# Molecular profiling of DNMT3A and ASXL1 in chronic myeloid leukemia and their association with response to treatment with tyrosine kinase inhibitors

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**Abstract.** The BCR-ABL1 fusion gene is the main driver of chronic myeloid leukemia (CML); yet, heterogeneity in treatment responses suggests there are other types of molecular events that contribute to disease progression. The present study investigated the expression of the DNA methyltransferase 3 alpha (DNMT3A) and additional sex combs-like 1 (ASXL1) genes and screened specific regions of both genes by targeted sequencing and assessing whether polymorphisms located within these genes are associated with disease susceptibility or resistance against tyrosine kinase inhibitors (TKIs). A total of 140 patients with CML (TKI-naïve, n=20; responders, n=60; and non-responders, n=60) and 20 age-sex matched healthy controls were included in the analysis. Gene expression analysis revealed the marked downregulation of DNMT3A expression (P=0.004) and the strong overexpression of ASXL1, even higher in non-responders (P=0.015). In addition, five DNMT3A single nucleotide polymorphisms were sequenced and four (rs2149275435, rs2149275458, rs25240928 and rs25240958) presented a significant association with disease susceptibility and response to therapy, whilst this did not occur with rs734693. The Hardy-Weinberg disequilibrium was observed for rs734693, rs2149275458, rs2149275435 (all $P<0.0001$) and rs25240928 (P=0.003), whereas rs25240958 was monomorphic in the controls and linkage disequilibrium analysis revealed a strong association between rs2149275435 and rs2149275458 (D'=1.0, $r^2=0.704$). An AAT haplotype analysis demonstrated a significantly elevated frequency of the AAT haplotype in patients (odds ratio, 9.81; $P<0.01$). There were no pathological mutations detected within ASXL1 exon 12, comparable transcriptional over expression was found, particularly in resistant groups. Taken together, these results support the downregulation of DNMT3A and overexpression of ASXL1 in addition to certain DNMT3A haplotypes as molecular markers involved in CML development and resistance against TKIs. The integration of such biomarkers in prognostic models may improve risk stratification and individualized treatment decisions.

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## Introduction

Chronic myeloid leukemia (CML) is a clonal form of chronic myeloproliferative neoplasm that is associated with the presence of the BCR-ABL1 fusion gene in the Philadelphia chromosome (1). The introduction of tyrosine kinase inhibitors (TKIs) has markedly improved patient survival; however, resistance remains a major clinical concern. Although ABL1 kinase domain mutations are the most well-studied resistance mechanism, it is important to note that 40% of cases may occur through BCR-ABL1-independent means (2,3), often with chromosomal instability or mutation in other commonly altered myeloid genes (4,5). Apart from the genetic mutations, accumulating evidence indicates to epigenetic deregulation as a key player in the pathogenesis of leukemia and response to treatment (5,6). Epigenetic modifications (such as DNA methylation and chromatin remodeling) are capable of impacting on transcriptional programs independent of the underlying sequence without altering the latter, enabling disease progression and resistance to targeted therapies (7,8).

The *de novo* DNA methyltransferase encoded by DNA methyltransferase 3 alpha (DNMT3A) is required for proper differentiation of hematopoietic stem cells. Short variants, present in 20-30% of acute myeloid leukemia (AML) cases can be classed as truncating and impact DNA methylation observed after a differentiation block and clonal outgrowth inducer (9-11). However, limited information is available on the expression of the DNMT3A gene in CML and its association with treatment outcomes. Another key regulator, sex combs-like 1 (ASXL1), is a chromatin-binding factor, which along with polycomb repressive complexes, can directly affect the transcriptional programs that are essential for stem cell function (12). ASXL1 mutations occur in 15-20% of myeloid disorders and are uniformly associated with a poor prognosis (12). In CML, mutations in ASXL1

have been found to be associated with rapid transformation and an inferior response to TKIs (13,14) Nevertheless, the precise roles of DNMT3A and ASXL1 in CML have not yet been fully elucidated, particularly in Middle Eastern countries with limited molecular data.

Regional analyses have emphasized the critical requirement for population-based investigations; for example, the study by Sabir *et al* (8) emphasized the importance of epigenetic regulation in patients with CML who are treated with TKIs. Based on this background, the primary objective of the present study was to explore whether DNMT3A and ASXL1 expression, in addition to DNMT3A polymorphisms are associated with CML in Iraqi patients and whether they may be indicators for susceptibility and response towards therapy.

## Patients and methods

*Patients and study design*. A cross-sectional analytical study was employed, adhering to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for cross-sectional studies. The study was conducted between January, 2024 and July, 2025. The study included 140 patients with confirmed CML and 20 age- and sex-matched healthy controls (with no history of hematological or other chronic diseases), for a total of 160 participants. All subjects were ≥18 years of age.

Patients were categorized according to their molecular response to therapy with TKIs, following the European Leukemia Net (ELN) 2020 guidelines (15). The study population was stratified into three groups as follows: i) 20 TKI-naïve patients (newly diagnosed, treatment-initiation group); ii) 60 responders; and iii) 60 non-responders/resistant cases. The molecular response was assessed by measuring BCR-ABL1 transcript levels (expressed as BCR-ABL1 on the international scale, BCR-ABL1^IS) at three standardized time points: 3 months (≤10%), 6 months (≤1%) and 12 months (≤0.1%), in accordance with ELN 2020 criteria. Responders were defined as patients who achieved ELN-recommended molecular milestones, whereas non-responders were defined as those with persistent failure to achieve ELN-defined response criteria across follow-up time points, rather than a single time-point deviation. To minimize potential confounding factors, patients with documented non-adherence to TKI therapy were excluded. In addition, patients who required treatment switching due to intolerance or adverse effects were also excluded from the study.

*TKI therapy*. All patients received TKI therapy as part of their standard clinical management for CML. The cohort comprised patients receiving the following TKI regimens: i) Imatinib mesylate (Gleevec®): A total of 60 patients (43%) received imatinib at a standard dose of 400 mg daily as first-line therapy. This group included 20 newly diagnosed patients, 20 responder patients and 20 non-responder patients. ii) Nilotinib (Tasigna®): A total of 40 patients (29%) received nilotinib at a standard dose of 300 mg twice daily. This group included 20 responder patients and 20 non-responder patients. iii) Bosutinib (Bosulif®): A total of 40 patients (29%) received bosutinib at a standard dose of 500 mg once daily as second-line therapy following imatinib failure or intolerance. This group comprised 20 responder patients and 20 non-responder patients.

