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Identification of Novel Alterations in the *ASXL1* Gene and Its Expression Profile in CML Patients

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ABSTRACT

Chronic myeloid leukemia is a hematopoietic stem cell malignancy characterized by excessive proliferation of white blood cells, primarily driven by the Philadelphia chromosome (t 9;22). Despite the availability of tyrosine kinase inhibitors, disease progression and therapy resistance remain major challenges, often linked to additional somatic mutations. **Objectives:** To find out the mutational status and expression variations of the *ASXL1* gene hotspot region in CML patients treated by TKIs. **Methods:** This retrospective cross-sectional study was conducted at the Department of Zoology, Division of Science and Technology, University of Education, Lahore, Pakistan. DNA and mRNA of 50 CML patients were analyzed alongside 10 healthy controls and statistically assessed using SPSS. **Results:** Results showed that sequence analysis of exon 13 identified hotspot mutations, including two novel missense alterations, G659S and D667E, within the coding region of *ASXL1*. In-silico analysis also suggested potential structural and functional relevance of these variants. The *ASXL1* gene expression showed a progressive but statistically non-significant down-regulation in CML patients across disease phases (p=0.662), disease duration (p=0.42), treatment with second-line versus first-line TKIs (p=0.412), and wild-type and mutant (p=0.544). **Conclusions:** It is concluded that two novel missense alterations, G6559S and D667E, were identified in the hotspot region, and *ASXL1* gene expression was downregulated non-significantly in CML patients.

INTRODUCTION

Chronic Myelogenous Leukemia (CML) is one of the Myeloproliferative malignancies that occur due to reciprocal translocation of a gene located on chromosome 9 named ABL1 (Abelson murine leukemia) gene sequence (q34) and Breakpoint Cluster Region (BCR) gene sequence (q11) located on chromosome 22, forming a Philadelphia chromosome (Ph) [1, 2]. This Ph chromosome encodes a tyrosine kinase, which is responsible for activating multiple signaling pathways that promote the uncontrolled proliferation, improper cell differentiation, and inhibit the apoptosis process [3, 4]. Based on the number of blasts cells, CML is classified as a triphasic disease. Chronic phase shows 10% blast cells, accelerated phase shows 10-19%, while blast phase shows above 20% blast cells [5]. Tyrosine Kinase Inhibitors (TKIs) have been used to treat the majority of CML patients for the past 20 years [6, 7]. The *ASXL1* gene, positioned on chromosome 20q, codes a protein of 1541 amino acids involved in transcriptional regulation and chromatin modifications. Mutations in the *ASXL1* gene are frequently observed in myeloid cancers, including MDS, CMML, MPN, and AML [8-10]. These mutations mostly change the chromatin remodeling

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functions, leading to deregulated gene expression. In CML, *ASXL1* mutations were reported in both chronic and advanced phases, and affect the treatment response and disease progression [11]. Crucially, new studies show that aberrant expression of the *ASXL1* gene mRNA may contribute to leukemogenesis by dysregulating normal epigenetics even in situations when there are no discernible gene abnormalities [8, 12]. This emphasizes the need of exploring both mutational status and expression levels of *ASXL1* in CML, particularly in conditions where disease heterogeneity and resistance to treatment are prominent.

The study aims to analyze the mutational alterations and expression profile of the *ASXL1* gene in CML patients.

