Indian J Hematol Blood Transfus
https://doi.org/10.1007/s12288-024-01808-9

ORIGINAL ARTICLE

Significance of Somatic Mutation Profiling in CML Beyond BCR-ABL: A Retrospective Study of the Indian Population

Pooja Chaudhary[1] · Spandan Chaudhary[1] · Falguni Patel[2] · Shiv Patel[1] · Dhiren Patel[1] · Lokesh Patel[1] · Nikha Trivedi[1] · Toral Vaishnani[1] · Ekta Jajodia[1] · Firoz Ahmad[1] · Neeraj Arora[1]

Received: 24 February 2024 / Accepted: 13 June 2024

Abstract Somatic mutation and fusion detection in acute myeloid leukemia to determine disease subtype and treatment regime is a common practice, but it's not yet employed in chronic myeloid leukemia (CML). CML is still monitored by routine quantitative determination of the BCR-ABL fusion transcript and treated with tyrosine kinase inhibitors (TKIs). Despite the availability of the three generations of TKIs, resistance and progression in CML pathogenesis suggest a strong role for somatic mutations. The present study aimed to identify the role of somatic mutation profiling in CML patients in disease management. 196 CML patient samples were used in this investigation, comprising 26 CML-BP, 8 CML-AP, and 162 CML-CP samples. Following cytogenetic analysis for confirmation, each sample was sequenced utilizing the Ion Torrent platform by a targeted panel. Of the 196 CML samples, 81 (41.33%) had 125 variations affecting 27 genes, while 115 (58.67%) harboured no mutations. The study revealed that *ASXL1* (31.2%), *ABL1* (14.4%), and *TET2* (8.8%) were the most frequently altered genes. These genes are recognized indicators of CML disease. Few samples found with mutated *GATA2, IDH1, NRAS, SETBP1, WT1, PHF6, KIT,* etc. and fusions like *RUNX1(5)-MECOM* (2) and *CBFB- MYH11* are indicative of disease progression. The outcome of this study suggests that mutational profiling of CML patients can help in the prognostication of disease. Based on the results of the study, the authors have also provided possible future risk stratification and diagnosis workflow for CML disease.

Keywords CML · Somatic mutations · NGS · Fusions · TKIs

Introduction

CML is a myeloproliferative neoplasm characterized by a translocation event t (9; 22) (q34; q11.2) that rearranges the Abelson murine leukemia viral oncogene homolog 1 (*ABL1*) and breakpoint cluster region (*BCR*) genes, which leads to the *BCR-ABL* fusion gene. This translocation is also called the Philadelphia (Ph) chromosome, which initiates the event of CML and is reported to be found in more than 95% of CML patients [1–7]. The *BCR-ABL* fusion gene generates a chimeric protein with constitutively active tyrosine kinase activity [8]. The tyrosine kinase inhibitors (TKIs) targeting the *BCR-ABL* fusion protein have proved to be a significant turnaround in the treatment of CML [9–11]. In comparison to other myeloid malignancies, CML patients have better long term survival. However, some patients develop resistance to these treatments through *BCR-ABL*- dependent or independent mechanisms, which increases the chances of progression to an accelerated phase (AP) or blastic phase (BP) of disease.

With the emergence of high-throughput sequencing technologies, numerous research studies conducted on other myeloid malignancies have revealed commonly mutated genes and the pathways affected by them [12–16]. As per the data published in 'The Cancer Genome Atlas (TCGA) Consortium', eight categories have been defined for the

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12288-024-01808-9.

✉ Pooja Chaudhary
poojashah.chaudhary@gmail.com

[1] Unipath Specialty Laboratory Ltd., Ahmedabad, Gujarat 380015, India

[2] Department of Biotechnology and Microbiology, Shri M.M.Patel Institute of Science and Research, Kadi Sarva Vishwavidyalaya, Gandhinagar, Gujarat 382015, India

Published online: 21 June 2024

diagnosis of acute myeloid leukemia (AML) cases, based on the biological pathways they effect. These pathways include activated signalling, chromatin modification, cohesion complexes, DNA methylation, myeloid transcription factors, tumor suppressors, Nucleophosmin-1 (*NPM1*), splicing machinery, etc. [12]. Various studies conducted on myelodysplastic syndromes, myeloproliferative neoplasms, CML, and atypical CML, etc. have reported that most patients with somatic mutations fall within the aforementioned pathway categories [13, 17–19].

In CML, BCR-ABL1, a single fusion gene, was believed to be responsible for initiating and maintaining the cancer phenotype. But the heterogeneity of the clinical outcomes of CML in TKI treatment suggests an unidentified role of BCR-ABL1-independent mechanisms in CML pathogenesis. Approximately 5–10% of CML patients show primary resistance, and 20–30% show secondary resistance to second- and third-generation TKIs [20, 21]. The primary reason for this resistance is acquired mutations in the kinase domain region in 60% of the cases [22]., whereas the rest are still unknown. Various mechanisms have been reported that lead to a decrease or loss of response to TKIs, but the acquisition of point mutations in the BCR-ABL1 kinase domain (KD) is the most important and probably the only actionable one [23]. The advancement in sequencing technologies and the availability of more data suggest the role of additional genetic events in CML pathogenesis. TKI-resistant clones either have *ABL1* KD mutations prior to treatment or develop later, which block the binding of TKI and eventually account for TKI resistance [2, 24–28]. There are a significant number of studies available that investigate *ABL1* KD mutations, but very little information is available about the mutation spectrum in other genes in TKI-resistant CML patients before and after treatment [29–34].

The acquisition of genetic or epigenetic abnormalities is also reported to be associated with the progression of CML into AP or BP, apart from BCR-ABL gene rearrangement [35–39]. Several studies have reported that the genes associated with chromatin modification and DNA methylation are enriched with somatic mutations, whereas the other pathways are not enriched in CML [35–37, 40]. The role of the somatic mutations, their origin, clinical implications, and association with BCR-ABL transcript changes are mostly unknown. However, few studies have reported the potential role of preleukemic somatic mutations in CML [40]. Preleukemia is a phase where cells carrying a subset of genetic or epigenetic mutations necessary for leukemogenesis are growing, subsequently leading to cancer in many patients. The acquisition of additional genetic lesions plays a vital role in leukemic transformation [41].

The present study aimed to investigate variants at three stages of CML disease, namely the chronic phase, blast phase, and accelerated phase, and whether they can be considered markers of the preleukemic stage, which will transform into the disease stage. Based on a review of the literature, very few such studies are available, and none of them include Indian patients. So, to the best of our knowledge, this is the first study from India aimed at mutational spectrum analysis in CML patient samples. In this study, 196 CML patient samples and six control samples have been studied using a targeted NGS panel, which covers 40 key DNA genes and 29 RNA fusion transcript driver genes, to check for the variants and fusions involved. This study gave important insights into the mutational profiling of CML patients.