The distribution of TKI therapy across the study groups was as follows: The TKI-naïve group (n=20) received no prior TKI therapy; the responder group (n=60) comprised equal proportions on imatinib (n=20; 33%), nilotinib (n=20; 33%) and bosutinib (n=20; 33%); the non-responder group (n=60) similarly received imatinib (n=20; 33%), nilotinib (n=20; 33%) and bosutinib (n=20; 33%).

*Treatment duration and follow-up period*. The duration of TKI treatment prior to enrollment varied between the study groups. Patients who were responders had received TKI therapy for a sufficient duration to achieve and maintain optimal molecular response (BCR-ABL1IS ≤0.1%) by the 12-month assessment milestone as defined by the ELN 2020 criteria (15). Patients who were non-responders had received prolonged TKI therapy for a minimum of 12 months and failed to achieve ELN-defined molecular milestones, with persistent BCR-ABL1IS levels >1% despite adequate adherence and dose optimization. Molecular response assessment followed the ELN 2020 guidelines, with BCR-ABL1 transcript levels monitored at 3, 6, and 12 months from the initiation of TKI therapy (15). Additional clinical follow-up and molecular monitoring were conducted as clinically indicated beyond the 12-month assessment point, with quarterly BCR-ABL1 transcript monitoring performed in accordance with ELN recommendations.

*Inclusion and exclusion criteria*. Inclusion criteria comprised adult patients (aged ≥18 years) with a confirmed diagnosis of CML based on clinical, hematological, cytogenetic and molecular analyses, including the detection of the BCR-ABL1 fusion gene, and who were in chronic phase at the time of enrollment. The healthy controls were age- and sex-matched individuals with no history of hematological malignancies or other chronic diseases, with normal complete blood count (CBC) and differential counts.

Exclusion criteria included patients with concurrent hematological malignancies other than CML, those with prior chemotherapy or stem cell transplantation before enrollment, patients with documented non-adherence to prescribed TKI therapy (defined as <80% adherence over the treatment period), patients who required treatment switching due to intolerance or severe adverse effects during the active study observation period, and individuals who declined to provide informed consent.

*CML diagnosis and baseline characterization*. A confirmed diagnosis of CML was established through integrated clinical, hematological, cytogenetic and molecular analyses. All patients underwent CBC, bone marrow aspiration and biopsy, cytogenetic analysis and quantitative polymerase chain reaction (qPCR) for BCR-ABL1 transcript measurement. All patients were confirmed to be in the chronic phase at diagnosis, with no evidence of accelerated phase or blast crisis. The study sample comprised all eligible patients who met the inclusion criteria and were available at the study center during the recruitment period, consistent with the exploratory nature of the present investigation. Healthy controls were age- and sex-matched individuals with no history of hematological malignancies or other chronic diseases, with normal CBC and differential counts.

*Ethical considerations*. The research protocol was approved by the Ethics Committee of the College of Science, Al-Mustansiriyah University, on December 30, 2023 (Ref. no. BCSMU/291/100477/2). The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki and the guidelines of the approving committee. Institutional Review Board (IRB) approval was obtained prior to participant enrollment. Written informed consent was obtained from all participants prior to enrollment. Participants were informed of the study objectives, procedures, potential risks, and their right to withdraw at any time without consequence. Confidentiality of all personal and clinical data was ensured throughout the study.

*Type of sampling and reasons for selection*. A consecutive sampling strategy was employed, whereby all eligible patients attending the National Center for Research and Treatment of Hematology, Mustansiriyah University, and the Center for Hematology and Bone Marrow Transplantation, Baghdad Teaching Hospital, Medical City, Baghdad, Iraq, during the defined recruitment period who satisfied the inclusion criteria were systematically enrolled. This approach ensured that participant selection was determined solely by eligibility and availability rather than by subjective judgment, thereby minimizing selection bias. The three clinical subgroups (TKI-naïve, responders and non-responders) were defined exclusively on the basis of objectively assessed molecular response criteria in accordance with ELN 2020 guidelines, rather than by investigator discretion (15).

*Blood sample collection*. Peripheral blood was obtained from each participant under aseptic conditions. For gene expression analysis, whole blood was mixed with TransZol Up reagent (TransGen Biotech) and processed to extract total RNA, using the manufacturer's protocol, and then subjected to reverse transcription-quantitative PCR (RT-qPCR) analysis of the DNMT3A and ASXL1 genes. Samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes, stored at -20˚C, and used for DNA extraction, PCR amplification and Sanger sequencing of the DNMT3A target exons.

*Study primers*. Primers were designed using Primer 3Plus (version 4) and verified through the UCSC and NCBI databases. Primers were synthesized and lyophilized by Alpha DNA Ltd. The specific primer sequences used for gene expression and sequencing analysis were designed to yield distinct product sizes. For gene expression analysis, the ASXL1 primers (forward, 5'-CGGCTTGAAGATCGTCAGTCCT-3'; reverse, 5'-GGCTGACCTTTAACCACCCAGG-3') generated a 146-bp product, whereas the DNMT3A primers (forward, 5'-TATTGA TGAGCGCACAAGAGAGC-3'; reverse, 5'-GGGTGTTCC AGGGTAACATTGAG-3') produced a 111-bp fragment. The reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was amplified using specific primers (forward, 5'-ACAACTTTGGTATCGTGGAAGG-3'; reverse, 5'-GCC ATCACGCCACAGTTTC-3'), resulting in a 101-bp product. For gene sequencing, the ASXL1 primers (forward, 5'-AGG TCAGATCACCCAGTCAGTT-3'; reverse, 5'-TAGCCCATC TGTGAGTCCAACTGT-3') yielded a 561-bp product, and the DNMT3A primers (forward, 5'-TCCATATCTGGGAGG CTCAG-3'; reverse, 5'-CAGGAGGCGGTAGAACTCAA-3') produced a 738-bp fragment.

*Gene expression analysis*. Total RNA was isolated from peripheral blood using the TransZol Up Plus RNA kit (TransGen Biotech) according to the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). Complementary DNA (cDNA) was synthesized by adding 5 $\mu$l EasyScript Reverse Transcriptase (TransGen Biotech) to the extracted RNA. qPCR was performed using a Rotor-Gene Q Real-Time PCR system (Qiagen, Hilden, Germany) with SYBR Green Master Mix (Qiagen GmbH). The cycling conditions included an initial denaturation at 94˚C for 30 sec, followed by 40 cycles of denaturation at 94˚C for 5 sec, annealing at 60˚C for DNMT3A and 58˚C for ASXL1 for 15 sec, and extension at 72˚C for 20 sec. Gene expression levels were normalized using GAPDH as the reference gene, based on its previously reported stability across different tissues and experimental conditions. The $2^{-\Delta\Delta Cq}$ method was used to analyze relative gene expression levels, and the $2^{-\Delta\Delta Cq}$ method was used to calculate fold changes between groups. The healthy control group was used as the calibrator (16).