METHODS

This retrospective cross sectional was conducted at the Department of Zoology, Division of Science and Technology, University of Education, Lahore, Pakistan. Approval was obtained from the Ethical Institutional Review Board of the UE Lahore (Batch No. 732) and the ERB of Allama Iqbal Medical College, Lahore (ERB172/12/10-10-2024/S1ERB). The study duration was from September 2024 to March 2025. The study was carried out in compliance with the Declaration of Helsinki. Written informed consent was obtained from all CML patients before sample collection. The study design was a retrospective cross sectional including CML patients and healthy controls. Sample size was calculated with the WHO Sample Size calculator [13]. Expected Population Prevalence was (p) =0.5 (50%), level of significance (z)=1.96 (C.I 95%), precision (d)=0.10 (10%, 0,.05), and the minimum sample size was 49. Accordingly, fifty CML patients, Philadelphia +ve (48) and –ve (02), diagnosed by physicians at Jinnah Hospital, participated in this study. None of them had any other reported malignancy, and all were treated with either imatinib (400 mg) or nilotinib (200 mg). Their age range was 25-70 years and represented all three phases of CML, with less than 15 years of treatment duration. Patient history, treatment strategy, hematological and cytogenetic reports were obtained through patient files and were recorded on designed data forms. Furthermore, 10 healthy individuals were included in the study as a control group, whose age and gender were matched with those of the patients. Healthy controls were selected based on the absence of hematological disorders, chronic illness, or malignancy. Demographic data were collected through interviews and confirmed via medical records. Hematological parameters, including hemoglobin (Hb), RBC, WBC, platelets, hematocrit (HCT), and differential counts (neutrophils, lymphocytes, eosinophils, monocytes), were measured using an automated hematology analyzer. Standard laboratory protocols were followed for sample collection and analysis. Blood samples were collected and preserved in TRIzol reagent for DNA and RNA extraction. Total RNA was isolated by TRIzol LS reagent and confirmed via GAPDH gene amplification. The cDNA synthesis was performed using ABScript II First-Strand cDNA Synthesis Kit. Primers for the *ASXL1* gene were designed using Primer Blast. Exons 12 and 13 of the ASXL1 gene were amplified from extracted DNA using PCR primers. Annealing temperature of 59ºC was used to amplify exon 12 (Forward Primer: GCCACTGCTTGGCACATACC), (Reverse Primer: AGTGAGACCAAGCAACTCCT) with product size (305), while 60ºC was used for exon 13 (Forward Primer: AGGTCAGATCACCCAGTCAG), (Reverse Primer: CTCACCACCATCACCACTGC) product in PCR amplifications. The PCR products were subjected to Sanger sequencing (Lab Genetix(®)). The sequence electropherogram was analyzed using Geneious Prime (version 2025.1.2) bioinformatics software for analysis, and all variations were reviewed manually. For expression analysis of the *ASXL1* gene in both groups, primers were designed using the Primer3 tool. Forward and Reverse Primers sequences were GTTGGGACCAAGCACAAACT and ACTGGTGGGTCACTTGGAAG, respectively. Their product size was 191bp, and the melting temperature Tm was 60°C. Primers were commercially sourced from Macrogen Lab (Korea). Synthesized cDNA was subjected to quantitative real-time PCR (qPCR) at Lab Genetix(®) using SYBR Green to quantify *ASXL1* gene expression. Cycle Threshold (Ct) value of the target gene (*ASXL1* gene) and the House Keeping gene (GAPDH) was calculated by Real Time PCR. Relative gene expression of *ASXL1* was quantified using the comparative Ct ($2^{-}\Delta\Delta Ct$) method, i.e., Livak method [14]. GAPDH was used as the internal housekeeping gene for normalization. The ΔCt was calculated by subtracting the Ct value of GAPDH from the Ct value of *ASXL1* for each sample. Relative expression levels were expressed as fold change values. Each qPCR reaction contained 1 µL cDNA template, 0.5 µM of each primer, and SYBR Green master mix in a total volume of 20 µL. All reactions were performed in triplicate. Fold change ($2^{-}\Delta\Delta Ct$) was calculated by comparing patient ΔCt to control ΔCt. Fold change values are reported in Table 4. Statistical analysis and graphical illustrations were performed with SPSS version 25. Independent T-test and One-way ANOVA were used to compare two and three groups, respectively. Chi-square test of association was employed, and Kaplan-Meier Survival Analysis was also performed. Detrimental effects of observed modifications (G659S and D667E) were determined using Mutation Taster, Align GVGD, SIFT, and Polypen-2. The following online tools were used for in-silico analysis. SOPMA: http://npsa-pbil.ibcp.fr/cgi-

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bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html. NetSurfP 2.0: https://services.healthtech.dtu.dk/services/NetSurfP-2.0/. UCSF Chimera: https://www.rbvi.ucsf.edu/chimera/. STRING database: https://string-db.org/ and RNAfold Webserver: https://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi. In-silico analysis of *ASXL1* mutations (G659S and D667E) was performed using SOPMA for secondary structure prediction, NetSurfP 2.0 for solvent accessibility, UCSF Chimera for 3D visualization, STRING database for protein-protein interactions, and RNAfold Web Server for mRNA secondary structure prediction. Each analysis was conducted according to the standard protocols of the respective tools. Results are presented as separate figure panels (Figure 3a-e) with corresponding explanations in the results section.

