePD-1(265). Additionally, CD80 (B7-1) which is expressed on the surface of CD8+ T cells interacts with the PDL-1 receptor. Although the signaling events that are started downstream of CD80 are still being studied, it has been demonstrated that they have effects on CD8+ T cell function similar to those of the signaling that is started downstream of the PD-L1/PD-1 connection(266–268).

2.3.4 PD-1/PD-L1 signaling pathways in cancer

When PD-1, PDL-1, and immune cells are bound together, a potent inhibitory signal is sent to the T cell, reducing cytokine production and suppressing T cell

proliferation, migration, and cytotoxic mediators. This decreases the ability of T cells to kill tumor cells and helps tumor cells evade immune detection(269–271).

The binding of tumor PDL-1 to its receptor PD-1 on T cell surface inhibits infiltrating T cell activation and subsequent lysis of tumor cells. PDL-1 expression in tumors strongly correlates with poor prognosis in gastric cancer, hepatocellular carcinoma, RCC, EC, PC, and ovarian cancer(211,272–274). Additionally, the PD-1 receptor in T cells and PDL-1, its ligand in tumor cells, reduce anti-tumor T cell responses and enable tumors to avoid these responses(275,276). Besides The PD-1/PDL-1 interaction plays a crucial role in immune escape in several malignancies and infectious illnesses, in addition to being a crucial immune regulation mechanism. The ability of antibodies to elicit remission in patients with advanced-stage solid tumors serves as an illustration of this(277–279). Lastly, when PD-1 binds to its ligand, the immune system is negatively regulated, preventing it from becoming overstimulated. Contrarily, excessive PDL-1 expression in cancer pathology aids tumors in depleting and suppressing the immune system, enabling them to successfully evade immune destruction(280).

2.3.5 PD-L1 and PD-1 blockade in cancer

Successful blocking of the PD-1 pathway enhances T-cell responses in vitro and encourages tumor regression in vivo in animals. However, antibody blockade of PD-1/PDL-1 increases anticancer immune responses by reducing the number and/or suppressive activity of regulatory T cells and by restoring the function of effector T cells in tissues and the tumor microenvironment(269).

Inhibiting PD-1/PDL-1 in a CML mouse model was observed to increase the survival of CML mice during a blast crisis, suggesting that the relationship may be significant as an immune suppressive mechanism in CML(281).

Both PD-1 and PD-L1-targeting antibodies have significantly reduced toxicity while producing strong anti-tumor activity. Antibody-based therapies targeting PD-1 on T lymphocytes and its main receptor PDL-1 on tumor cells have been the main focus of efforts to reactivate latent anti-tumor immunity(282).

Therefore, PD-1-blocking in vitro increases the production of IFN-g and IL-2 by certain T lymphocytes by controlling their death and proliferation. Blocking PD-1 signaling in vitro increases T and natural killer (NK) T cell proliferation and cytokine production in response to antigen stimulation(283–285).

2.3.6 Detection of PDL-1 in the laboratory

Numerous methods, including immunoassays like the enzyme-linked immunosorbent assay test, can be used to measure PDL-1 levels in blood and plasma. These tests have limited repeatability and sensitivity for clinical applications, and they are challenging to carry out. Several precise, sensitive, and quick detection techniques have been created(286).

2.4 Previous Studies

A few studies have examined the plasma levels of PDL-1 in patients with Chronic Myeloid Leukemia resistant to Imatinib Mesilate and correlate with Tyrosine Kinase Domain Mutation (T315I). For instance, a study conducted by Elkhawanky *et al.* in Egypt in 2017, was aimed to assess the plasma level of PDL-1 in patients with chronic myeloid leukemia and its correlation with prognostic parameters and response to first line therapy (Imatinib mesylate). 40 patients with Chronic Myeloid Leukemia in the chronic phase of the disease were divided were divided into three subgroups: 17 patients responding to Imatinib mesylate, 12 resistants to Imatinib Mesilate, and 11 newly diagnosed CML patients. Testing the plasma PDL-1 by using ELISA technique. They found that the plasma levels of soluble PDL-1 increased significantly in all groups (newly diagnosis responding and resistant) when compared to the control group, with means±SD of 2.6±0.8 ng/mL, 2.4±0.7 ng/mL and 2.4±0.4 ng/mL, and 1.06±0.8 ng/mL in controls. However, they failed to determine a cutoff for Imatinib Mesilate resistance because they found that there no different in PDL-1 levels between Imatinib Mesilate-responding and Imatinib Mesilate resistant patients. All patients were subjected to laboratory responding and resistant patients(21).

In a different study conducted by Noor Abdul Razaq*et al.*, in Iraq in 2021 was test the plasma levels of PDL-1 and CD25 in patients with CML and correlate with response to the first line of therapy, tyrosine kinase inhibitor (Imatinib Mesilate). 66 patients with Chronic Myeloid Leukemia in the chronic phase of the disease were divided into three subgroups; 20 new diagnoses before starting treatment, 30 Imatinib therapy responders, 16 Imatinib Mesilate resistant patients, and 20 controls. Testing the plasma PDL-1 by using ELISA technique. According to their findings, plasma levels of PDL-1 were significantly higher in CML patients than in controls. with a P value*(P=0.000*). They

discovered that there was a significant difference (P 0.002) in the plasma level of PD-L1 between those with a recent diagnosis of CML and those who were Imatinib-resistant. Furthermore, there is no difference between those who respond to Imatinib and resistant patients.(*P = 1.0*)(287).

In addition, there is no clear defined cut-off value to distinguish between high and low levels of PD-L1 in patients with CML diseases. *Elkhawanky et al* in their study failed to determine the cut-off of the plasma PDL-1 levels for predictive risk factor for CML disease progression. However, one study conducted in Australia in 2019 by Aghajani *et al.,* in cohort study, 101 patients with papillary thyroid cancer, testing pre-treatment levels of plasma and serum of PD-L1 by using ELISA method, to determine the cut-off for the median disease-free survival(DFS) in papillary thyroid cancer (PTC) patients. Where use the median PD-L1 level in a corresponding healthy cohort and ROC curve models. The optimal cut-off was 0.19ng/mL, with an area under the curve of 0.564 (95% confidence interval: 0.435–0.694, P=0.375, and The cut-off for serum PDL-1 derived from the ROC curve was 0.44ng/mL, with an area under the curve of 0.647 (95% confidence interval: 0.521–0.773, P=0.043. Their results were the survival analysis revealed that patients with high plasma PDL-1 showed shorter DFS than those with low plasma PDL-1, but the difference was not significant (15 months vs 19 months, P=0.263). and the patients with high serum PDL-1 had significantly shorter DFS compared with those with low PDL-1 levels (14 months vs 23 months, P=0.010) (288).

A descriptive cross-sectional study conducted in Sudan by *Wagass et al.,* on 50 patients with CML treating with Imatinib Mesilate to detect BCR-ABLT315I mutation gene by using Allele Specific Oligonucleotide Polymerase Chain Reaction (ASO-PCR) during the period from June 2006 up to December 2008, were found the prevalence of BCR-ABLT315I mutation gene in Sudanese patients was 5(10%)(289).