*Sequencing and single nucleotide polymorphisms (SNP) genotyping*. PCR amplification was performed using primers specifically designed to amplify the clinically relevant hotspot regions of DNMT3A exon 23 and ASXL1 exon 12. The rationale for targeting exon 23 of DNMT3A stems from cumulative evidence demonstrating that this exon harbors the most recurrently mutated codon in myeloid malignancies arginine 882 (R882), which alone accounts for >60% of all reported DNMT3A mutations (17). For ASXL1, exon 12 represents the predominant site of pathogenic mutation across the spectrum of myeloid disorders, most notably the frameshift variant G646WfsX12, which has been identified as the canonical hotspot mutation in this gene (18,19). While comprehensive full-gene sequencing would have been ideal, resource limitations restricted the analysis of these well-characterized hotspot regions.

The PCR amplification protocol comprised of an initial denaturation at 94˚C for 5 min, 35 cycles including a denaturation step at 94˚C for 30 sec, annealing at 58˚C for 30 sec, and extension at 72˚C for 50 sec, with a final elongation step at 72˚C for 5 min. The product were confirmed by electrophoresis on a 2% agarose gel prepared in 1X TBE buffer and stained with ethidium bromide. A 100-bp DNA ladder was used as a molecular size marker, and the electrophoresis was performed at 90 V for 60 min. The bands were visualized under UV illumination using a gel documentation system, and the expected amplicon size was confirmed by comparison with the DNA ladder. Representative gel images for DNMT3A and ASXL1 PCR amplification are presented in Fig. 1. No densitometric quantification was performed, as agarose gel electrophoresis was used only for qualitative confirmation of PCR amplification prior to sequencing. Sequencing analysis of the purified PCR products was performed using an ABI 3730XL Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.) at Macrogen Inc. The sequences were analyzed using Chromas v2. 6 (Technelysium Pty Ltd.) and mapped to the human reference genome (GRCh38). Gene-specific reference sequences were retrieved from NCBI GenBank (ASXL1: NG_027868.1;

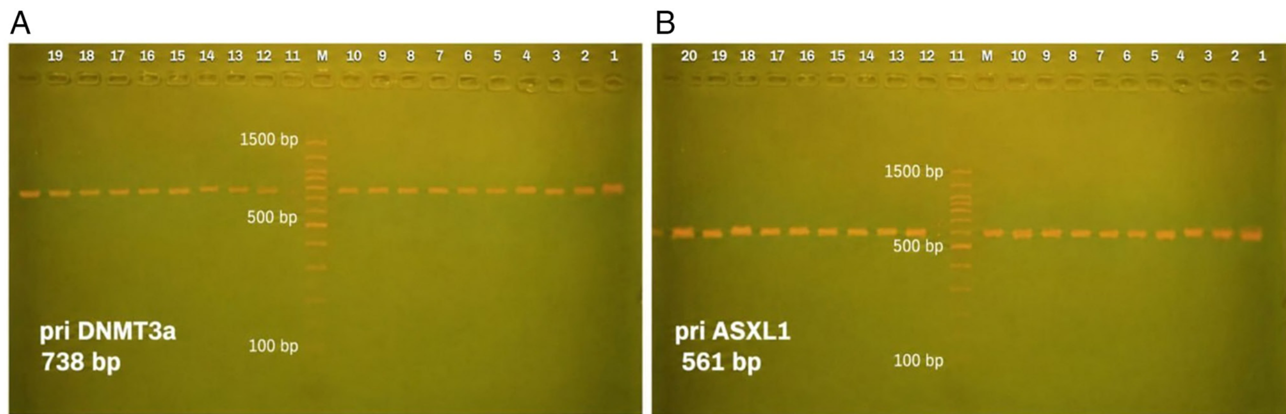


Figure 1. Agarose gel electrophoresis of PCR products. (A) DNMT3A amplicon (738 bp) and (B) ASXL1 amplicon (561 bp) resolved on a 2% agarose gel in 1X TBE buffer, stained with ethidium bromide, and visualized under UV illumination. Lane M, 100-bp DNA ladder; lanes 1-5, representative healthy controls; lanes 6-10: representative patients newly diagnosed with chronic myeloid leukemia; lanes 11-15, representative treatment-responders; lanes 16-20, representative non-responders.

DNMT3A: NG_029465.2). Variants were also confirmed using NCBI BLAST and annotated by both the dbSNP and ClinVar databases.

*Accuracy, reproducibility and quality control*. Several measures were implemented to ensure data accuracy and reproducibility. RNA and DNA concentrations and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.) prior to downstream applications. PCR amplification products were confirmed by agarose gel electrophoresis before sequencing. Sanger sequencing was performed by an accredited external facility (Macrogen Inc.) using an ABI 3730XL Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Representative Sanger sequencing chromatograms were reviewed to confirm sequencing quality. A representative chromatogram demonstrating clear peak resolution and reliable base calling is presented in Fig. 2. Sequence analysis was conducted using Chromas v2.6 (Technelysium Pty Ltd.) and variants were cross-validated against the human reference genome (GRCh38) using NCBI BLAST and annotated through both the dbSNP and ClinVar databases. Gene expression analyses were performed in accordance with standardized RT-qPCR protocols, and the reference gene, GAPDH, was used as an internal control to normalize expression data.

*Statistical analysis*. Statistical analyses were performed using IBM SPSS Statistics version 29 (IBM Corp.). Quantitative data for gene expression are expressed as the mean ± standard deviation (SD) and compared using one-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test for pairwise comparisons where appropriate. Genotype and allele frequencies of DNMT3A polymorphisms were calculated by direct counting and analyzed using Pearson's Chi-squared test or Fisher's exact test, reporting odds ratios (ORs) and 95% confidence intervals (CIs). Haplotype construction and linkage disequilibrium (LD) were assessed using the SHEsisPlus online platform (20), and haplotype distributions between patients and controls were compared using the Chi-squared test with ORs and 95% CIs. A two-tailed P-value of <0.05 was considered to indicate a statistically significant difference.

## Results

The demographic and clinical characteristics of the study participants are summarized in Table I. There were no significant differences in age distribution among the study groups (P=0.292). However, a significant difference in sex distribution was observed (P=0.039).

qPCR amplification and the melt curves of GAPDH, DNMT3A and ASXL1 confirmed efficient and specific amplification (Figs. 3-5).