Methodology

Sample Collection

In this study, total of 202 samples have been used, out of which 196 were CML patients of different disease stages. Six control samples (3 males and 3 females) of healthy individuals have also been considered to rule out any possibility of false variants. CML patient samples were selected and classified into chronic, accelerated, and blast crisis phases as per WHO 2016 guidelines [42]. However, the accelerated phase has been removed from recent WHO 2022 guidelines, but similar studies published earlier have considered three phases, so for the comparative aspects, we have also divided samples into three phases. This is a retrospective study that used left-over samples, and patient details are anonymized. All the samples included in the study were confirmed CML patients, which means all samples were found to have a *BCR-ABL1* major (P210) fusion transcript. The data about the features of the patients included in the study is presented in Table 1, and a detailed study flow chart is depicted in Fig. 1a. This study was approved by the Sangini Hospital Ethics Committee (ERC/147/Inst/GJ/2013/RR-19).

Table 1 Features of the samples included in the study

Features	Samples (n)
Total samples	202
CML patients	196
Healthy controls	6
Female patients	86 (43.88%)
Male patients	110 (56.12%)
CML-CP	162 (82.65%)
CML_BP	26 (13.27%)
CML-AP	8 (4.08%)
Control-male	3
Control-female	3
Median age	42

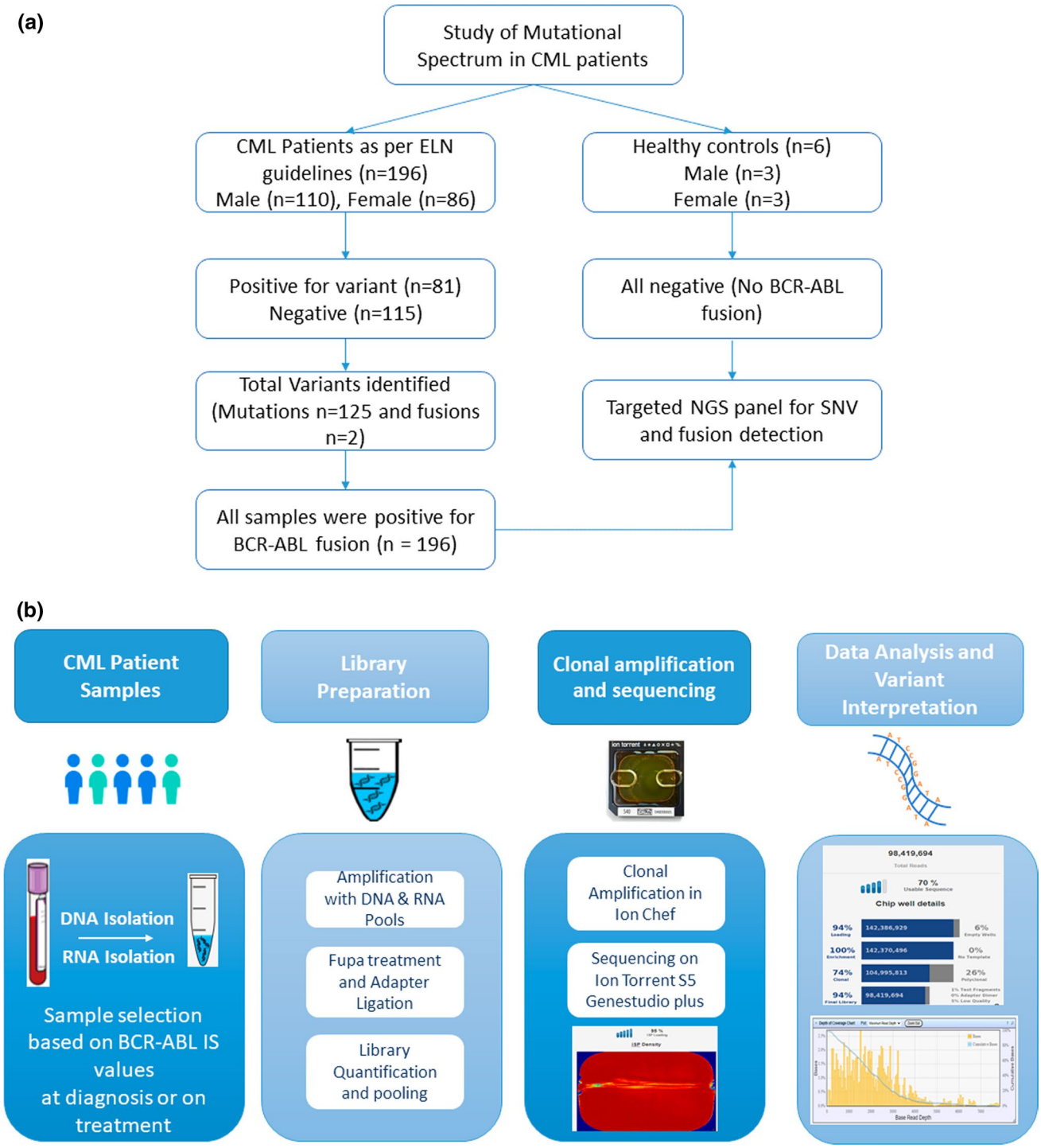


Fig. 1 **a** Study flow chart. **b** Schematic representation of overall workflow of the study. All the samples with BCR: ABL translocation were selected for the study. Samples are subjected to DNA and RNA isolation followed by respective library preparation using targeted NGS panel. Libraries were pooled based on quantification by qPCR and sequenced using Ion torrent S5 genestudio plus sequencer followed by analysis using Ion reporter software

Sample Preparation

All the patients' samples and control blood samples were subjected to RNA isolation using the QIAamp blood mini kit (Cat. No. 51104) and DNA isolation using the Alexgen Blood gDNA Extraction Kit (Cat. No. 1002) as per the user manual, followed by quality check using gel electrophoresis.

Library Preparation and Sequencing

Library was prepared using the Oncomine™ Myeloid Research Assay (Cat. No. A36940). This assay contains the Oncomine™ Myeloid DNA Panel and the Oncomine™ Myeloid RNA Panel. This panel covers 40 key DNA target genes and 29 driver genes, which cover CML and other myeloid malignancies. The presence of 23 hotspot genes, 17 full genes (exonic regions), 29 fusion driver genes, 5 expression genes, and 5 expression control genes makes this a comprehensive panel. Commonly reported genes involved in CML patients of all three stages, i.e., CP, BP, and AP, include *RUNX1, ASXL1, TET2, IDH1, IDH2, TP53, IKZF1, JAK2, CBL, CBLB, GATA2, UBE2A, DNMT3A, BCOR, WT1,* etc. [43–45], and most of these are covered by the panel used for this study. However, some studies have reported a few extra epigenetic modifier genes that are not included in this panel. All the genes covered in the panel used in the present study are mentioned in Table 2.

Briefly, 20 ng of extracted RNA was converted to cDNA as per kit protocol and used for the amplification, whereas DNA was quantified and 10 ng was used directly for amplification using the respective targeted panels. Prepared cDNA and DNA samples were subjected to targeted amplification using three vials of oligos provided as DNA pools (2 vials) and RNA pools (one vial). Amplicons of both the DNA pools were pooled together before partial digestion of oligoes using FuPA reagent (supplied with the kit), followed by indexing and adapter ligation; however, the RNA library was prepared separately for the same steps. Prepared DNA and RNA libraries were quantified using the Ion Library TaqMan™ Quantitation Kit (Cat no. 4468802) as per the user manual. Libraries were pooled together in equal concentration, and 100 pM of the pool was subjected to the Ion Chef™ System for clonal amplification and chip loading. The loaded chip was subjected to sequencing using an Ion S5 sequencing kit with a 400-bp read length on the Ion GeneStudio™ S5 Plus System (Thermofisher) to generate 1.5 million reads per sample at an average sequencing depth of around 1500x. A schematic representation of the entire study workflow is provided in Fig. 1b.