RESULTS

Hematological parameters, including hemoglobin, white blood cells (WBCs), Eosinophils, and platelet count, showed no significant difference. However, red blood cells (RBCs count, Hematocrit (HCT), Neutrophils, and lymphocytes showed a significant difference (p=0.004*) between CML patients and the healthy control group. The difference between the hematological parameters of CML patients bearing wild-type *ASXL1* and those harboring mutations was not statistically significant, except for Blast cells (p=<0.001). Mean HB, RBC, WBC, platelets, Hematocrit, Neutrophils, and lymphocytes were slightly lower in the mutant group, while Eosinophils and monocytes were higher in the mutant group compared to the wild-type CML patient (Table 1). Chi-square test indicated that there was no significant association between phase of the disease and proportion of the blast cell (χ^2 = 5.357, p= 0.69). To provide a clear understanding of *ASXL1* mutational status, we assessed the distribution of wild-type and mutant variants in patients and controls. Among the 50 CML patients, 5 (10%) carried heterozygous *ASXL1* mutations, while the remaining 45 (90%) were wild-type. No homozygous mutations were detected. All healthy controls (n=10) exhibited the wild-type genotype (Table 1).

Table 1: Comparison of Hematological Parameters Between Healthy Controls (n=10) and CML Patients (n=50), and Comparison of CML Patients with Wild-type (n=45) and Mutant (n=05)

Parameters	Healthy Control	CML Patients	p-value	*ASXL1* Wild Type	*ASXL1* Mutant	p-value
	Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM	
Hemoglobin	11.50 ± 3.64	12.07 ± 1.71	0.901	12.31 ± 1.84	10.78 ± 4.83	0.144
WBCs	12.73 ± 4.03	8.33 ± 1.18	0.069	4.52 ± 0.67	4.43 ± 1.99	0.228
RBCs	4.26 ± 1.35	4.46 ± 0.63	0.004*	8.45 ± 1.26	7.86 ± 3.53	0.155
Platelets	337.20 ± 106.71	257.20 ± 36.38	0.528	254.49 ± 37.98	233.80 ± 104.84	0.719
Eosinophils	3.44 ± 0.37	5.00 ± 1.00	0.211	3.20 ± 0.48	5.00 ± 2.24	0.528
Monocytes	5.30 ± 0.55	8.00 ± 1.37	0.41	5.28 ± 0.79	8.00 ± 3.59	0.849
Neutrophils	60.35 ± 2.56	50.40 ± 6.44	0.017	56.24 ± 8.39	50.40 ± 22.60	0.755
Lymphocytes	33.84 ± 1.92	27.40 ± 7.95	0.018	33.81 ± 5.05	27.40 ± 12.29	0.592
HCT	42.87 ± 1.49	35.26 ± 4.25	0.039	37.52 ± 5.60	35.26 ± 15.81	0.793
Blast Cells	NA	NA	NA	1.89 ± 0.28	22.80 ± 10.22	<0.001*

The present study covered hotspot regions of *ASXL1*, including exons 12 and 13. According to sequencing data, there are five heterozygous mutations (57%), one silent mutation (14%), and two novel missense mutations (8%). All of these mutations were observed in the *ASXL1* gene's exon 13 that effect protein's function. No mutant variants were detected in the control group. All control samples showed the wild-type *ASXL1* genotype, with no homozygous or heterozygous mutations observed. One missense mutation caused substitution of Glysine (GGT) by Serine (AGT) (A), while the second mutation, in which aspartic acid (GAT) changed into glutamic acid (GAG) (B) (Figure 1).

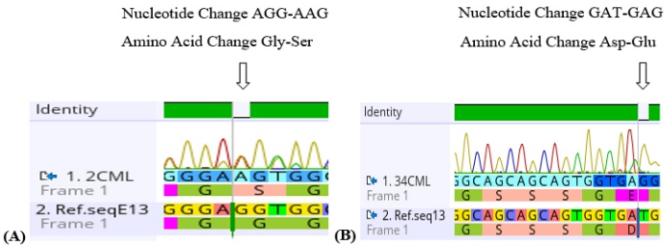

Figure 1: Single Nucleotide Mutations in the ASXL1 Gene Exon 13 Shown by Electropherogram

There was no significant association (χ^2 = 1.389, p=0.499) between phases of the disease and mutation status. Two novel missense mutations had been observed in which a single nucleotide replacement resulted into amino acid change (Table 2).