DNMT3A expression was significantly downregulated in all CML subgroups compared with the healthy controls (P=0.004), with the greatest reduction observed in the newly diagnosed and responder patients (Table II and Fig. 6A). By contrast, ASXL1 expression was significantly upregulated, exhibiting a progressive increase across disease stages and peaking in non-responders (P=0.01) (Table III and Fig. 6B).

DNMT3A and ASXL1 genotyping was performed by PCR amplification and Sanger sequencing. A representative Sanger sequencing chromatogram confirming the sequencing quality is presented in Fig. 2. The investigation of DNMT3A polymorphisms revealed significant variations in genotype and allele frequencies between the patients with CML and the controls. In total, four SNPs (rs2149275435, rs2149275458, rs25240928 and rs25240958) exhibited a strong association with CML susceptibility, whereas no significant association was found for rs734693. Homozygous mutant genotypes (AA) at rs2149275435 and rs2149275458 were identified exclusively in patients and were associated with markedly elevated disease risk (OR, >20; P<0.01). The CC genotype of rs25240928 and the AC genotype of rs25240958 were similarly enriched in cases, suggesting potential pathogenic relevance (Table IV).

In addition, significant deviations from the Hardy-Weinberg Equilibrium (HWE) were observed for rs734693, rs2149275458, rs2149275435 and rs25240928 in the control group, whereas rs25240958 was monomorphic (Table V).

Linkage disequilibrium analysis revealed a strong non-random association between rs2149275435 and rs2149275458 (D'=1.0, $r^2$=0.758), supporting the presence of

Table I. Demographic characteristics of the study population.

| | Groups | Mean | Std. Deviation | Std. Error | P-value |
|---|---|---|---|---|---|
| Age, years | | | | | |
| | Control (n=20) | 42.66 | 13.17 | 5.37 | 0.292 (NS) |
| | Newly (n=20) | 45.11 | 11.93 | 2.89 | |
| | Response to therapy (n=60) | 48.72 | 12.72 | 1.73 | |
| | Non-response to therapy (n=60) | 50.57 | 10.41 | 1.812 | |
| | Sex, n (%) | Male | Female | Chi-squared test value | P-value |
| | Control (n=20) | 14 (70%) | 6 (30%) | 8.350 | 0.039[a] |
| | Newly (n=20) | 9 (45%) | 11 (55%) | | |
| | Response to therapy (n=60) | 26 (43.33%) | 34 (56.67%) | | |
| | Non-response to therapy (n=60) | 39 (65%) | 21 (35%) | | |

Data are presented as the mean ± SD. One-way ANOVA was used for continuous variables and the Chi-squared test for categorical variables. [a]Indicates a statistically significant difference (P<0.05). NS, not significant.

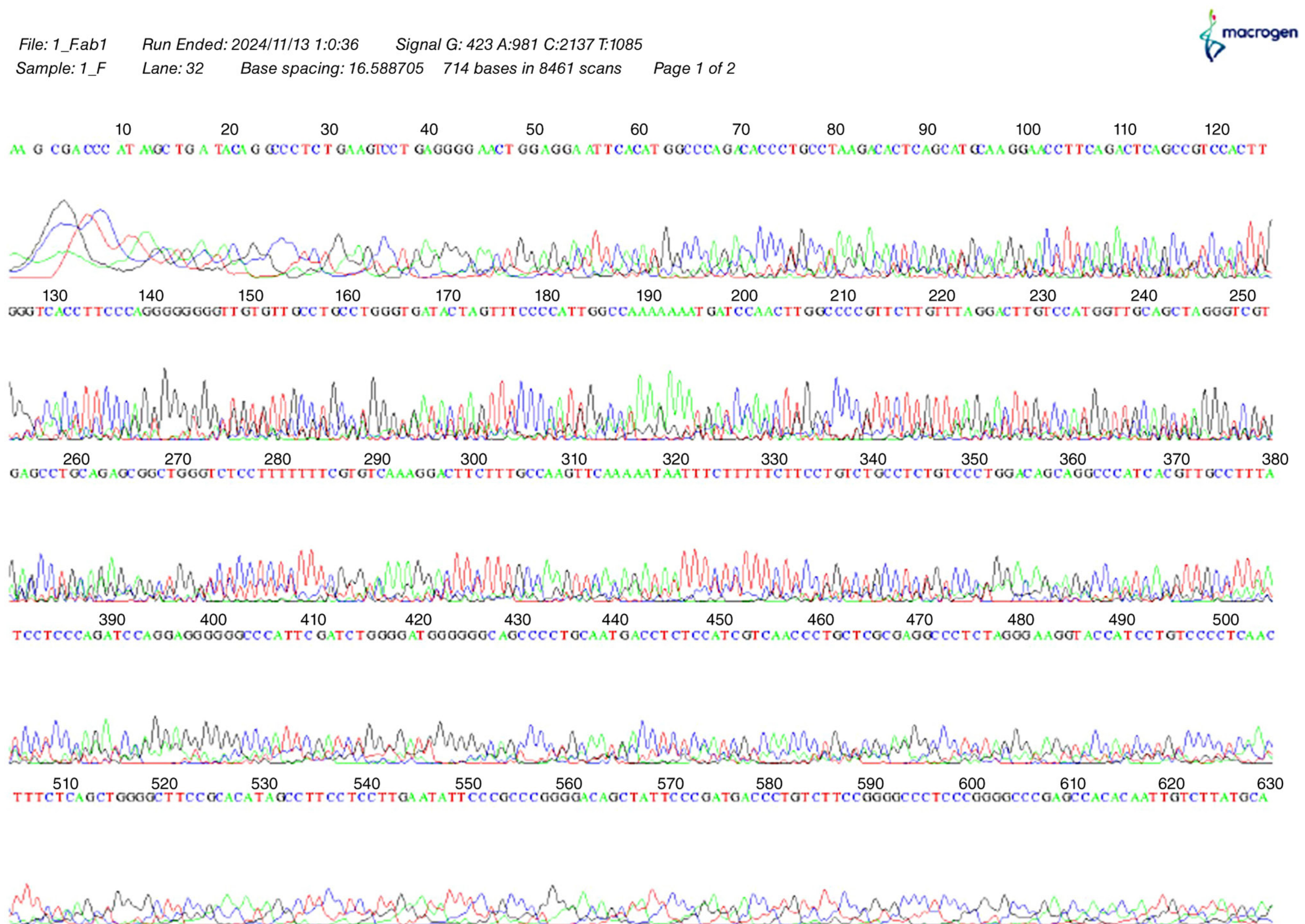


Figure 2. Representative Sanger sequencing chromatogram of the amplified DNMT3A region. The chromatogram illustrates clear peak resolution and reliable base calling, confirming the quality of the Sanger sequencing analysis.

a haplotype block. Haplotype analysis revealed significant associations of specific DNMT3A haplotypes with CML susceptibility and treatment response. The C A A A C haplotype was overrepresented in newly diagnosed patients and non-responders, while the T A A A C haplotype was more frequent in responder and non-responder groups compared with controls. In addition, the T G A A C haplotype was strongly associated with newly diagnosed CML patients.