Data Analysis

Demultiplexing, quality check of the sequencing reads, mapping on the hg19 human reference genome, variant calling, and annotation were carried out with IonReporter™ (IR) Software 5.18.2.0. This software uses different databases for the identification and characterization of gene-associated variants. The annotation for the variants was derived using the disease database ClinVar. Population frequency information from databases like 1000 genomes, the Exome Aggregation Consortium (ExAC), the Genome Aggregation Database (GnomAD), and the Exome Sequencing Project (ESP) was used for the elimination of common variants and polymorphisms. For the prediction of the possible impact of coding non-synonymous single nucleotide variants (SNVs) on the structure and function of a protein, PolyPhen-2 and SIFT scores were used. Variants with an allele frequency of less than 5% were not considered in the final analysed data, as the protocol also suggests a validated detection limit for somatic variants of up to 5% allele frequency (as recommended by the user manual). In the present study, we have considered majorly pathogenic and likely pathogenic variants called by the default filter of the ion reporter software and also verified them using the Franklin and Clinvar mutation databases.

Table 2 List of genes covered by the targeted panel used in this study

Hotspot genes (23)		Full genes (17)		Fusion driver genes (29)			Expression genes (5)	Expression control genes (5)
ABL1	*KRAS*	*ASXL1*	*PRPF8*	*ABL1*	*HMGA2*	*NUP214*	*BAALC*	*EIF2B1*
BRAF	*MPL*	*BCOR*	*RB1*	*ALK*	*JAK2*	*PDGFRA*	*MECOM*	*FBXW2*
CBL	*MYD88*	*CALR*	*RUNX1*	*BCL2*	*KMT2A*	*PDGFRB*	*MYC*	*PSMB2*
CSF3R	*NPM1*	*CEBPA*	*SH2B3*	*BRAF*	*(MLL)*	*RARA*	*SMC1A*	*PUM1*
DNMT3A	*NRAS*	*ETV6*	*STAG2*	*CCND1*	*MECOM*	*RBM15*	*WT1*	*TRIM27*
FLT3	*PTPN11*	*EZH2*	*TET2*	*CREBBP*	*MET*	*RUNX1*		
GATA2	*SETBP1*	*IKZF1*	*TP53*	*EGFR*	*MLLT10*	*TCF3*		
HRAS	*SF3B1*	*NF1*	*ZRSR2*	*ETV6*	*MLLT3*	*TFE3*		
IDH1	*SRSF2*	*PHF6*		*FGFR1*	*MYBL1*			
IDH2	*U2AF1*			*FGFR2*	*MYH11*			
JAK2	*WT1*			*FUS*	*NTRK3*			
KIT								

Results

Out of 196 patient samples, 162 were of CML-CP, 26 were of CML-BP, and 8 were of CML-AP. All the samples from the CML-CP stage were at the diagnosis stage, whereas the BP and AP stage samples were ongoing treatment samples. Sequencing performed on ion torrent platform resulted in > 90% chip loading with 28–31% of polyclonal wells, which is optimum for the Ion torrent S5 genestudio sequencing platform. The read length histogram showed a mean read length of around 170–190 bp in all the samples, indicating the quality of the reads is good and non-fragmented.

A total of 81 samples out of 196 had mutations (41.33%), which includes 125 variants involving 27 genes, whereas 115 samples (58.67%) harboured no mutations (data available in Additional file 1: Table 1). Mutational profiling of all the positive samples is presented as a graph in Fig. 2a, b. Overall, the *ASXL1* gene was found to be most frequently mutated (31.2%), followed by *ABL1* (14.4%) and *TET2* (8.8%). A schematic representation of mutations, gene structure, and a graphical presentation of mutations with Variant allele frequency (VAF) are provided in Fig. 3a, b for the *ASXL1* and *ABL* genes, respectively. Among all the samples in the study, the 22 out of 26 blast phase samples were found to have 37 mutations in 16 genes, resulting in an average of 1.68 mutations per sample. Whereas, 52 out of 162 chronic phase samples showed 81 mutations from 20 genes, which is an average of 1.68 mutations per sample. Seven out of eight accelerated phase samples resulted in only 7 mutations, the lowest in comparison with chronic and blast phase samples. Among all the samples, two samples of CML-BP were found to have rare fusions, *RUNX1(5)-MECOM (2)* and *CBFB-MYH11* apart from *BCR-ABL*. Out of 125 variants identified, 54 (43.2%) were missense mutations and 35 (28%) were frameshift mutations. Chronic phase samples (n = 14) were found to have a high mutation rate (77.78%) in the *ABL1* gene compared to mutations (22.22%) in blast phase samples (n = 4). Whereas *ASXL1* gene mutations were found with a ratio of 56.41% (22 variants), 10.26% (four variants), and 33.33% (13 variants) in CML-CP, CML-AP, and CML-BP, respectively. *TET2* gene mutations were found prevalently in CML-CP (81.82%; nine variants) compared to CML-BP (18.18%; two variants) or CML-AP (no variant). Few genes, like *KIT, WT1, NRAS, SETBP1*, and *NF1*, were found to have no mutations in CML-CP samples but were mutated in CML-BP phase samples except for one *KIT* gene mutation, which was found in the CML-AP sample. Mutations in the aforementioned genes also indicate disease progression, as they act as markers for acute myeloid leukemia. Mutations found in the entire study are presented in Fig. 2b.

ASXL1, ABL1 and *TET2* Gene Mutations

In our study, we found 68 out of 125 variants (54.4%), mainly in the three genes *ASXL1, ABL1*, and *TET2*. Mutational abundance found in all three stages of the CML disease in the three aforementioned genes is provided in pie chart form in Fig. 4a. *ASXL1, ABL1,* and *TET2* genes were found to have 39, 18, and 11 variants respectively, out of 125 variants. Out of 18 variants of the *ABL1* gene, 14 were identified in chronic phase samples, four in blast phase samples, and none in accelerated phase samples. The mutational distribution for the *ASXL1* gene was 22 in the chronic phase samples, 13 in blast phase samples, and four mutations in accelerated phase samples. The *TET2* gene had nine mutations in chronic phase, two in the blast phase, and no mutation was found in the accelerated phase samples (data is provided in Additional file 1: Table 1). In Fig. 4a, it is visible that CML-CP samples have a high prevalence of mutations in the *ASXL1, ABL1,* and *TET2* genes (57%, 78%, and 82%, respectively) compared to the other two phase samples, which represents the role of these genes and somatic mutations in the emergence of the disease. Imatinib resistance is linked to mutations in the ABL gene, while clonal hematopoiesis of indeterminate potential (CHIP) may be the source of mutations in *ASXL1* and *TET2*. The condition known as CHIP refers to the clonal growth of hematopoietic stem cells in people who do not exhibit cytopenia, dysplasia, or hematologic malignancy but instead have leukemogenic mutations [46]. The possibility that *ASXL1* and *TET2* mutations are age-related and may not necessarily contribute to CML cannot be disregarded. According to studies, the frequency of CHIP mutations in people who are at least 70 years old is between 5 and 6% [47]. The patients in this investigation, however, had a median age of 42 years, and the variations under consideration had already been found to be pathogenic or likely to be pathogenic with an allele frequency of greater than 5%. Taking into account all the information and studying the patient samples at various time points of disease progression, we can verify the distinction between CHIP mutations and oncogenic mutations. Discussion about the results of mutations in these genes is presented separately and in depth, as they are the most frequently mutated genes in the CML disease [43].