Another descriptive cross-sectional study, conducted in Sudan on 100 patients with CML who visited RICK hospital between May 2018-2019, to detect the BCR-ABLT315I mutation gene by using RT/PCR and followed by RLFP. They found that the BCR-ABLT315I mutation gene was 43(43.4%)(20).

Chapter Three: Materials and Methods

3.1 Study design:

This a cross-sectional case control study.

3.2 Study duration and study area:

This study was conducted during the period from January 2018 to November 2022 in Khartoum Oncology Hospital in Khartoum state - Sudan.

3.3 Study population:

104 hospitalized patients with a Chronic Myeloid Leukemia positive Philadelphia Chromosome, resistant to Imatinib Mesylate treatment according to the IRIS study definitions or ELN recommendations and 50 apparently healthy controls.

3.4 Sample size:

The following Equation was used to estimate the sample size:

$N=Z^2/d^2p\ (1-p)$ where:

Z is deviation standard and equal 1.96.

d is margin of error and equal 5% (0.05).

P is proportion of population and equal 0.025

Cases =104 and Controls = 50 Total= 150 samples.

3.5 Inclusion criteria:

- Ages eligible for study: Age of 20 to 80 years.
- Sex eligible for study: both sexes.
- Diagnosed CML by (Clinical presentation, morphological criteria of blood and bone marrow film and confirmed by molecular studies.
- Previous treatment: Treated with Imatinib Mesilate 400mg or 600mg for 6 months (patients group).

3.6 Exclusion criteria:

- CML patients with negative Philadelphia chromosome.
- Patient with leukemoid reaction.
- Patient with other myeloproliferative diseases.
- Patient with other types of hematological malignancies.
- Patients who treated with second and third generation of Tyrosine Kinase Inhibitor.
- Recent blood transfusion.
- All patients who had received an allogeneic transplant.

3.7 Method of Data collection:

We use questionnaire to obtain information from the participants includes; age, gender, duration of diseases and treatment seen Appendix 1.

3.8 Sample collection:

One hundred blood samples were collected from patients who were attending Khartoum Oncology Hospital- Khartoum-Sudan. And fifty blood samples from healthy volunteers used as control group. By using sterile syringe, tourniquet will be used to make the veins more prominent. Puncture sites will be cleaned with 70% ethanol and collect five milliliters of blood were obtained in EDTA tube, 2.5 mL for molecular techniques and the rest 2.5 mL were centrifuged at 1000g for 10mins at room temperature to separate plasma from whole blood, then stored as 500µL aliquots at −20°C until analysis. The diagnosis for patients was made by the consultant medical staff at the clinic of oncology, based Complete Blood Count (CBC), Bone Marrow Aspiration and Biopsy, Cytogenetic Analysis FISH (Fluorescence In Situ Hybridization) and Polymerase Chain Reaction (PCR).

3.9 Statistical analysis and presentation

Data were analyzed and computed with the statistical analysis Graph Pad Prism version 8.0.1 and in Excel to determine the descriptive parameters (mean and median) and standard deviation were calculated for numerical variables.

The Student's t test was performed to determine the association between the mean value of PDL- levels in the CML cases and controls. In addition, Frequency table was carried out to assess the frequencies of T315I mutations in Sudanese patients with Imatinib resistant. Moreover, the point biserial correlation coefficient analysis was used to assess the strength of association between categorical and continues variables (correlation between plasma concentration levels PDL-1 and T315I mutation). ROC (receiver operating characteristic) Curve to predictor variables of interest and provide a useful way to evaluate the sensitivity and specificity for quantitative diagnostic measures. A p value was considered significant if <0.05 at confidence interval 95%.

3.10 Programmed Death Ligand-1 ELISA Detection.

Detection of human programmed death ligand-1 plasma concentrations by use sandwich ELISA test using commercially available kits.

3.10.1 Materials used for PDL-1 detection.

3.10.1.1 Instruments.

1. ELISA Machine Microplate Strip Washer ELx50^{TM} (BioTek, Winnooski, Vermont 05404-0998, SN 236964, USA)

2. ELISA Machine Absorbance Microplate reader ELx800^{TM} (BioTek, Winnooski, Vermont 05404-0998, SN 237006, USA)

3. Centrifuge Heraeus Labofuge 200 T (Thermo Fisher Scientific, Shanghai, China)

4. Incubator muve oven KD 200 (DAFCO – Dar-Al-Farabi, Germany)

3.10.1.2 Equipment:

1. Microplate reader capable of measuring absorbance at 450 nm.

2. Microplate washer or washing bottle.

3. 5mL and 10mL graduated pipettes.

4. 5μL to 1000μL adjustable single channel micropipettes with disposable tips.

5. 50μL to 300μL adjustable multichannel micropipette with disposable tips.

6. test tubes for dilution.

3.10.1.3 Reagents:

Seen Appendix 2

3.10.2 Methods used for PDL-1 detection

3.10.2.1 Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for PDL-1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any PDL-1 present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for PDL-1 is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of PDL-1 bound in the initial step. The color development is stopped and the intensity of the color is measured.

(AmoyDx, Xiamen, Fujian, China)

3.10.2.2 Specimen preparation

The plasma samples were stored as 500μL aliquots at −20°C until analysis than we prepare the plasma for thawing at room temperature (25°C).

3.10.2.3 Reagent preparation

1. All kit components and samples were brought to room temperature (20-25°C) before use.

2. Fresh standard was prepared for each assay. Used within 4 hours and discarded after use.

3. standards was reconstituted carefully according to the instruction, and foaming avoided and gently mixed until the crystals had completely dissolved. To minimize imprecision caused by pipetting, small volumes was used and ensure that pipettes was calibrated. It was recommended to suck more than 10μl for once pipetting.

4. Distilled water was recommended to be used to make the preparation for reagents or samples. Contaminated water or container for reagent preparation will influence the detection result.

- Biotin-antibody(1x)- Centrifuge the vial before opening.

Biotin-antibody requires a 100-fold dilution. A suggested 100-fold dilution is 10μl of Biotin-antibody + 990μl of Biotin-antibody Diluent.

- HRP-avidin (1x)- Centrifuge the vial before opening.

HRP-avidin requires a 100-fold dilution. A suggested 100-fold dilution is 10μl of HRP-avidin + 990μl of HRP-avidin Diluent.

Wash Buffer (1x)- If crystals have formed in the concentrate, warm up to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 ml of Wash Buffer Concentrate (25 x) into deionized or distilled water to prepare 500 ml of Wash Buffer (1 x). (AmoyDx, Xiamen, Fujian, China)

3.10.2.4 Standard dilution preparation

- The standard vial was centrifuged at 6000-10000 rpm for 30s.
- The Standard was reconstituted with 1.0 ml of Sample Diluent.