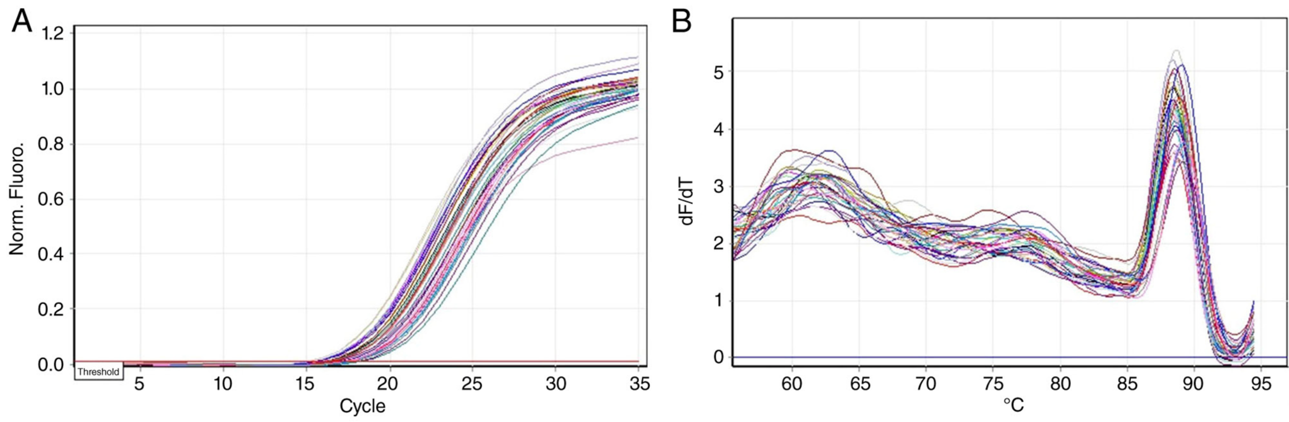


Figure 3. Amplification and dissociation (melt) curves of GAPDH across all study groups. (A) The amplification plot shows uniform sigmoidal kinetics, confirming stable efficiency, while (B) the melt curve demonstrates a single sharp peak, verifying specificity and absence of nonspecific amplification.

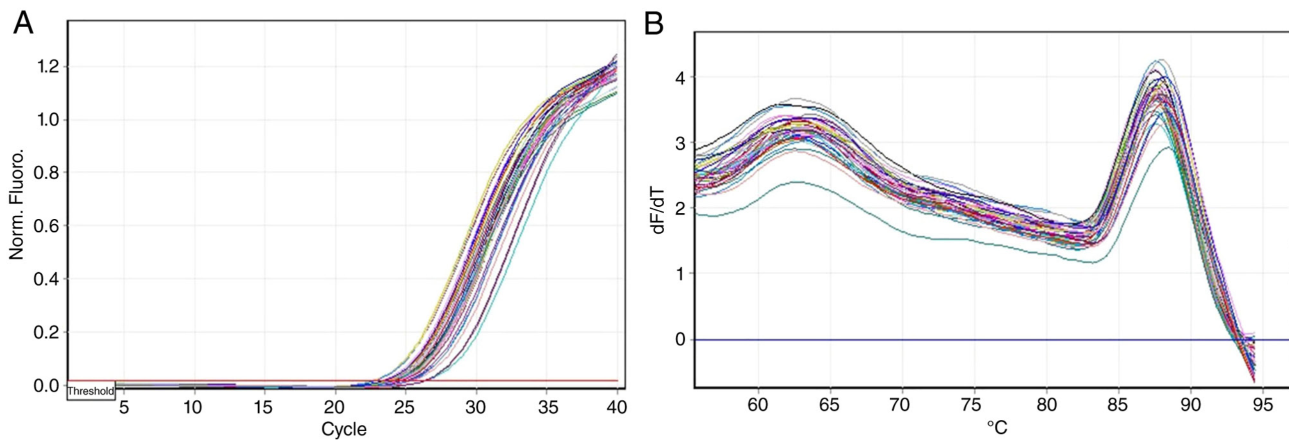


Figure 4. Amplification and dissociation (melt) curves of DNMT3A. (A) Amplification curves display consistent exponential growth across samples, while (B) the melt curve shows a single defined peak, confirming specific amplification and excluding primer-dimer formation.

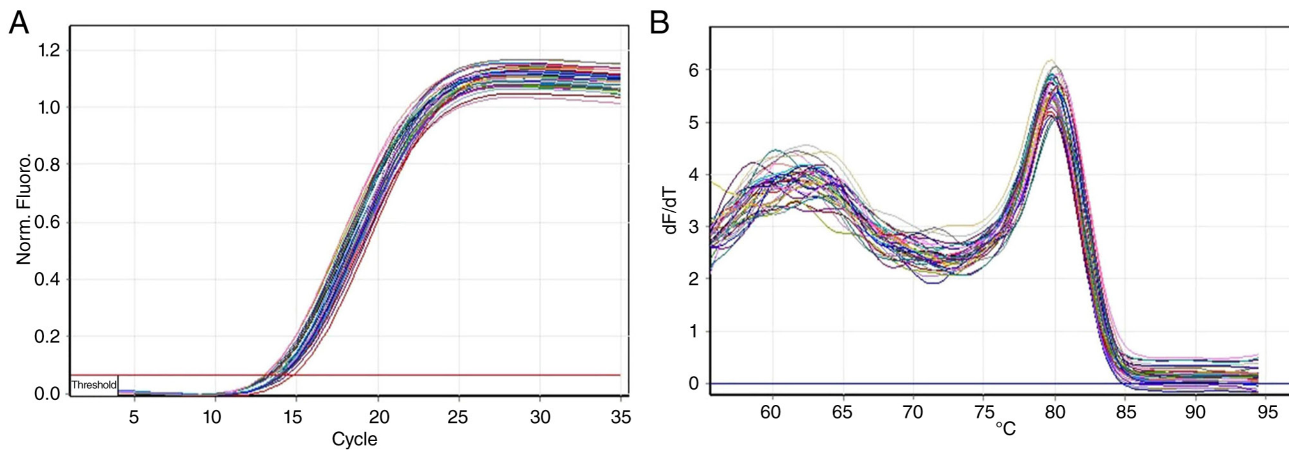


Figure 5. Amplification and dissociation (melt) curves of ASXL1. (A) The amplification plots exhibit clear sigmoidal profiles among patient and control groups, while (B) the melt curve reveals a distinct single peak, validating product specificity and ruling out nonspecific products.