Other Gene Mutations Found in this Study

In this study, variants were found in a total of 27 genes, out of which three genes (*ASXL1, BCOR, and PHF6*) were common in the samples of all three stages of the disease (CML-CP, CML-BP, and CML-AP). Mutations in ten genes (*CBL, DNMT3A, EZH2, IKZF1, KRAS, RB1, SRSF2, STAG2, CALR, and ZRSR2*) were exclusive

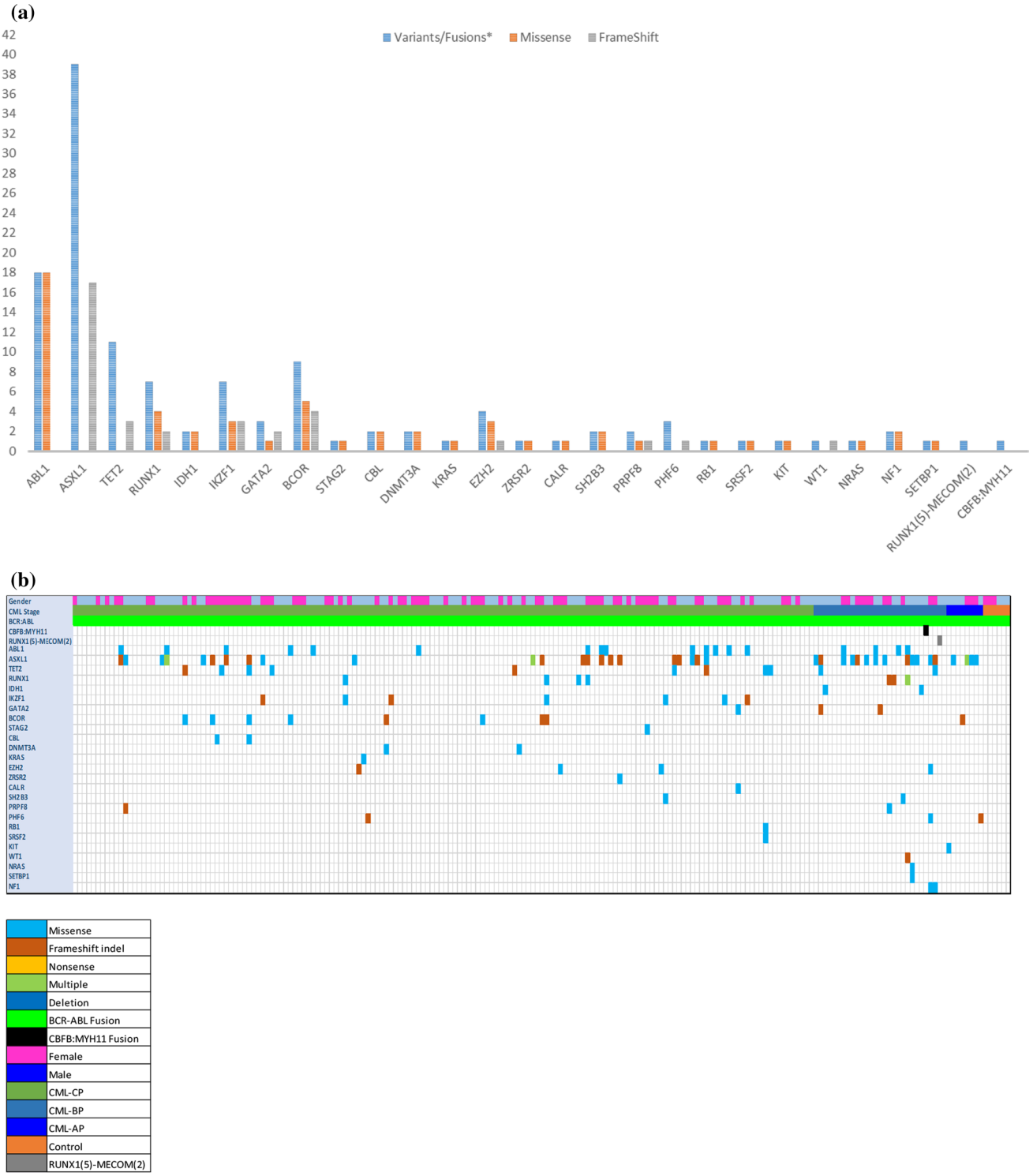


Fig. 2 **a** Mutational profiling of all the CML samples found with mutations. This graph also represents two categories of mutations: missense and frameshift mutations. Y-axis represents the number of times mutations were found and X-axis represents genes in which mutations were observed. *Fusions other than BCR-*ABL1* are only presented in this figure, as all the samples had BCR: *ABL1* fusion, it's not included in this representation. **b** Mutational profiling of the all the CML mutations found in this study in the respective genes along with the fusions. This figure represents overall results of all the positive and negative samples, age and disease stage of the patient samples, included in this study. Color coding is explained in the small table provided with the figure. In this figure, each block represents one mutation/fusion

to CML-CP stage samples; eight genes (*GATA2, IDH1, CBFB-MYH11, NRAS, SETBP1, RUNX-MECOM, WT1, NF1*) were exclusive to CML-BP stage samples; and no exclusively mutated gene was found in the CML-AP stage sample. Apart from two fusions *(CBFB-MYH11 and RUNX-MECOM*), six genes *(GATA2, IDH1, NRAS, SETBP1, WT1, NF1, and KIT)* were exclusively found mutated in accelerated and blast-phase samples, which are already known to have mutations in other progressive forms of leukemia like acute myeloid leukemia. An illustration of the same is represented in Fig. 4b with a Venn diagram along with the mutational frequency of each stage, i.e., CML-CP, CML-AP, and CML-BP. This image represents the patterns of gene mutations that occur and progress during the various stages of the CML disease.