- This reconstitution produces a stock solution of 1000 ng/mL.
- The standard was mixed to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

250µl of Sample Diluent was pipetted into each tube (S0-S6). Use the stock solution to produce a 2-fold dilution series (below). each tube was mixed thoroughly before the next transfer. The undiluted Standard serves as the high standard (10 ng/mL). Sample Diluent serves as the zero standard (0 ng/mL). (AmoyDx, Xiamen, Fujian, China)

Table 3.1 Serial standard concentration of PDL-1

Tube No	**S7**	**S6**	**S5**	**S4**	**S3**	**S2**	**S1**	**S0**
ng/mL	10	5	2.5	1.25	0.625	0.312	0.156	0

3.10.2.5 Procedure

- All reagents and samples were brought to room temperature before use. the sample centrifuged again after thawing before the assay.
- All reagents, working standards, and samples were prepared as directed in the previous sections.
- The assay layout Sheet was referred to determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and unused wells were stored at 4°C.
- 100µl of standard and sample were added per well. Then the wells were covered with the adhesive strip. The wells were incubated for 2 hours at 37°C. A plate layout was provided to record standards and samples assayed.
- The liquid was removed from each well, don't wash.
- 100µl of Biotin-antibody(1x) was added to each well. Then the wells were covered with a new adhesive strip. The wells were incubated again for 1 hour at 37°C.

(Biotin-antibody(1x) may appear cloudy. Warmed up to room temperature and mixed gently until solution appears uniform.

- Each well was aspirated and washed, repeating the process two times for a total of three washes, each well was washed with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or auto washer, and the well was lifted on stand for 2 minutes, complete removal of liquid at each step is essential to good performance. After the last wash, any remaining was removed using wash Buffer by aspirating or decanting. the plate was inverted and blotted against clean paper towels.
- 100µl of HRP-avidin (1x) 1x) 1x) added to each well. Then the microtiter plate covered with a new adhesive strip and incubated for 1 hour at 37°C.
- The aspiration/wash process Repeated for five times.
- 90µl of TMB substrate added to each well and incubated for 15-30 minutes at 37°C. the wells protected from light.
- 50µl of stop solution added to each well, gently the plate tapped to ensure thorough mixing.
- The optical density determined of each well within 5 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.
- The detection rage was 0.156 ng/ mL – 10ng/mL.(AmoyDx, Xiamen, Fujian, China)

3.10.2.6 Calculations of the Result.

Each sample was tested in duplicate. The PDL-1 level was determined using a standard curve. The minimum detectable level of PDL-1 was 0.156 ng/ml and the detection range was 0.156ng/ml - 10ng/ml. Create the standard curve known concentration of human PDL-1 standards and corresponding reading optical density is plotted on the log scale (x-axis) and the log scale (y-axis). The concentration of human PDL-1 in sample is determined by plotting the sample's optical density on the y-axis.

we calculation of the patients and controls samples by obtain this equation:

PDL-1 concentration(ng/mL) = O.D T/O. D SDT x Conc of SDT (5).

3.11 DNA extraction from Whole blood.

Total cellular DNA was extracted using commercially available kits from EDTA whole blood sample by using Genomic DNA extraction Bioneer reagent (AccuPrep K-3032).

3.11.1 Materials used for DNA extraction

3.11.1.1 Instrument

We use centrifuge DAFCO- Sigma Lab- Germany- Dar-Al Farabi corporation

Speed 200 – 14800 rpm. engineer@dafcotech.com

3.11.1.2 Equipment

1. 1 mL and 10 mL graduated pipettes with disposable yellow and blue tips
2. 20µL adjustable graduated pipette with disposable yellow tips.
3. 100µL to 1000µL variable pipette with disposable yellow tips.
4. Collection tube for filtration
5. AccuPrep Binding column-1

3.11.1.3 Regents:

Seen in appendix 3

3.11.2 Methods used for DNA extraction

3.11.2.1 Procedure of DNA extraction

- 20μL of Proteinase K was added into a clean 1.5 ml tube.
- 200μL of whole blood was added to the Proteinase K tube.
- 200μL of GB buffer added then mixed immediately by vortex mixer.
- Solutions incubated at 60c for 10 mins.
- 400μL of absolute ethanol added and again mixed well by pipetting.
- The lysate Carefully transferred into the upper reservoir of binding column tube (fit in a 2ml tube filtration tube) without wetting the rim.
- The tube Closed and centrifuged at 8.000 rpm for 1 min.
- The solution discard from the collection tube and discard the collection tube reused
- 500μL of Washing buffer1 added without wetting the rim, the tube closed, and centrifuged at 8.000 rpm for 1min.
- The tube opened and transferred the binging column tube to a new 2ml tube for filtration.
- 500μL of Washing buffer2 added without wetting the rim, the tube closed and centrifuged at 8.000 rpm for 1 min.
- The solution discarded from collection tube and the collection tube reused.
- Centrifuged once more at 13.000 rpm for 1min to completely remove ethanol and check that there is no droplet clinging to the bottom of binding column tube.
- The binding column tube Transferred to a new 1.5 ml tube for elution, 200μL of Elution buffer added onto binding column tube and wait for at least 1min at RT (15-25C). until elution buffer is completely absorbed into the glass fiber of binding column tube.

- Centrifuged at 8.000 rpm for 1 min to elute.
- The eluted genomic DNA was stable and can be used directly. Or stored at 4 C for later analysis.
- About 6µg of DNA in 200µL of eluent obtained from 200µL of whole blood contain $5x10^6$.

3.11.3 Visualize DNA in agar gel

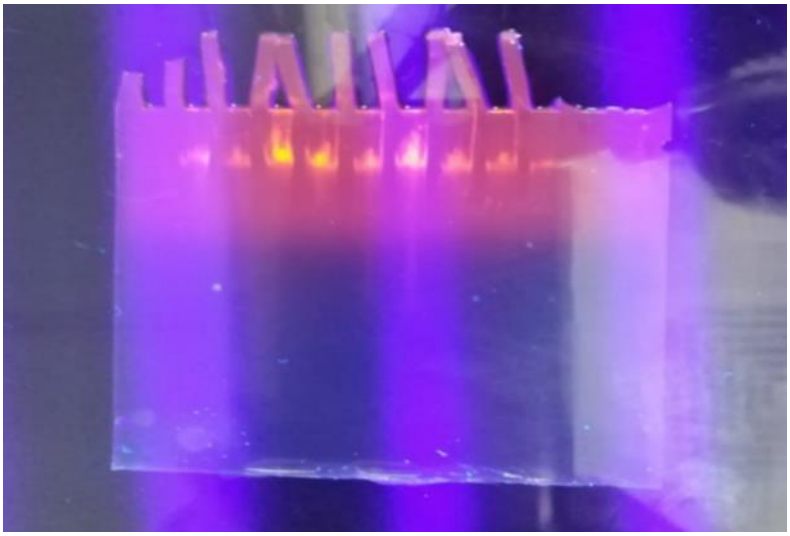

Fig. 3.1 DNA sample in agarose gels to examine the DNA quality

3.12 Molecular detection of T315I mutation

Detection of the T315I mutation in the BCR-ABL gene using a qualitative qPCR AmoyDx (AmoyDX Diagnostics Co., LTD, China) BCR-ABL T315I Mutation Detection Kit. It is extremely sensitive and selective in detecting the T315I mutation in the BCR-ABL gene. However, the proprietary method of AmoyDx enables the detection of 1% mutant DNA in a background of 99% normal DNA. Moreover, this kit uses novel, proprietary primers and probes in a real-time PCR assay to detect BCR-ABL mutations in human genomic DNA. The mutant BCR-ABL gene is amplified by the specific primers, and detected by the novel probes. http://www.amoydiagnostics.com