These haplotype distributions and their corresponding OR and P-values are presented in Table VI).

Furthermore, there is a significant increase in values of the AAT and CAAAC haplotype frequencies among the patient groups compared to the controls (Table VII).

## Discussion

In the present study, there was no difference in the GAPDH mRNA levels between each of the groups investigated which confirms its adequacy as an internal reference gene for CML

Table II. DNMT3A gene expression of patient and control groups.

| DNMT3A fold expression | Mean | Std. Deviation | Std. Error | P-value |
|---|---|---|---|---|
| Control | 1.042[b] | 0.29727 | 0.13294 | 0.004[a] |
| Newly | 0.428[c] | 0.29482 | 0.13185 | |
| Response to therapy | 0.435[c] | 0.15537 | 0.04012 | |
| Non-response to therapy | 0.619[c] | 0.40855 | 0.10549 | |

[a]P<0.01, statistically significant difference determined using one-way ANOVA. Different letters (b and c) denote homogeneous subsets; groups sharing the same letter are not significantly different from each other.

Tables III. ASXL1 gene expression of patient and control groups.

| ASXL1 fold expression | Mean | Std. Deviation | Std. Error | P-value |
|---|---|---|---|---|
| Control | 1.0360[b] | 0.29670 | 0.13269 | 0.015[a] |
| Newly | 1.3100[b] | 0.41755 | 0.18674 | |
| Response to therapy | 2.0360[c] | 1.27139 | 0.32827 | |
| Non-response to therapy | 3.4860[d] | 2.40202 | 0.62020 | |

[a]P<0.01, statistically significant difference determined using one-way ANOVA. Different letters (b, c and d) denote homogeneous subsets; groups sharing the same letter are not significantly different from each other.

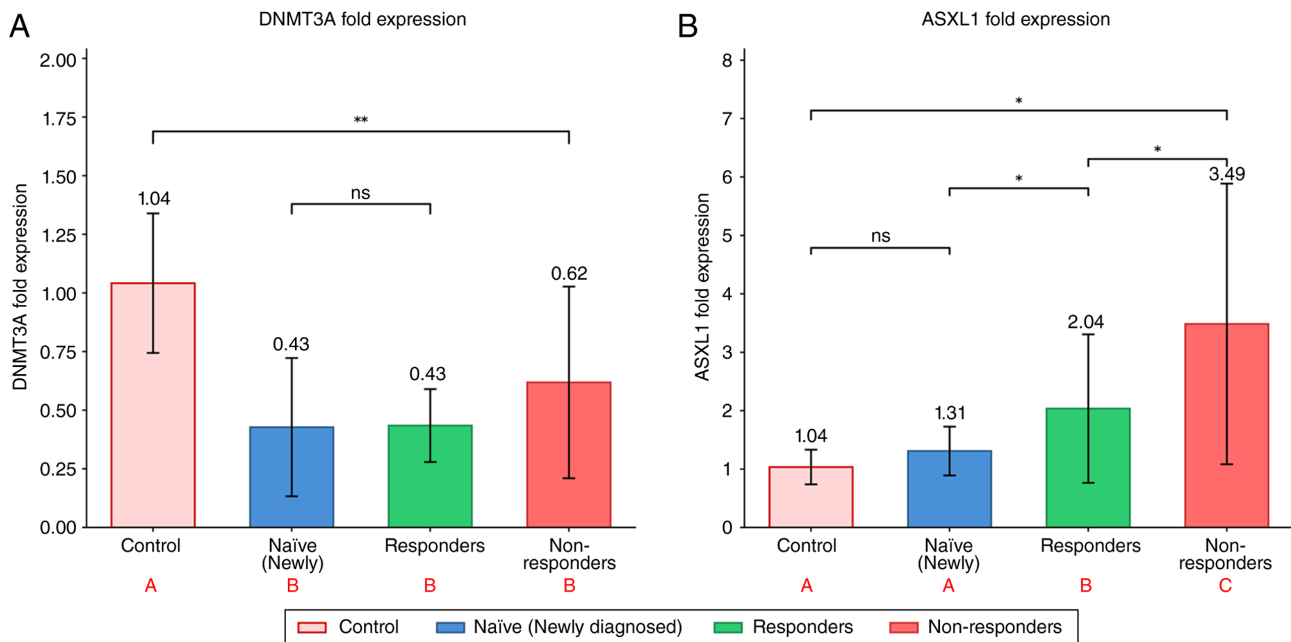


Figure 6. Fold expression of DNMT3A and ASXL1 genes across patient groups and controls. (A) DNMT3A fold expression levels in control subjects, naïve (newly diagnosed) patients, responders to therapy, and non-responders to therapy. All three patient groups exhibited significantly decreased DNMT3A expression compared to the control group (P=0.004, one-way ANOVA). No significant difference was observed among the three patient subgroups). (B) ASXL1 fold expression levels in control subjects, naïve (newly diagnosed) patients, responders to therapy, and non-responders to therapy. ASXL1 expression was progressively upregulated across groups, with non-responders, exhibiting showing the highest expression (3.49±2.40), followed by responders (2.04±1.27), naïve patients (1.31±0.42) and controls (1.04±0.30); overall ANOVA, P=0.015. Data are presented as the mean ± standard deviation. Statistical analysis was performed using one-way ANOVA followed by Tukey's HSD post hoc test. Different letters in red (A, B and C) denote homogeneous subsets; groups sharing the same letter are not significantly different from each other. *P<0.05 and **P<0.01, significant difference; ns, not significant.

expression profiling. This is in line with previous evidence supporting the suitability of using GAPDH as a reference gene in different tissues and pathologies (21). DNMT3A encodes a *de novo* DNA methyltransferase that is required for silencing, hematopoietic differentiation and the maintenance of genomic stability. There is a general downregulation of DNMT3A in