Fusions in CML Other Than BCR-ABL

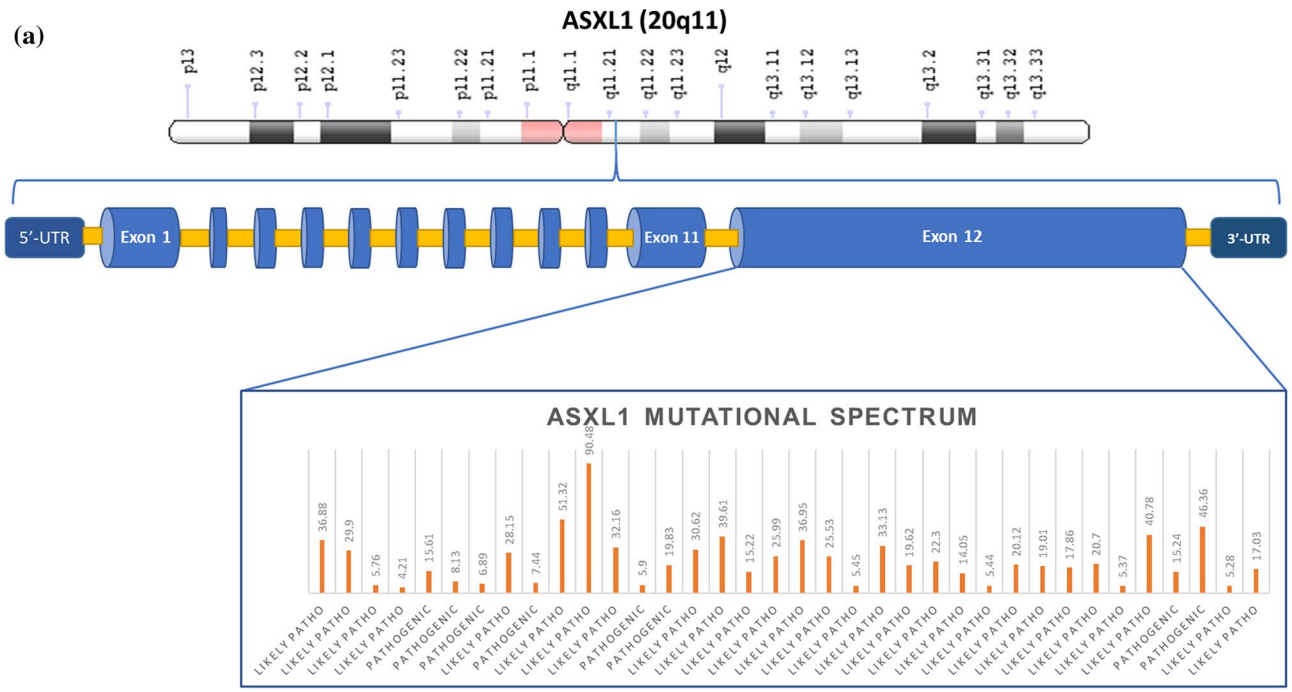
Apart from SNVs, two samples were found with fusions in this study, which were *RUNX1(5)-MECOM (2)* and *CBFB-MYH11*. *RUNX1-MECOM* was reported in an elderly patient who had AML-MRD with t (3;21) (q26: q22) and subsequent *RUNX1-MECOM* (previously AML1-MDS1-EVI1). This rare recurring translocation is reported in therapy-related myeloid neoplasm, CML with accelerated or blastic phase, and, rarely, in de novo AML [48]. Recently, many studies [49–52] have found this fusion in various AML cases, confirming its role in disease transformation from the chronic to the blast phase or even further. Studies have already established the role of the identification of such fusion transcripts in the diagnosis, risk stratification, and clinical management of the affected patients [53]. *CBFB-MYH11* fusion is usually observed in AML and predicts a favorable prognosis [54]. However, the same fusion is also reported in elderly CML patients [55] with primary blast crises. Interestingly, one case report has studied the blastic transformation of *BCR-ABL1* positive chronic myeloid leukemia through the acquisition of the *CBFB-MYH11* fusion transcript. In this case study, a CP-CML patient was diagnosed with a co-occurrence of *CBFB-MYH11*, which progressed to myeloid BP-CML under treatment with nilotinib after acquiring three different in-frame indel mutations in exon 8 of *KIT* [56]. In the present study, this patient with *CBFB-MYH11* fusion has also progressed to AML.