3.12.1 Materials used for detection of T315I mutation

3.12.1.1 Instrument

1, Real-Time PCR STRATAGENE- Agilent Technologies, 76337 Waldbrenn Model No.: 401513 Serial No.: DE31701884. Germany

2. Centrifuge DAFCO- Sigma Lab- Germany- Dar-Al Farabi corporation

Speed 200 – 14800 rpm. engineer@dafcotech.com

3.12.1.2 Equipment:

1. 1µL to 100µL variable micropipettes with disposable white tips.

2. 200µL to 500µL variable micropipettes with disposable white tips.

3. 100µL to 1000µL variable micropipettes with disposable white tips.

3.12.1.3 Regents:

Seen in appendix 4

3.12.2 Methods used for detection of T315I mutation

3.12.2.1 Procedure

The mutation assay for sample and control must be analyzed within the same PCR run to avoid run-to-run variations in threshold settings. It is recommended that the BCR-ABL Mixed Standard (STD) should be analyzed during each PCR run, along with no-template controls (NTC). Frozen samples, BCR-ABL reaction Mix and Mixed standard were thawed and incubated at 37^{o}C. then briefly centrifuge BCR-ABL Tag DNA polymerase, Reaction Mix and Mixed Standard prior to use.

0.25µL BCR-ABL Taq DNA Polymerase added to 35µL BCR-ABL reaction Mix per sample, the appropriate amount of BCR-ABL Taq DNA Polymerase transferred and BCR-ABL Reaction Mix into a sterile tube. each solution mixed for approximately 15 seconds and then centrifuged for 15 seconds. 35µL of the master mix transferred into PCR

reaction wells. And 5µl sample (DNA) added and 5µl BCR-ABL Mixed standard, or 5µl deionized water to appropriate PCR reaction wells. Then seal the PCR tubes.

Spin the PCR tubes gently in a centrifuge to collect the reagents at the bottom of wells.

Place the PCR tubes into the real-time PCR instrument. Carry out real-time PCR using the cycling conditions mention in the table.

Table 3.2 PCR Cycles

Temperature	Time	Cycle
Stage 1		
95 ºC 5 min	**1**	
Stage 2		
95 ºC25 s **60 ºC20 s** **70 ºC20 s**	**15**	
Stage 3		
93 ºC 25 s **56 ºC35 s** **72 ºC20 s**	**31**	

3.12.2.2 Result.

Based on different mutant Ct values, the detection results are divided into strong positive, weak positive or negative.

1) Negative: If the sample FAM Ct value is greater than or equal to 29 (the critical negative value), the sample is classified as negative or below the detection limit of the kit.

2) If the FAM Ct value is less than 29

a) Strong Positive: If the sample FAM Ct value is less than 26 (critical positive value), the sample is classified as strong positive.

b) If the sample FAM Ct value is greater than or equal to 26 (critical positive value), the ΔCt of the reaction tube is calculated to confirm the result.

I) Weak positive: If the ΔCt value is less than10.

II) Negative or below the detection limit of the kit: If the ΔCt value is greater or equal to 10.

The calculation of ΔCt: ΔCt = mutant FAM Ct value – internal control HEX (or VIC) Ct value.

3.12.2.3 Quality Control

For quality control, samples were processed together with one positive and one no template control (NTC) each time. If the amplification curve was not classic S-curve or if Ct value was ≥29, the result was determined as wild type (mutation negative). This kit provides a highly sensitive molecular technique to detect as low as 1% BCR-ABL T315I mutation DNA in background of 99% normal DNA at 5 ng sample DNA amount, while ensuring that false negatives are minimized. www.amoydx.com/en

Chapter Four: Results

One hundred and fifty participants were involved in this study, out of them 100 patients with Chronic Myeloid Leukemia in chronic phase of diseases, (68 males, 32 females), were conducted from Khartoum Oncology Hospital-Khartoum- Sudan during (2018-2023). All patients treated with Imatinib Mesilate for at least 6 months, with IM dose of 600 mg, while 50 apparently healthy controls (30 males and 20 females). in **(Table 4.1 and Figure 4.1).**

Their age in the cases group range from 23 to 77 years old with a mean ages in case group were (54.2±12.4) years, males (54.2±13.9 years) where the females (54.1±11.9 years). their mean ages in control group were (46.8±9.1) years, males (46.8±8.4 years) where the females (46.8±8.49 years). There were significant differences between the two groups concerning ages in **(Table 4.1 and Figure 4.2).**

Results showed that in **(Table 4.2)**, the mean plasma levels of PDL-1 in Sudanese patients with Chronic Myeloid Leukemia resistant to Imatinib Mesilate was significantly increased (0.613ng/ml) when compared to healthy control group (0.336 ng/ml), with P-value < 0.0001. However, the comparison of the mean plasma levels of programmed death ligand-1 between Imatinib resistant CML cases and controls groups were showed in box plot graph in **(Figure 4.3)**.

A significant association was observed between age and plasma levels of programmed death ligand-1 the result shoewed that the plasma levels of PDL-1 Low than 0.595 ng/mL in age groups less than 54 years were 40% and in ages more than an equal 54 years old were 10%. While the plasma levels of PDL-1 more than or equal 0.595 ng/mL in age groups less than 54 years were 10% and in ages more than an equal 54 years old were 40% with P-value <0.0001 in **(Table 4.3)**. To rule out this strong effect of age, two age groups were formed (< 54 and ≥ 54). Furthermore, there association was

observed between gender and plasma levels of programmed death ligand-1 with P-value 0.471in **(Table 4.3)**.

The prevalence of BCR-ABLT315I mutation in Sudanese patients with Chronic Myeloid Leukemia resistant to Imatinib Mesilate was presented in (**Figure 4.4**), were positive in chronic phase 11 (11%), (8 males and 3 females) and negative in 89 (89%) (60 males and 29 females). However, all 50 controls were negative (100%) in **(Table 4.4)**.

The results also showed a statistical analysis revealed the correlation between the plasma levels PDL-1 and T315I mutation status in Sudanese patients with Chronic Myeloid Leukemia resistant to Imatinib Mesilate, showed a positive correlation between T315I mutation and plasma levels PDL-1 concentrations, with correlation coefficient (0.3713) indicates moderate correlation and p-value 0.0001. That means there was a positive correlation between T315I mutation and plasma levels PDL-1 concentrations in **(Table 4.5)**.