Table IV. Significant genotype and allele associations.

| SNP | Genotype/allele | Odds ratio (OR) | 95% CI | P-value |
|---|---|---|---|---|
| rs2149275435 | AA | 21.67 | 3.14-484.86 | <0.01 |
| rs2149275458 | AA | 41.82 | 5.72-931.15 | <0.01 |
| rs25240928 | CC | 33.85 | 4.74-754.74 | <0.01 |
| rs25240958 | AC | 11.92 | 1.77-268.65 | <0.01 |
| rs734693 | - | 1.57 | 0.35-6.43 | 0.6 (NS) |

P-values were calculated using Fisher's exact test. NS, not significant (P≥0.05). SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Table V. Hardy-Weinberg equilibrium analysis for DNMT3A SNPs in the control group.

| SNP | Observed (Wild/Het/Mut) | Expected (Wild/Het/Mut) | $\chi^2$ | P-value |
|---|---|---|---|---|
| rs734693 | 0/20/0 | 5/10/5 | 20.000 | <0.0001 |
| rs2149275458 | 0/20/0 | 5/10/5 | 20.000 | <0.0001 |
| rs2149275435 | 0/20/0 | 5/10/5 | 20.000 | <0.0001 |
| rs25240958 | 20/0/0 | 20/0/0 | - | Monomorphic |
| rs25240928 | 0/16/4 | 3.2/9.6/7.2 | 8.889 | 0.003 |

Hardy-Weinberg equilibrium was assessed using the Chi-squared test. Monomorphic loci preclude Hardy-Weinberg equilibrium calculation. Wild, homozygous major allele; Het, heterozygous; Mut, homozygous minor allele; SNP, single nucleotide polymorphism.

Table VI. Complete linkage disequilibrium analysis across DNMT3A SNP pairs in CML cohorts and controls.

| SNP pair | Newly vs. control (D', $r^2$) | Response vs. control (D', $r^2$) | Non-response vs. control (D', $r^2$) |
|---|---|---|---|
| rs734693-rs2149275458 | 0.388, 0.058 | 0.259, 0.031 | 0.058, 0.001 |
| rs734693-rs2149275435 | 0.235, 0.010 | 0.281, 0.039 | 0.282, 0.016 |
| rs734693-rs25240958 | 1.000, 0.076 | 1.000, 0.041 | 1.000, 0.059 |
| rs734693-rs25240928 | 0.389, 0.035 | 0.212, 0.019 | 0.348, 0.027 |
| rs2149275458-rs2149275435 | 1.000, 0.474 | 1.000, 0.943 | 1.000, 0.758 |
| rs2149275458-rs25240958 | 0.999, 0.195 | 0.999, 0.088 | 0.998, 0.080 |
| rs2149275458-rs25240928 | 0.564, 0.189 | 0.871, 0.676 | 1.000, 0.682 |
| rs2149275435-rs25240958 | 0.999, 0.075 | 0.999, 0.083 | 0.495, 0.072 |
| rs2149275435-rs25240928 | 1.000, 0.796 | 0.933, 0.732 | 0.815, 0.598 |
| rs25240958-rs25240928 | 0.999, 0.095 | 1.000, 0.099 | 0.458, 0.025 |

D', standardized linkage disequilibrium coefficient; $r^2$, correlation coefficient. Strong LD was considered when D' >0.8 and $r^2$ >0.3. SNP, single nucleotide polymorphism; CML, chronic myeloid leukemia.

numerous hematological malignancies, particularly AML, which is frequently attributed to loss-of-function mutations or epigenetic silencing. Such reduction results in global DNA hypomethylation, abnormal activation of oncogenic signaling, and compromised lineage fidelity (22-24). DNMT3A inhibition is associated with an unfavorable prognosis and impaired stem cell differentiation (25), as well as resistance to apoptosis and self-renewal capability in DNMT3A-null cells (26). These findings underscore the tumor-suppressive contribution of DNMT3A and point to its silencing as a potential mechanism facilitating leukemic escape during treatment with TKIs. In the present study, ASXL1 expression, however, was markedly higher in the CML groups than the controls and also gradually increased between the CML groups; ASXL1 expression exhibited a progressive increase, increasing from 1.31-fold in the newly diagnosed patients to 2.04-fold in the

Table VII. Haplotype analysis across CML patient groups compared with controls.

| Haplotype | Newly vs. control (freq %, OR, P-value) | Response vs. control (freq %, OR, P-value) | Non-response vs. control (freq %, OR, P-value) |
|---|---|---|---|
| C A A A C | 25.0%, OR 7.31, P=0.009 | 5.0%, OR 1.21, P=0.85 | 20.0%, OR 5.48, P=0.033 |
| T G A A C | 45.0%, OR 13.69, P<0.001 | - | - |
| T A A A C | - | 62.5%, OR 32.19, P<0.001 | 47.0%, OR 14.84, P<0.001 |
| T A A C C | 15.0%, not estimable, P=0.011 | 20.0%, not estimable, P=0.002 | 5.0%, not estimable, P=0.15 |
| T A G C T | 10.0%, not estimable, P=0.040 | 7.5%, not estimable, P=0.070 | 8.0%, not estimable, P=0.068 |

Only haplotypes with frequency ≥5% or significant associations are shown. OR was not estimable when the haplotype was absent in either group. CML, chronic myeloid leukemia; OR, odds ratio.

responders (Table III). ASXL1 deregulation and mutations have been widely implicated in the transformation of myeloid malignancies, including myelodysplastic syndromes (MDS), AML and CML (12,27). Recent clinical studies consistently position ASXL1 among the most relevant adverse genetic markers in CML, particularly concerning treatment refractoriness. For instance, Bidikian *et al* (13) identified ASXL1 as the most frequently mutated gene in chronic-phase CML and the sole independent predictor of inferior event-free survival. Similarly, the TIGER trial by Schönfeld *et al* (14) demonstrated that newly diagnosed patients harboring ASXL1 mutations exhibited inferior molecular responses to nilotinib, confirming that its adverse prognostic impact extends beyond imatinib-treated cohorts. These findings align with broader evidence that variants in epigenetic modifier genes predict response failure to TKIs and maintain their prognostic significance even under proactive therapeutic strategies (28). Consistent with this adverse role, the present study observed a progressive increase in ASXL1 expression from newly diagnosed to resistant patients, further underscoring its strong link to treatment failure. However, no pathogenic mutations were identified within the sequenced region of ASXL1 exon 12 in the present study cohort. This absence suggests that the observed upregulation is likely driven by alternative, non-mutational mechanisms, such as transcriptional or epigenetic deregulation. Ultimately, these findings support the emerging consensus that whether through genetic mutation or alternative overexpression mechanisms, ASXL1 contributes to TKI resistance by reprogramming and activating alternative pro-survival signaling pathways (12,14,29).