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Table 2: Novel *ASXL1* Exon 13 Alterations Detected in CML Patients (n=50)

ASXL1 Exon	Variation Detected	Amino Acid Change	Nucleotide Change	NCBI Accession Numbers
ASXL1 Exon 13	Novel	G659S	c.1975G>A	SCV006278281.1
ASXL1 Exon 13	Novel	D667E	c.2001T>G	SCV006278281.1

Different mutation types and their frequency were found in the different phases of the disease (Table 3).

Table 3: *ASXL1* Mutation Types and Their Frequency among CML Patients in Different Phases (n=50)

Chronic Phase	Accelerated Phase	Blast Phase
Chronic Phase	5%	Missense, Heterozygous & Silent
Accelerated Phase	16%	Heterozygous
Blast Phase	25%	Missense

Relative *ASXL1* gene expression levels were lower in CML patients than controls, not statistically significant (p=0.433). It was observed to be the least in blast crisis as compared to chronic and accelerated phase patients (p=0.662). Chi-square test of association also showed no significant association (χ^2 = 1.705, p=0.426) between phases of disease and expression. The *ASXL1* gene expression in patients receiving first-line treatment (Imatinib) and second-line treatment (Nilotinib) was lower as compared to healthy controls (p=0.412). It decreased over time in CML patients (p=0.42). There was no significant association (χ^2 = 0.126, p=0.722) observed between expression of the *ASXL1* gene and treatment lines (1st and 2nd) as per the Chi-square test (Table 4).

Table 4: Comparative analysis of *ASXL1* Gene Expression Between Control and CML Groups in Relation to Clinical and Treatment Characteristics

Comparisons	Groups	Gene Expression (Mean ± SEM)	p-value
Wild Type vs. Mutant	Wild type	1.33 ± 0.43	0.544
	Mutant	1.74 ± 1.51	
Control vs. CML Patient	Control	1.98 ± 0.488	0.433
	CML Patient	1.37 ± 0.412	
Control vs. Phases of CML	Control	1.982 ± 0.488	0.662
	Chronic	1.588 ± 0.519	
	Accelerated	0.669 ± 0.115	
	Blast	0.331 ± 0.0141	
Control vs. Treatment Duration of CML	Control	1.98 ± 0.488	0.422
	1-5 Years of CML Patients	1.883 ± 0.63	
	5-10 Years CML Patients	0.891±0.58	
	10-15 Years CML Patients	0.192 ± 0.35	
Control vs. Treatment Group of CML	1st Line Treated	1.89 ± 0.75	0.412
	2nd Line Treated	1.97 ± 0.44	

(A): Comparison of *ASXL1* gene mRNA expression between Healthy Controls and CML patients. (B): Comparison of *ASXL1* gene mRNA expression between Healthy Controls and Triphase (Chronic, Accelerated & Blast Chronic) CML patients. (C)Comparison of *ASXL1* gene mRNA expression between Healthy Controls and Treatment Duration Groups of CML patients. (D) Comparison of *ASXL1* gene mRNA expression between Healthy Controls and Treatment Type of CML patients (Figure 2).

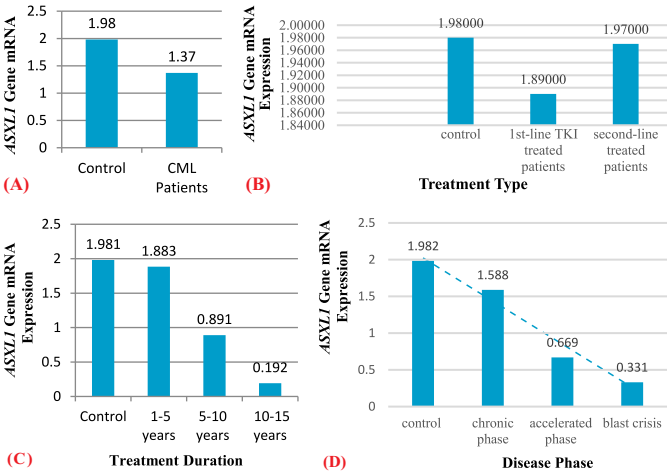

Figure 2: Graphical Illustration showing Comparison of *ASXL1* Gene Expression

The Kaplan-Meier survival curve indicated that the survival rates of CML patients receiving first-line and second-line treatments decline over time (Figure 2). Patients receiving second-line treatment demonstrated a slightly higher survival probability compared to those receiving first-line treatment (p = 0.798) (Figure 3).