The present study also showed that the cut-off value result, when use the median plasma level of PDL-1 in CML patients was significant increase were compared to healthy controls (0.595ng/mL vs 0.335ng/ng/mL) with p value 0.0001 in **(Table 4.6)**. Also, the cut-off value result of the median the patients with positive BCR-ABLT315I mutation was a significant increase were compared to those negative BCR-ABLT315I mutation (0.718 ng/Ml vs 0.592 ng/mL) with p value 0.0001. in **(Table 4.7)**.

In addition, the study showed that the cut-off values derived from the ROC curve, the plasma levels of PD-L1 was significantly increased in CML patients, showed high accuracy AUC (AUC=0.877). At best cut off value (>0.593ng/mL), sensitivity was 100%, specificity was 66.91% and p value <0.001 in (**Table 4.8**). and increased plasma levels of PD-L1 was significantly increase in positive T315I mutation patients showed high accuracy AUC (AUC=0.808). At best cut off value (>0.685ng/mL), sensitivity was 63.64%, specificity was 87.64% and p value <0.001 in (**Table 4.9**).

Table 4.1 Demographical data distribution of the CML cases and controls groups.

Variables		Cases (n=100)	Controls (n=50)	P. value
Age(years) (Mean ±SD)		54.1±12.45	46.8±9.10	**0.0003**
Gender	Males	68 (68%)	30 (60%)	**0.1634**
	Females	32(32%)	20 (40%)	**0.0003**

- **The table shows the mean ± Standard Deviation (descriptive analysis)**
- **Independence t-test with two tail (two categorical nominal variable)**
- **Level of significance (alpha) is 0.05**
- **P value less than 0.05 considered significant**

Table 4.2 Comparison of the plasma levels of programmed death ligand-1 concentration between Imatinib resistant CML cases and controls.

Parameter	Participants	Mean	Std. Deviation	P-value
sPDL-1ng/mL	Cases (n=100)	0.623	0.092	< 0.0001
	Controls (n=50)	0.336	0.023	

- **The table shows the mean ± Standard Deviation**
- **T. test (independent t test) used to calculate P value**
- **Level of significance (alpha) is 0.05**
- **P value less than 0.05 considered significant**

Table 4.3a Comparision of the plasma levels of programmed death ligand-1 with Age.

Age group	sPDL-1		P-value
	Low(<0.595 ng/mL)	**High(≥0595 ng/mL)**	
< 54	40 (80%)	10 (20%)	<0.0001
≥ 54	10 (20%)	40 (80%)	

Table 4.3b Comparision of the plasma levels of programmed death ligand-1 with Gender.

Gender	sPDL-1		P-value
	Low(<0.595 ng/mL)	High(≥0595 ng/mL)	
Male	33 (34.68) [0.08]	35 (33.32) [0.08]	0.471
Female	18 (16.32) [0.17]	14 (15.68) [0.18]	

Table 4.4 Frequency of T315I mutations in patients with imitinib mesilate resistant and healthy controls.

Participants	T315I mutation				Total
	Positive		Negative		
	Males	Females	Males	Females	
Cases	8(72.7%)	3 (27.3 %)	60 (68%)	29 (32%)	100 (100%)
Controls	0 (60%)	0 (40%)	30 (60%)	20 (40%)	50 (100%)
Total	**11(7.3%)**		**139(92.7)**		**150(100%)**

- **Frequency table**
- **Use percentage**

Table 4.5 Correlation between plasma levels of programmed death ligand-1 and T315I mutation.

Parameter	T315I mutation	Number	Correlation coefficient (r)	P-value
sPDL-1ng/mL	Positive	11	0.3713	0.0001
	Negative	89		

- **The table shows correlation coefficient (r) and p value.**
- **point biserial correlation coefficient test**
- **Level of significance (alpha) is 0.05**
- **P value less than 0.05 considered significant**

Table 4.6 Cut- off value plasma levels of programmed death ligand-1 between Imatinib resistant CML cases and controls groups.

Parameter	Participants	Median	P-value
sPDL-1ng/mL	Cases (n=100)	0.595	0.0001
	Controls (n=50)	0.335	

- **The table shows the median and p value**

Table 4.7 Cut- off value plasma levels of programmed death ligand-1 between T315I mutation status.

Parameter	T315I mutation	Median	P-value
sPDL-1ng/mL	Positive (n=11)	0.718	0.0001
	Negative (n=89)	0.592	

Table 4.8 ROC curve shows the Cut-off value of Plasma levels of PDL-1 between CML cases and control groups (cut-off, sensitivity, specificity, accuracy and AUC)

	Cut-off	Sensitivity %	Specificity %	Accuracy %	AUC %
sPDL-1	0.593ng/mL	100.00	66.91	66.91	87.7

Table 4.9 ROC curve shows the Cut-off value of Plasma levels of PDL-1 in the cases group whose Positive and Negative T315I mutation (cut-off, sensitivity, specificity, accuracy and AUC)

	Cut-off	Sensitivity %	Specificity %	Accuracy %	AUC %
sPDL-1	0.685ng/mL	63.64	87.64	51.28	80.8