As regards the genotype distributions, HWE testing in the control group revealed significant deviations for rs734693, rs2149275458 and rs2149275435 (all P<0.0001) and rs25240928 (P=0.003), which may reflect population stratification or small sample size effects. Notably, rs25240958 was monomorphic in the control group, precluding HWE calculation. Therefore, association results for these SNPs should be interpreted with caution and require replication in larger, independently validated cohorts.

Sequencing data of DNMT3A indicated that four variants (rs2149275435, rs2149275458, rs25240928 and rs25240958) were associated with CML risk, whereas no significant association was found for rs734693. Notably, rs2149275435 and rs2149275458 have not been previously reported in the context of CML or myeloid malignancies in any published literature, at least to the best of our knowledge, representing potential population-enriched variants in the Iraqi cohort that merit further functional characterization. The enrichment of homozygous mutant genotypes, such as AA at rs2149275435 and rs2149275458, and CC or AC at rs25240928 and rs25240958, suggests pathogenic effects. Consistent observations in AML and MDS have associated DNMT3A polymorphisms with reduced DNA methylation, clonal hematopoiesis and adverse clinical outcomes (23,25). In the present study, linkage disequilibrium analysis indicated that rs2149275435 and rs2149275458 were in significant non-random association (D'=1.0, $r^2$=0.758), forming a risk haplotype block. Haplotype analyses demonstrated that the CAAAC and TGAAC haplotypes were significantly enriched in newly diagnosed and non-responder patients, suggesting that the additive effects of allele combinations promote leukemia persistence rather than individual alleles acting in isolation (30). Analogous haplotype-level associations have been observed in myeloid malignancies, with DNMT3A haplotypes implicated in the modification of DNA methylation and disease susceptibility (31-33).

Recent literature has further strengthened the clinical importance of somatic mutation profiling beyond canonical kinase-domain analysis. Contemporary reports indicate that ASXL1 abnormalities at diagnosis continue to be associated with inferior outcomes and may even be associated with a higher risk of acquiring ABL1 kinase domain mutations during therapy, suggesting that epigenetic dysregulation may create a permissive background for subsequent evolutionary adaptation (34). In parallel, broader outcome analyses across age groups have confirmed that ASXL1, DNMT3A and TET2 remain among the most recurrently altered epigenetic regulators in adolescent, young adult, and older adult CML populations (35). Taken together, these studies reinforce the interpretation that the expression abnormalities and DNMT3A variants detected in the present study cohort are clinically meaningful and fit within the current understanding of CML as a genetically and epigenetically heterogeneous disease.

Overall, the results of the present study support a model in which a reduced DNMT3A activity and an increased ASXL1 expression contribute to an epigenetic state that favors leukemic persistence, attenuated therapeutic response, and possible clonal selection under TKI pressure. Although BCR-ABL1 remains the defining lesion in CML, accumulating evidence

indicates that additional epigenetic abnormalities strongly influence the disease trajectory (13,23,28,36). Consequently, the incorporation of epigenetic biomarkers into future prognostic algorithms may improve risk stratification and guide individualized treatment decisions, especially in patients with unexpected resistance or those considering treatment discontinuation. These findings await confirmation in larger, ethnically diverse populations and should be integrated with sequencing of regulatory regions to establish true prognostic value and therapeutic opportunities.

Given the exploratory nature and modest sample size of the present study, the findings should be regarded as preliminary. The elevated ORs observed for certain genotypes should be interpreted with caution, as these estimates may be influenced by the limited sample size and warrant confirmation in larger, prospective, and ethnically diverse independent cohorts.

Several limitations should be acknowledged when interpreting the findings of the present study. First, the relatively small sample size, including a limited control group (n=20), and the single-center design reduce statistical power and limit generalizability, underscoring the need for external validation in independent, ethnically diverse cohorts. Second, although the cross-sectional design precludes formal establishment of temporal associations, the consistent and statistically significant expression patterns observed across all patient groups strongly support the biological relevance of the findings. Third, molecular profiling was necessarily restricted to the highest-yield hotspot regions of DNMT3A (exon 23) and ASXL1 (exon 12); while comprehensive next-generation sequencing would further enrich these findings, the associated costs rendered this approach unfeasible in the context of the present self-funded investigation. Additionally, BCR-ABL1 kinase domain mutation analysis was not performed; however, the differential expression patterns identified herein represent independent epigenetic alterations contributing insights beyond canonical resistance pathways. The use of GAPDH as a sole reference gene, although widely adopted, may introduce normalization bias and should be considered when interpreting fold-change values. Finally, ROC curve analysis was not performed, as the primary objective was to characterize molecular expression patterns rather than establish diagnostic thresholds, and such analysis would be more appropriately conducted in future prospective studies. Notwithstanding these limitations, the present study provides novel insight into the epigenetic dysregulation of CML in an underrepresented population, contributing to the growing evidence implicating DNMT3A and ASXL1 in disease susceptibility and response to TKIs.

In conclusion, the downregulation of DNMT3A and the upregulation of ASXL1 expression, along with specific DNMT3A polymorphisms, present a combined epigenetic and genetic signature associated with CML susceptibility and resistance to TKIs. Integrating these biomarkers into prognostic models may enhance risk stratification and guide personalized therapeutic strategies in CML management.

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## Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to patient confidentiality considerations, but are available from the corresponding author upon reasonable request. All sequence data were mapped to the human reference genome (GRCh38), and gene-specific reference sequences were retrieved from NCBI GenBank (ASXL1: NG_027868.1; DNMT3A: NG_029465.2).

## Authors' contributions

AMA conceptualized the study. AMA and ANAR were involved in the study methodology, and in the writing, reviewing and editing of the manuscript. All authors (AMA, ANAR and AFA) were involved in data validation, investigation and in the writing and preparation of the original draft of the manuscript. AFA contributed to the formal analysis, data curation and figure preparation. AMA provided laboratory facilities, reagents, instruments and technical support. AFA supervised the study. AMA was involved in project administration. All authors have read and agreed to the published version of the manuscript.

## Ethics approval and consent to participate

The study protocol was approved by the Institutional Review Board (IRB) of the College of Science, Al-Mustansiriyah University (Ref. No. BCSMU/291/100477/2). The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the guidelines of the approving committee. Participants provided written informed consent before being recruited into the study.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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