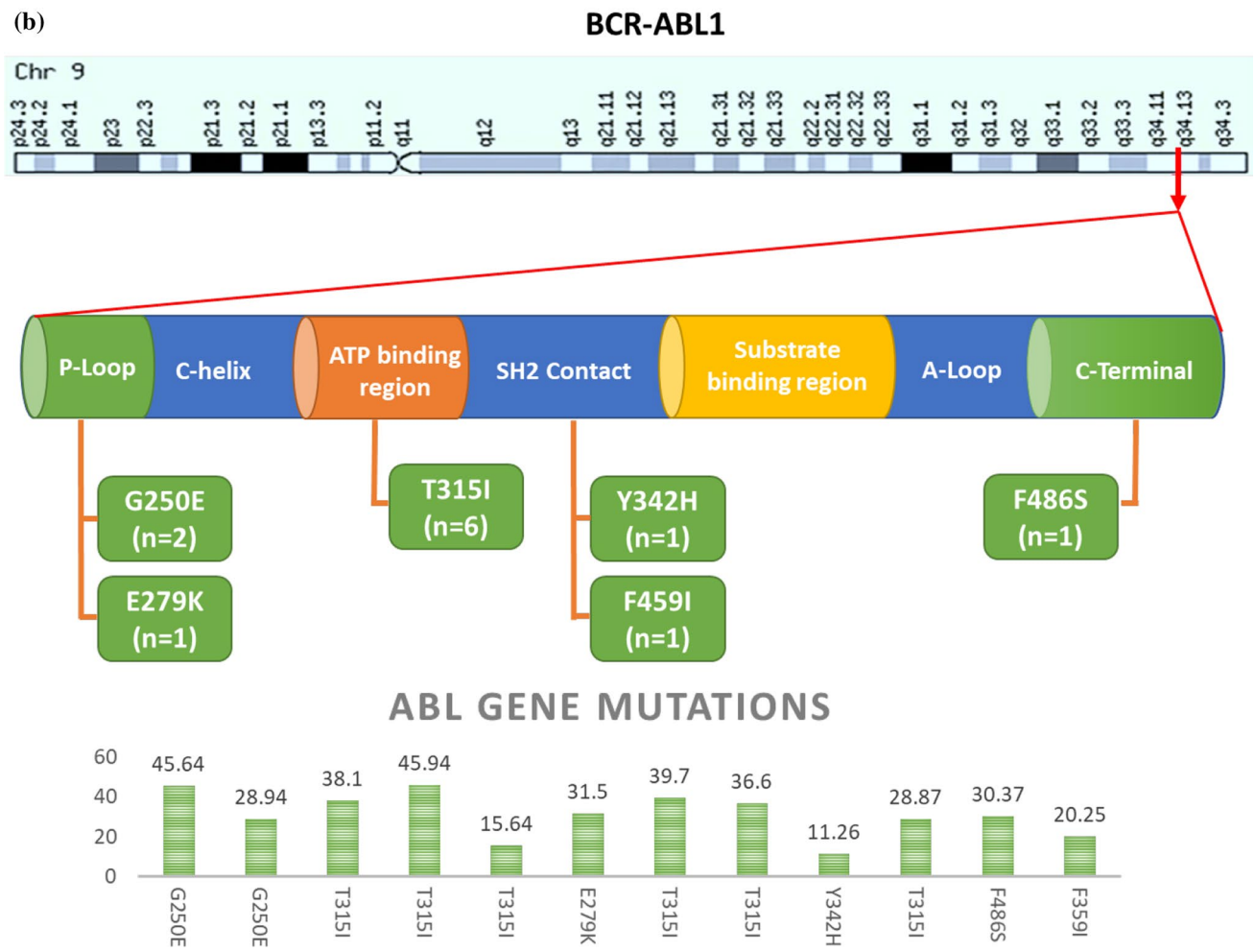
Discussion

CML progression occurs in three stages; the majority of CML patients are diagnosed in the chronic phase, which will progress through the accelerated phase to a crisis if they do not get treatment. However, as per the new WHO 2022 guidelines, the accelerated phase is no longer in the classification; samples with up to 20% blasts in blood or bone marrow are considered in the chronic phase, and samples with more than 20% blasts are classified as blast phase [57]. In the present study, a total of 196 patient samples have been used, out of which 41.33% showed mutations in one or more genes, whereas 58.67% had no mutations. Based on the review of the literature, there are very limited studies available that were focused on CML mutational analysis. A study of 71 samples revealed that 32.40% (n = 23) of the samples had mutations, whereas 67.60% (n = 48) of the samples had none at all [58]. Another study that employed 300 serial samples drawn from 100 samples of CML patients revealed that 37% of the samples contained mutations, whereas 63% did not [45]. It can be inferred that somatic mutations may be present in 32–41% of CML samples on average. Although there are, of course, far too few studies to draw any firm conclusions from, the ones that are included here were conducted on various populations in various parts of the world.

The presence of the *ABL* fusion transcript is definitive for the CML and was present in all the patient samples studied (data not provided). This fusion is considered oncogenic as it generates a leukemic phenotype. So theoretically, there should be very few or no mutations found in chronic stage samples, at least in the predominant clone. Moreover, CML is a disease predominantly affecting the elderly, so there are chances of the presence of a significant number of passenger variants [59]. In contrast, very few partially characterized oncogenic variants are responsible for the impairment of the myeloid differentiation machinery, leading to the evolution of BC from CP. Progression of CP to BC occurs within 2–3 years after the onset of the disease, which spares less time for the accumulation of passenger variants. Therefore, the expected number of somatic mutations functionally associated with cancer is higher in BC than in CP, which is true for this study as well. In the present study, the average mutation detected per sample for CML-CP and CML-BP was derived as 1.56, 1.68, respectively (Additional file 1: Table 2).

In the present study, the most number of mutations (39 mutations) were found in the *ASXL1* gene in all three categories (CP, BP, and AP), which coincides with earlier reported studies [43, 44, 58]. Despite being commonly found in mutated genes in CML patients, along with *ABL* gene mutations, the exact mechanism and role of *ASXL1* gene mutations are still not well understood. Earlier studies have tried investigating the significance of *TET2, IDH1, IDH2*, and *ASXL1* mutations in CML and found that *TET2, IDH1,* and *IDH2* may not represent a major genetic event in CML transformation, but *ASXL1* mutations can be an early event in CML leukemogenesis [16]. The second most frequently mutated gene found in our study was *ABL1* (19 variants). From all the CML patients, around 50–90% of patients


(a)
ASXL1 (20q11)
p13
p12.3
p12.2
p12.1
p11.23
p11.22
p11.21
p11.1
q11.1
q11.21
q11.22
q11.23
q12
q13.11
q13.12
q13.13
q13.2
q13.31
q13.32
q13.33
5′-UTR
Exon 1
Exon 11
Exon 12
3′-UTR
ASXL1 MUTATIONAL SPECTRUM
36.88
29.9
5.76
4.21
15.61
8.13
6.89
28.15
7.44
51.32
90.48
32.16
5.9
19.83
30.62
39.61
15.22
25.99
36.95
25.53
5.45
33.13
19.62
22.3
14.05
5.44
20.12
19.01
17.86
20.7
5.37
40.78
15.24
46.36
5.28
17.03
LIKELY PATHO
PATHOGENIC


(b)
BCR-ABL1
Chr 9
p24.3
p24.2
p24.1
p23
p22.3
p21.3
p21.2
p21.1
p13.3
p11.2
q11
q12
q13
q21.11
q21.12
q21.13
q21.31
q21.32
q21.33
q22.2
q22.31
q22.32
q22.33
q31.1
q31.2
q31.3
q32
q33.1
q33.2
q33.3
q34.11
q34.13
q34.3
P-Loop
C-helix
ATP binding region
SH2 Contact
Substrate binding region
A-Loop
C-Terminal
G250E (n=2)
E279K (n=1)
T315I (n=6)
Y342H (n=1)
F459I (n=1)
F486S (n=1)
ABL GENE MUTATIONS
60
40
20
0
45.64
28.94
38.1
45.94
15.64
31.5
39.7
36.6
11.26
28.87
30.37
20.25
G250E
G250E
T315I
T315I
T315I
E279K
T315I
T315I
Y342H
T315I
F486S
F359I

◂**Fig. 3** **a** Mutational profiling of the *ASXL1* gene in CML patient samples. This figure represents the mutations identified in the *ASXL1* gene with their variant allele frequencies in the bar chart. This image also represents the schematic structure of the *ASXL1* gene and location of the mutations identified. This image does not depict the size of the introns and exons of the *ASXL1* gene. **b** Mutational profiling of the *ABL1* gene in CML patient samples. This figure represents the mutations identified in the *ABL1* gene with their respective variant allele frequencies. This image also represents the schematic structure of the *ABL1* gene and location of the mutations identified. This image does not depict the size of the various domains of the *ABL1* gene

who experience hematologic relapse on Imatinib have been reported to harbour kinase domain (KD) mutations [3, 25, 28, 60]. And out of all the reported point mutations, just twelve mutations (M244, G250, Q252, Y253, E255, V299, F311, T315, F317, M351, F359, and H396) account for most resistance-associated KD mutations [60]. These mutations affect the TKIs binding on different segments of the tertiary structure, such as the phosphate-binding loop (P-loop),