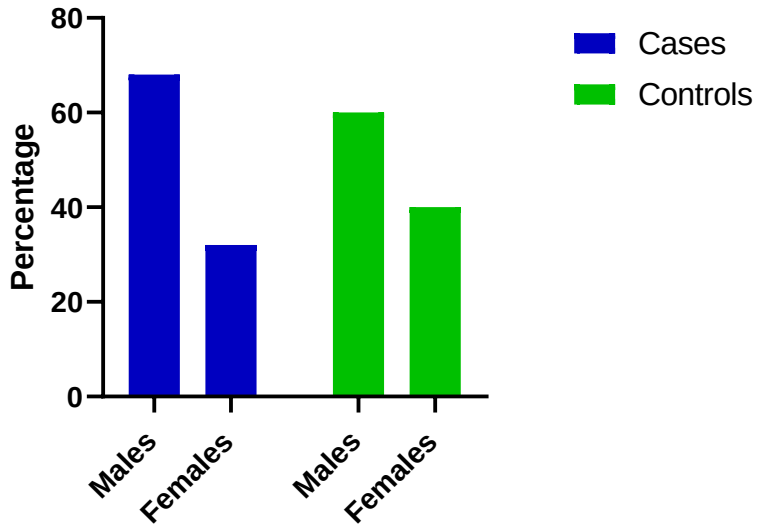

Fig. 4.1 Percentage of male and female in CML cases and controls

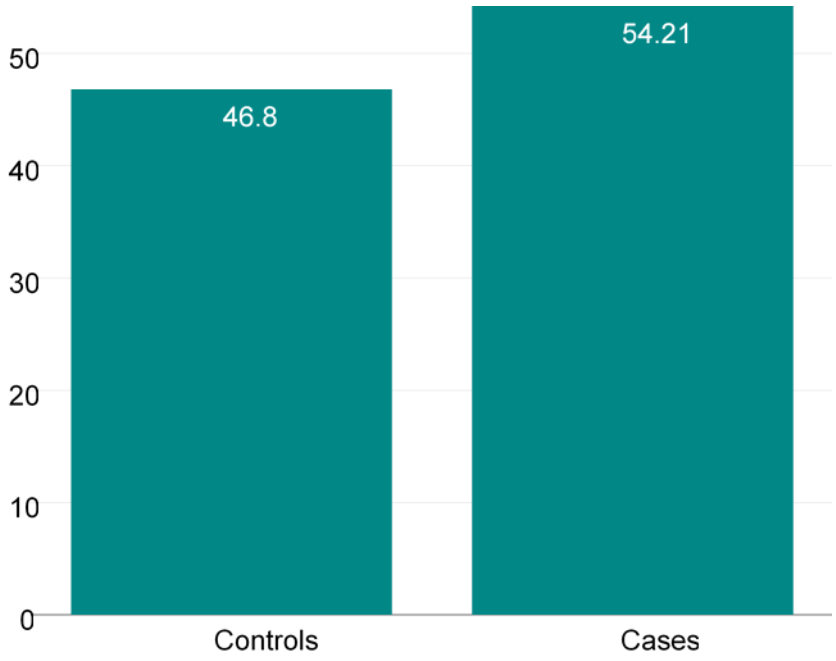

Fig. 4.2 Mean ages in CML Controls and Cases

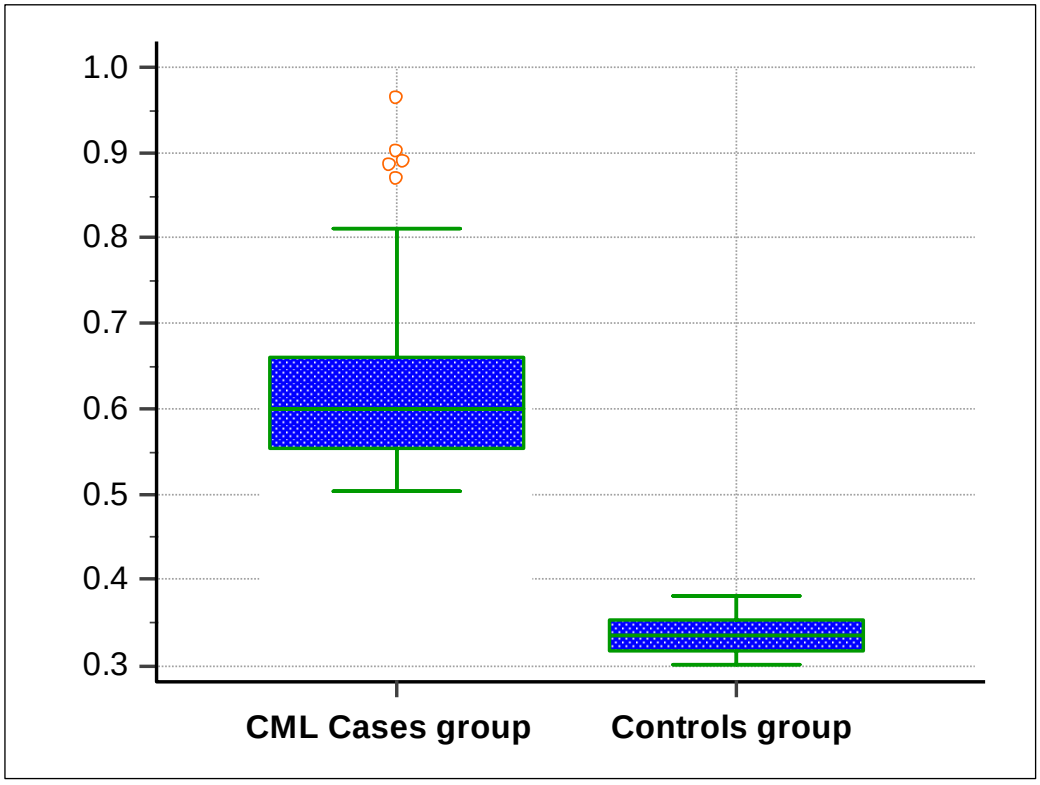

Fig. 4.3 Comparison of plasma levels programmed death receptor ligand-1 between cases and control.

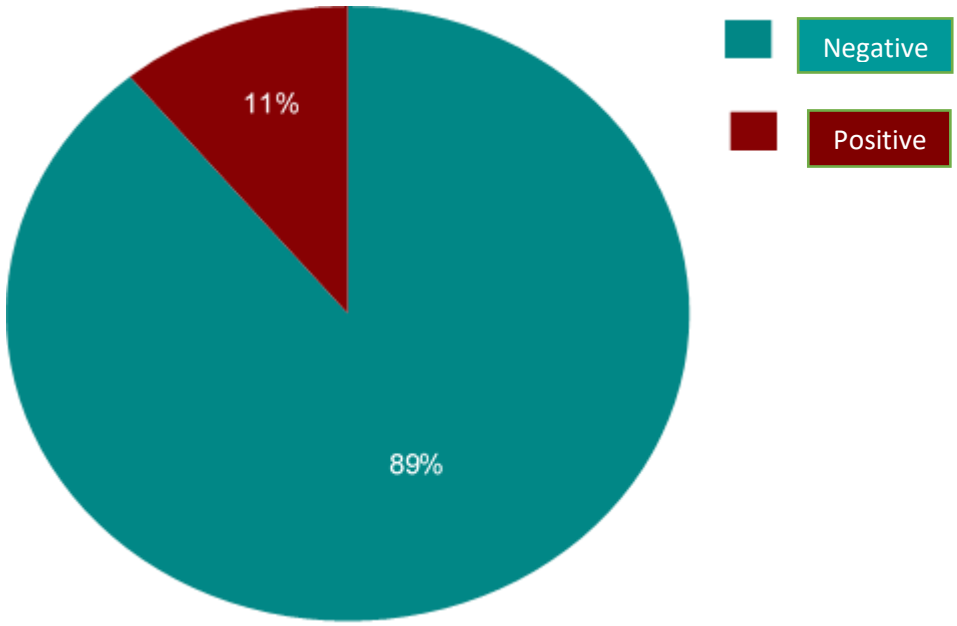

Fig. 4.4 Frequencies of BCR-ABL-1T315I mutation in Sudanese imitinib resistant CML Patients