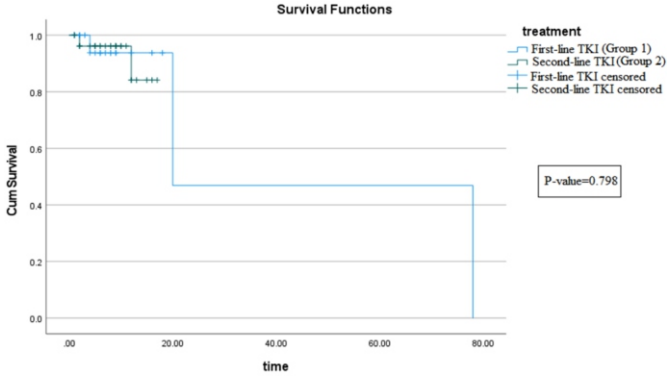

Figure 3: Depiction of Survival Analysis of CML Patients Based on Treatment

SIFT, a bioinformatics tool, showed that both mutations have a damaging effect on protein structure. Mutation G659S is predicted to be deleterious, while the other mutation D667E is predicted to be less damaging according to Mutation Taster, Align GVGD, and polyphen-2. MUpro and I-Mutant showed that the structural stability of mutated proteins was reduced. SOPMA predicted secondary structure properties like alpha-helix, Extranded strand, beta turn, and random coil of the *ASXL1* gene (Figure 3d). NetSurfP 2.0 showed that both the G659S and D667E mutant proteins represented the coil structure. Both mutated protein's structure was in the exposed region (a).

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Using Swiss-Model, the 3D structures of wild-type and mutant *ASXL1* proteins were predicted and downloaded in PDB format. These structures were then visualized and compared using UCSF Chimera, enabling a detailed analysis of conformational changes and structural deviations between the wild-type and mutant proteins (b). STRING database evaluated that *ASXL1* is functionally associated with several other proteins like TET2, EZH2, BAP1 9 (c). Any modification in the *ASXL1* protein directly affects the function of these proteins. RNA Fold Web Server presented the secondary structure of mRNA of both mutations, showing abnormal RNA folding, which altered mRNA localization and inhibited protein translation. (a) NetSurfP 2.0 predicted both mutant proteins to adopt coil structures in exposed regions. (b) UCSF Chimera visualized structural alterations showing side-chain orientation and hydrogen bond differences between wild-type and mutant proteins. (c) STRING analysis revealed functional associations of the *ASXL1* gene with interacting proteins, including TET2, EZH2, and BAP1. (d) SOPMA predicted secondary structure properties of the *ASXL1* gene (e) RNA fold Web Server (Vienna Package) predicted altered mRNA secondary structures, indicating abnormal folding and potential translational interference (Figure 4).

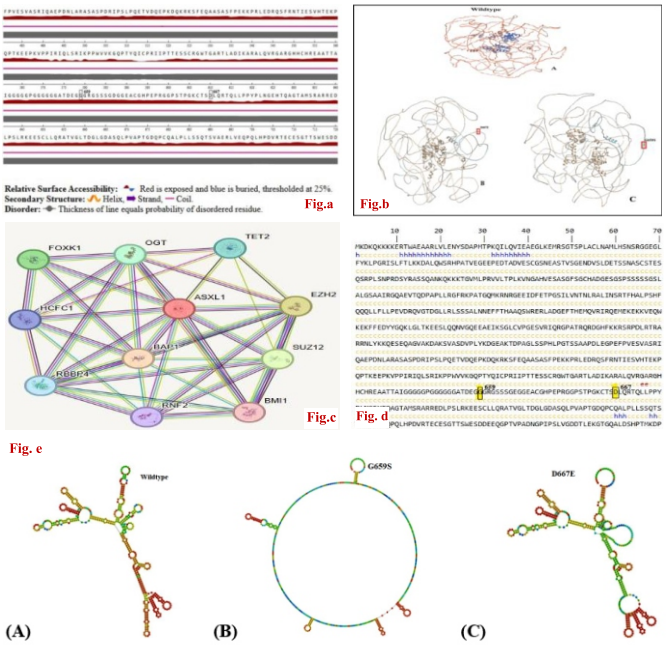

Figure 4: In-silico Analysis of *ASXL1* Gene Mutations (G659S and D667E)