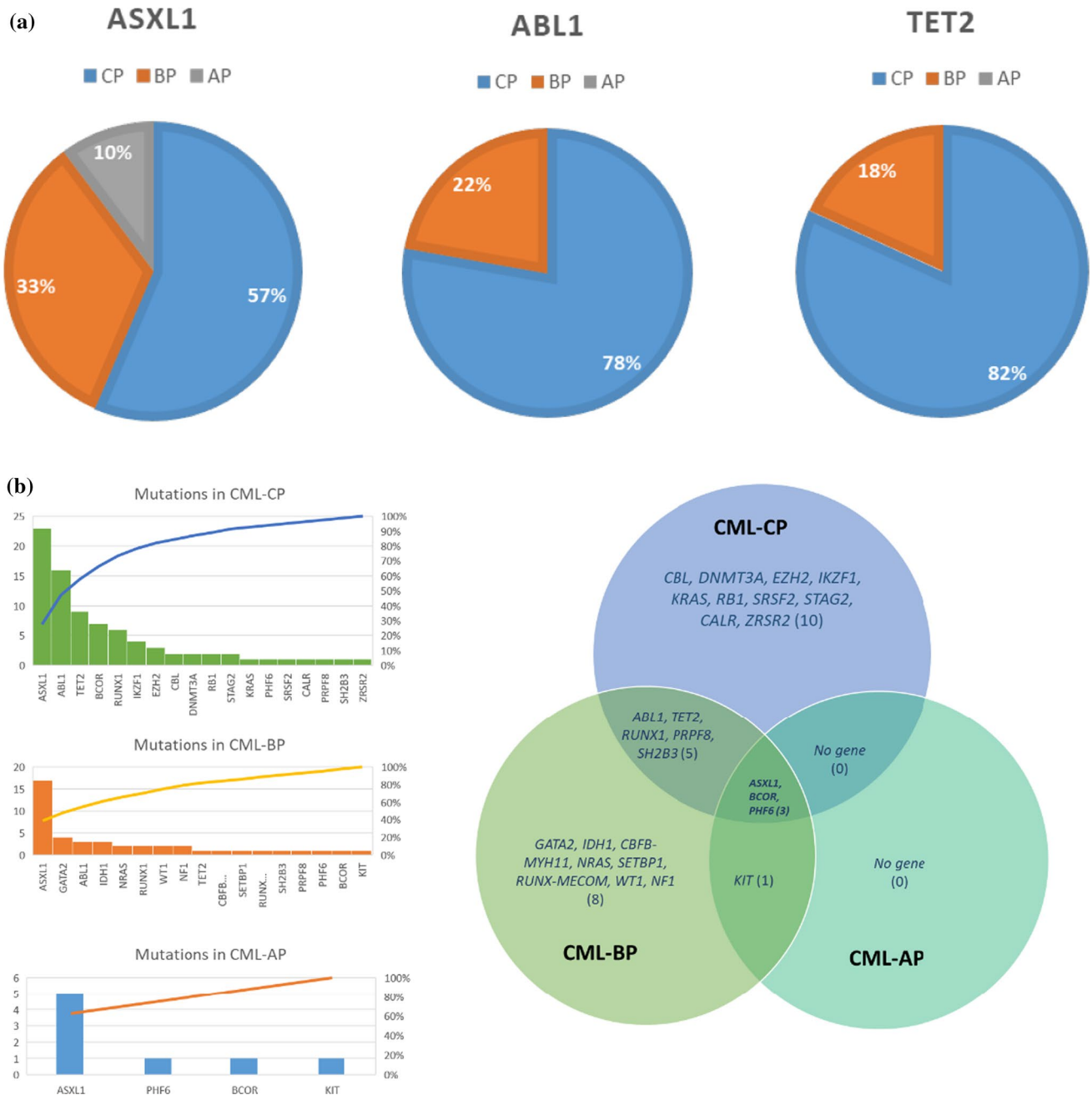


Fig. 4 **a** Mutational frequency of *ASXL1*, *ABL1* and *TET2* genes in CML-CP, CML-BP and CML-AP samples. **b** Mutational frequency of in CML-CP, CML-BP and CML-AP samples along with genes found mutated in each stages of the disease. Venn diagram represents common and uniquely mutated genes in CML CP, AP and BP

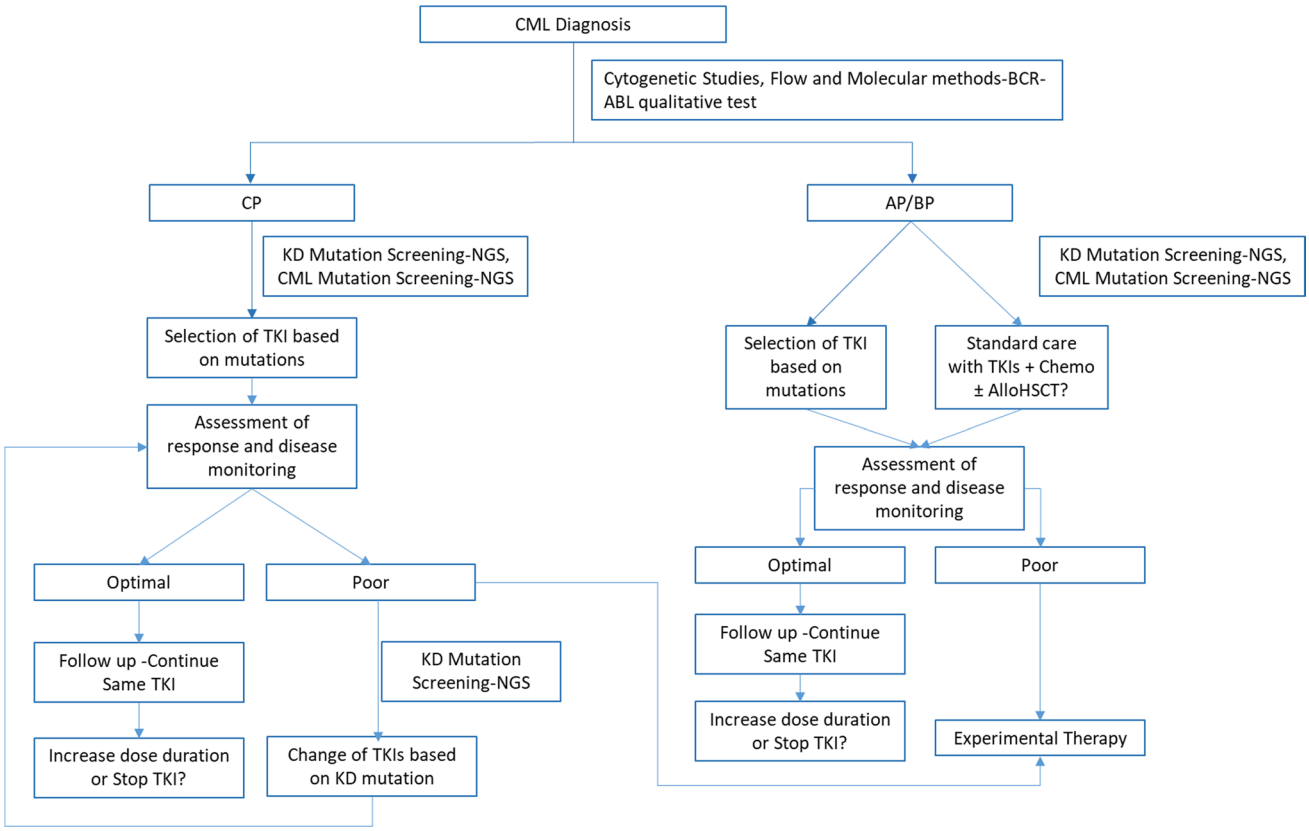


Fig. 5 Possible CML disease stratification and diagnosis workflow recommendation for future use

C-helix, ATP-binding cleft, the activation loop (A-loop), or C-terminal region. Among the mutations detected in the *ABL1* gene, 84.21% (16) mutations were found in CML-CP samples and 15.79% (three) in blast phase, whereas no mutations were found in accelerated phase (AP) samples, which might be due to the smaller number of samples (n = 7) in AP. The study, which reported a higher number of mutations in the BP or AP stage, used 297 patient's samples with primary or acquired resistance to Imatinib and found KD mutations in 27% of CP patients, 52% of AP patients, 75% of myeloid BC patients, and 83% of lymphoid BC patients [61]. Out of 19 mutations identified in the *ABL1* gene, 36.84% (seven variants) were variants of uncertain significance (VUS), whereas 63.16% (12) were either pathogenic or likely pathogenic mutations. One very important point to note here is that these mutations in the *ABL1* gene are detected from the DNA as a template and not from the fusion transcript, which means that the actual VAF at which these mutations are present in the sample can be higher than this if detected from fusion transcripts. Because it's a well-established fact that DNA-based mutation screening would mainly have untranslocated *ABL1* as a substrate, which dilutes the mutation level [62]. Out of all 12 mutations in the *ABL1* gene, 50% were T315I, which implies the change of a threonine for an isoleucine in codon 315, preventing the efficient interaction of the TKI with the protein and impairing Imatinib and most second-generation TKI activity. The other identified *ABL1* gene mutations include two instances of the G250E mutation, as well as the E279K, Y342H, F486S, and F359I mutations. The poor clinical outcome and negative impact of P-loop mutations, including G250E and E279K, are already widely demonstrated [3, 63].