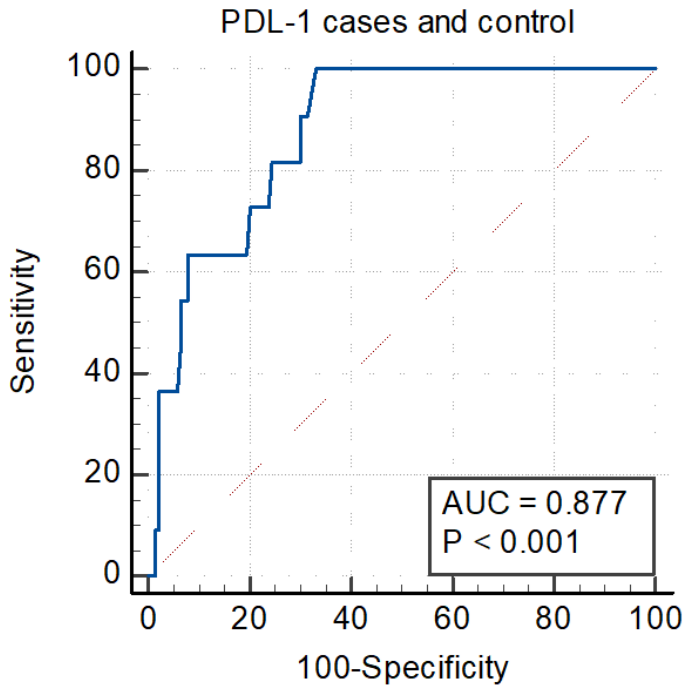

Fig. 4.5 ROC curve shows the Cut-off value of Plasma levels of PDL-1 between CML cases and control groups

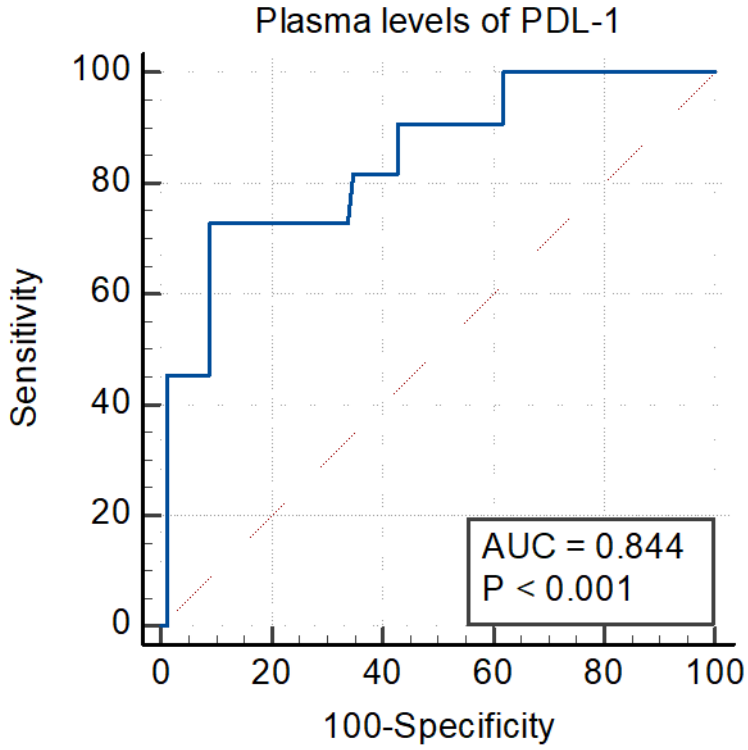

Fig. 4.6 ROC curve shows the Cut-off value of Plasma levels of PDL-1 in the cases group whose Positive and Negative T315I mutation

Chapter Five: Discussion, Conclusion and Recommendations

5.1 Discussion

Immune system releases programmed death ligand 1 (PDL-1) protein acts as a co-inhibitory molecule for T cells. by binds to the programmed death receptor1 (PD-1) receptor on T cells which prevents T cells itis activation, migration, proliferation, and cytokine secretion. However, PDL-1 protein may be produced by cancer cells, and this interaction causes leukemia cells to evade the immune system and T cells becoming latent and inactive(270,290,291).

To our knowledge, no studies have been reported in Sudan examine the plasma levels concentration of PDL-1 and BCR-ABLT315I mutation detection in patients with chronic myeloid leukemia resistant to Imatinib Mesilate treatment. In the present study, we examined the plasma level of PDL-1 and BCR-ABLT315I mutation, then compared with apparently healthy controls. The mean ages in the cases group were 54.2±12.4 years, males (54.2±13.8 years) where the females (54.1±11.8 years). The controls ages were 46.8±9.1 years, males (46.8±8.4 years) where the females (46.8±8.5 years). This finding was not consistent and slightly increased in the mean ages, when compare with a descriptive study conducted in 2021 by Noah Awad *et al.* were tested one hundred CML patients, the mean ages were 43.9 years, males were 46.2 years and females were 41.7 years. Also, in a different cross-sectional case-control study conducted in 2021 by Ezeldine Abdalhabib et al. were tested one hundred and eighty-six CML patients, the mean age of cases was 46.15±13.9 years. The average age of the controls was 44.9 ± 9.0 years.(292,293) Our results, which are slightly higher than two studies done by Ezeldine Abdalhabib and Noah Awad.

In the present study, the plasma levels of PDL-1, showed a statistically significant difference in cases when compared to the control group (P =0.001). these results were

similar and totally agrees with a case control study carried out on 80 individuals, 40 patients with CML in chronic phase and 40 control healthy participants in Egypt by Elkhawanky *et al.,* were found that plasma levels of PDL-1 in the treatment group and the control group were statistical different with P value = 0.001(21). Also, in another study was conducted in Iraq by Noor Jaleil *et al.* during 2019 in a case control study on 66 patients with CML in the chronic phase and 20 healthy individuals, found that Plasma PDL-1 levels were significantly higher in adult patients with CML compared with healthy subjects ($P < 0.001$)(287). these levels were significantly higher in patients with CML who are newly diagnosed compared with Imatinib treatment responders and resistant counterparts ($P < 0.001$).

The present study has shown that the PDL-1 levels were low than 0.595 ng/mL in age groups less than 54 years were 40% and in ages more than an equal 54 years old were 10%. While the plasma levels of PDL-1 more than or equal 0.595 ng/mL in age groups less than 54 years were 10% and in ages more than an equal 54 years old were 40%.

In Addition, this study showed that the frequency of the BCR-ABLT315I mutation in patients with chronic myeloid leukemia resistant to Imatinib Mesilate treatment was found to be 11% (11/100), males were 8 (8%) while females were 3 (3%). Several studies had identified and reported the frequency of BCR-ABLT315I in Sudanese CML patients. A descriptive cross-sectional study was conducted in 2011 by Waggas Elaas *et al*. carried out on 50 CML patients. They found that the frequency of BCR-ABLT315I mutation in Sudanese patients was 10% (5/50)(289). Moreover, in other descriptive cross-sectional study were conducted between May, 2018-2019. by Hala Khair *et al.* Which they found the frequency of the BCR-ABLT315I mutation in Sundanese was 43.4% 43/99 (20). However, a lower frequency was observed in our study among Imatinib Mesilate resistant Sudanese patients with CML, this variation in BCR-

ABLT315I mutation frequencies may be due to deferent types of methods used for BCR-ABLT315I mutation detection.

The present study revealed that the correlation between the analyzed the plasma levels of programmed death ligand-1 with the BCR-ABLT315I mutation status, a positive correlation was found between plasma levels programmed death ligand-1 concentration and BCR-ABLT315I mutation status, with regression 0.3081 indicates moderate correlation and p-value 0.0018. Which indicate our results suggest that elevated plasma levels PDL-1 is significantly associated with increased risk of BCR-ABLT315I mutation present in patients with chronic myeloid leukemia resistant to Imatinib Mesilate treatment.