DISCUSSION

Chronic Myeloid Leukemia (CML) is a myeloproliferative neoplasm that is predominantly associated with the oncogenic fusion of BCR: ABL1 [15]. The majority (80 percent) of the patients were diagnosed with chronic phase, fewer with accelerated (12%), and blast stage, which is in line with a previous study [16]. Two new exonic missense mutations, like G659S and D667E, were observed in patients of CML on Nilotinib therapy (200 mg/day). In one patient, with the D667E variant, the possible prognostic importance of *ASXL1* gene mutations in the emergence of aggressive disease phenotypes and TKI resistance. These findings were correlated with the study of Kim *et al.* [17]. Missense single-nucleotide variations have functional significance because they interfere with epigenetic control through amino acid changes. These variants were uploaded to ClinVar with accession number SCV006278281.1. The present study was in line with the findings of Gao *et al.* 2022 and Bamusa *et al.* [9, 18]. Tools (MutationTaster, Align GVGD, SIFT, and polyphen-2) predicted that variation G659S is more harmful than variation D667E. Mutant *ASXL1* gene increases vulnerability to myeloid transformation and plays important roles in leukemogenesis; these results correlated with the reported study [9, 19]. In the present study, MUpro and I-Mutant's results showed that practically every alteration was shown to reduce the protein's structural stability, which correlated with the study of Burgess *et al.* [20]. The evolutionary conservation (predicted by NetSurf), revealed that both of the variants were present in the exposed region and had a good score. It increases the possibility of carcinogenesis [21]. In this investigation, all *ASXL1* gene mutations resulted in aberrant RNA folding, which inhibited RNA localization and affected protein translation, as demonstrated by the Vianna package. This is in accordance with the study of Keita *et al.* [22]. STRING database demonstrated an association between the *ASXL1* and other epigenetic regulator genes like TET2, BAP1, and EZH2. It is reported that myeloid transformation and hematological abnormalities result from BAP1 loss or mutation [23]. In this study, the relative gene expression of the *ASXL1* gene between CML patients and healthy controls was compared. It was not significantly decreased in CML patients compared to healthy controls (p=0.433). It was not significantly down-regulated (p=0.662) with the advancement of disease phases in CML. It was also not significantly down-regulated (p=0.42) in CML patients. The *ASXL1* gene acts as a chromatin regulator and plays a role in controlling other genes and complexes, such as the Polycomb Repressive Complex (PRC). This complex is directly linked to H3K27me3, which involves the trimethylation of lysine 27 on the H3 protein. It leads to suppression of nearby genes and cellular activity. Current findings are correlated with a study conducted in the USA on CMML [24]. The Kaplan- Meier plots indicated that the second-line treated group experienced more reduced survival rates compared to the first-line treated group, which was not significantly different. This is in line with the study of Specchia *et al.* [25]. However, these survival

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findings should be interpreted cautiously due to the lack of statistical significance.

Furthermore, this study was cross sectional due to limited resources, also sample size was small. A longitudinal follow up study on large sample size is required to assess long-term impact of these findings in patients. Future studies with larger, multi-center cohorts and longitudinal follow-up are needed to validate the clinical relevance of *ASXL1* mutations in CML progression and TKI resistance. Functional in vitro and in vivo experiments should be conducted to elucidate the mechanistic impact of the identified variants (G659S and D667E) on leukemogenesis and epigenetic regulation. Additionally, integrating multi-omics approaches (e.g., methylation profiling, chromatin immunoprecipitation) could clarify how *ASXL1* dysregulation cooperates with BCR-ABL1 in disease evolution. Such efforts may uncover novel biomarkers for risk stratification and inform targeted therapeutic strategies in CML.

CONCLUSIONS

It is concluded that two novel missense mutations (G659S and D667E) were identified in the hotspot region (exon 13) of the *ASXL1* gene in CML patients, and *ASXL1* gene expression was found to be down-regulated in CML. However, the down-regulation in *ASXL1* expression levels across disease phases, duration, and TKI therapy was not statistically significant.

Authors Contribution

Conceptualization: RTM, AZ
Methodology: AMA, AZ
Formal analysis: KSK
Writing and Drafting: RTM, KSK, AMA, SH, AZ
Review and Editing: RTM, KSK, AMA, SH, AZ

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

The authors declare no conflict of interest.

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