There is currently no defined cut-off value to distinguish between high and low of plasma levels of PD-L1 in patients with CML resistant to Imatinib Mesilate treatment.

In present study we suggest cut-off value for plasma PDL-1 for diagnosis of Imatinib resistant CML patients. By two types of statistical analysis. First use the median plasma levels of PDL-1 in heathy controls which was 0.335ng/mL compare with the median plasma levels of PDL-1 in the cases was 0.595ng/mL and cut-off value for plasma PDL-1 for positive BCR-ABLT315I mutation by use the median plasma levels of PDL-1 in negative BCR-ABLT315I mutation which was 0.592ng/mL compare with the median plasma levels of PDL-1 in positive T315I mutation was 0.718ng/ mL. Second use the analysis of ROC curve for determine the cut-off of Plasma levels of PDL-1 in the healthy and Imatinib resistant CML patients, for prediction of BCR-ABLT315I mutation occurrence. Showed that PDL-1 levels a sensitivity of 100%, specificity of 66%, AUC was 0.877, and the best cut off value was established as 0.593ng/mL. Moreover, PDL-1 levels were excellent predictor for BCR-ABLT315I mutation and use the Plasma levels of PDL-1 the positive/negative BCR-ABLT315I mutation Imatinib resistant CML patients for prediction of T315I mutation occurrence. Showed that PDL-1 levels a

sensitivity of 100%, specificity of 66%, AUC was 0.877, and the best cut off value was established as 0.593ng/mL. Furthermore, the most interesting result, we determine the cut-off value for plasma levels of PDL-1 for diagnosis of Imatinib resistant CML patients. In the present study we use the median of the plasma levels concentrations of PDL-1 in heathy controls which was 0.335ng/ mL as cut-off value.

Limitation of the study

This study has several limitations, which should be addressed in future studies. First, the difficult in collection of samples from the CML patients because the majority of hospitals and clinical centers had closed due to affected by the COVID19 pandemic. However, it is one of the main obstacles to faced. After hospitals and clinics reopened, we finished the sample collection. Second, many samples were affected while being stored as a result of repeated electrical power failures.

5.2 Conclusion:

According to our results we concluded that:

- Plasma levels of PDL-1 were increased in patients with CML disease resistant to Imatinib Mesilate treatment when compared to control group.
- Plasma levels of PDL-1 were increased in patients with CML disease had positive T315I mutation when compared with to negative T315I mutation.
- The suggestive cut-off value for diagnosis of CML harboring T315I mutation when use the median and receiver-operating characteristic (ROC) analysis for cut-off value from the healthy controls and mutation status respectively.

5.3 Recommendations:

According to our results we recommended that:

1- Plasma levels PDL-1 test should be considering in CML diagnosis in patients showed resistance to Imatinib Mesilate treatment.

2- Screening of BCR-ABLT315I mutation tests in chronic phase is recommended for patients showed resistance for Imatinib Mesilate treatment.

3- Further studies should be done to determine the frequency of the BCR-ABLT315I mutation within accelerating and blast crises phases of CML diseases.

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Appendices

Appendix 1. Questionnaire

B. Others information.
Please answer by Yes or No.

المعلومات التحليلية		
Smoking	Yes ( )	No ( )
Snuff	Yes ( )	No ( )
Family History of Cancer	Yes ( )	No ( )
Past Medical History	Yes ( )	No ( )
Diabetes or abnormal blood-sugar tests	Yes ( )	No ( )
High blood pressure	Yes ( )	No ( )
Anemia	Yes ( )	No ( )
Bleeding	Yes ( )	No ( )
Take Chemotherapy	Yes ( )	No ( )
Bone marrow or Stem cell transplant	Yes ( )	No ( )

1

بسم الله الرحمن الرحيم
Republic of Sudan
Ministry of Higher Education and Scientific research
Shendi University
Faculty of Graduated Studies and Scientific Research

The Title:

Assessment of the Role of Programmed Death Ligand-1 in Sudanese patients with Chronic Myeloid Leukemia Patients Resistance to First-Line Therapy (Imatinib Mesylate) in the Presence of Tyrosine Kinase Domain Mutation (T315I).

Questionnaire

A. General information.

Name		Date of Birth	
Gender		Place pf Birth	
Family Structure		Occupation	
Address		Contact number	
Disease phase	1- Chronic phase ( ) 2- Accelerated phases ( ) 3- Blast (blast crisis) phase ( )		
Duration of disease (Years)	1- 1 to 3 years ( ) 2- 3 to 5 years ( ) 3- More than 5 years ( )		
Treatment	1- First Line Treatment with Imatinib Mesylate ( ) 2- First Line Treatment with 2nd generation TKIs ( ) 3- First Line Treatment with 3rd generation TKIs ( )		
Control of treatment	1- Continue ( ) 2- Discontinue ( )		

1

Appendix 2. ELISA reagents for PDL-1 detection:

	Reagent	LOT	Company
1	**Assay plate (12x8 coated Microwells) 1(96 Wells)**	**9B0101**	AmoyDx
2	**Standards2**	**9C0454**	AmoyDx
3	**Biotin antibody(100xconcentrate)-1x120µl.**	**9C0101**	AmoyDx
4	**HRP-avidin (100 x concentrate)- 1x120µl.**	**9C0101**	AmoyDx
5	**Biotin-antibody Diluent-1 x 15 ml**	**9C0151**	AmoyDx,
6	**HRP-avidin Diluent-1 x 15 ml**	**9C0151**	AmoyDx
7	**Sample Diluent-1 x 50 ml**	**9C0011**	AmoyDx
8	**Wash Buffer (25 x concentrate)-**	**9C0034**	AmoyDx
9	**TMB Substrate-1 x 20 ml**	**9C0354**	AmoyDx
10	**Stop Solution-1 x 10 ml**	**9C0292**	AmoyDx

Appendix 3. DNA extraction reagent:

	Reagent	Quantity	REF	LOT	Company
1	Proteinase K	1x25 mg	KB-0111	2001B	BioNEER
2	Binding buffer(GB)	25 ml	KB-2041	1903J	BioNEER
3	Absolute ethanol	2.5 ml	M/4056/17	2064631	Fisher Scientific
4	Washing buffer1 (W1)	40 ml	KB-3013	1904K	BioNEER
5	Washing buffer2 (W2)	80 ml	KB-4012	1903J	BioNEER
6	Elution buffer (EA)	25 ml	KB-8012	1903J	BioNEER

Appendix 4. T315I mutation detection reagents and equipment:

No	Tube / Reagents Supplied	Volume	Channel
1	T315I Reaction Mix	1000 µL	FAM, HEX/VIC
2	BCR-ABL Taq DNA Polymerase	15 µL	
3	BCR-ABL Mixed Standard	150 µL	
4	Sterile, nuclease-free H_2O	2000 µL	
No	Equipment	Amounts	
1	Dedicated pipette	250	
2	filtered pipette tips for handling DNA samples.	250	