The third frequently mutated gene in the study was *TET2*, which contained a total of 11 mutations, with the majority (81.82%) of them from CML-CP (n = 9) samples with 3 frameshift variants. *TET2* mutations are common in myeloid malignancies, including CML. It regulates epigenetic modification of DNA via regulation of cytosine methylation, which is crucial for stem cells and progenitor cells' self-renewal and leukemia prevention. The mechanism and role of *TET2* polymorphism among myeloid neoplasms are unknown, but studies have linked acute leukemia to various *TET2* polymorphisms, indicating its probable role in transforming CML from CP to BP [64]. In the present study, we found eight mutations (four missense and four frameshift mutations) of *BCOR* genes in CML-CP stage patients and one frameshift mutation in an accelerated stage patient (Fig. 2b) and seven mutations in *RUNX1* gene,

which coincide with similar published data [44, 58]. *RUNX1* plays a key role in the regulation of hematopoietic cell differentiation. In early studies, it was found to be mutated in the blast phase of CML disease. Mutated *RUNX1* is found in multiple CML studies and is reported as a frequent and aggressive driver of hematologic malignancies. But it is not always associated with a poor outcome of the disease, and it is very rare [65].

Important Aspect of CML Mutation Profiling

Mutational profiling of all the genes, found in this study, is represented in Fig. 2a. Roche-Lestienne et al. examined four cancer genes, *TET2, IDH1, IDH2,* and *ASXL1*, in 91 CML patient samples collected at the time of diagnosis and at other time points. All four aforementioned genes are commonly reported in BCR-*ABL1*-negative hematological cancers. One of the 91 patients had *TET2* mutations at the time of diagnosis, and this patient transformed into a myeloblastic crisis four months later. In our study, we reported 11 variants in *TET2* genes, and two of them are in the BP stage, whereas the rest are in the CP stage. This means that CML-CP patients with *TET2* gene mutations may exhibit aggressive behaviour during disease progression. The same study reported no mutations in the *IDH1* or *IDH2* genes, indicating rare variants of CML. Interestingly, we identified two variants in the *IDH1* gene, and both samples were CML-BP, indicating the role of *IDH* gene mutations in disease progression [66]. Most published studies on CML mutational profiling have found *ASXL1, ABL, RUNX1,* and *TET2* genes as frequently mutated genes, but other genes are more important than these genes. Among other genes found with lesser events in the study were *IDH1, IKZF1, GATA2, BCOR, STAG2, CBL, DNMT3A, KRAS, EZH2, ZRSR2, CALR, SH2B3, PRPF8, PHF6, RB1, SRSF2, KIT, WT1, NRAS, NF1, and SETBP1.* All these genes are reported to be involved in various AML disease subtypes. This also means that patients with mutations in these genes have more chances of progressing to AML than other CML patients, which proves the importance of NGS-based mutation profiling in CML patients at an early stage of disease.

Conclusion

This study was aimed at studying the mutational profiling of CML patients and to look for prognostic marker of preleukemic stage, which will transform into disease stage to check if it can play role in diagnosis or therapy regime. One of the most important maker identified was *ASXL1*, which was also described by many other studies to have role in transforming the disease form CP to BP. In addition to that, CML mutational profiling also provided significant information about *ABL1* gene mutations which confers resistance towards TKIs and is very helpful in early decision of diseases prevention and appropriate TKI selection. Few rare mutations in important AML related genes and fusions (like *RUNX1(5)-MECOM (2)* and *CBFB: MYH11)* etc. in co-occurrence with BCR-ABL were found, which if identified at early stage of the disease, can aid in early detection of possible diseases transformation. The outcome and findings of this study suggest mutational profiling of CML patients can be very helpful to decipher TKI selection and disease transformation, which was also highlighted in recent studies [43, 67]. In view of the findings of this study, the authors have tried to represent the new possible disease risk stratification and diagnosis workflow (Fig. 5) for early and effective management of CML patients. The selection of the sequencing method, like whole genome sequencing (WGS), whole exome sequencing (WES), or pre-designed targeted panel sequencing, required sequencing depth, sample type (i.e., sorted vs. whole blood), germline control samples, etc., are still debatable and can be a limiting factor in determining the results.

Acknowledgements Authors are thankful to the management of Unipath Specialty Laboratory Ltd. for providing all the necessary consumables and infrastructure required for the study.

Data Availability Data can be made available upon suitable request to corresponding author.

Declarations

Ethical Approval This study was approved by the Sangini Hospital Ethics Committee (ERC/147/Inst/GJ/2013/RR-19).

Conflict of Interest Authors declare no conflict of interest.

References

1. Elias MH, Baba AA, Azlan H et al (2014) BCR-ABL kinase domain mutations, including 2 novel mutations in imatinib resistant Malaysian chronic myeloid leukemia patients—Frequency and clinical outcome. Leuk Res 38:454–459
2. Branford S, Rudzki Z, Walsh S et al (2002) High frequency of point mutations clustered within the adenosine triphosphate–binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. Blood J Am Soc Hematol 99:3472–3475
3. Branford S, Rudzki Z, Walsh S et al (2003) Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. Blood 102:276–283

4. Jackson P, Baltimore D (1989) N-terminal mutations activate the leukemogenic potential of the myristoylated form of c-abl. EMBO J 8:449–456
5. Nam H-J, Haser WG, Roberts TM, Frederick CA (1996) Intramolecular interactions of the regulatory domains of the Bcr-Abl kinase reveal a novel control mechanism. Structure 4:1105–1114
6. Daley GQ, Van Etten RA, Baltimore D (1990) Induction of chronic myelogenous leukemia in mice by the P210 bcr/abl gene of the Philadelphia chromosome. Science (80-) 247:824–830
7. Willis SG, Lange T, Demehri S et al (2005) High-sensitivity detection of BCR-ABL kinase domain mutations in imatinib-naive patients: correlation with clonal cytogenetic evolution but not response to therapy. Blood 106:2128–2137
8. Rowley JD (1973) A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 243:290–293
9. O'Brien SG, Guilhot F, Larson RA et al (2003) Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med 348:994–1004
10. Lombardo LJ, Lee FY, Chen P et al (2004) Discovery of N-(2-chloro-6-methyl-phenyl)-2-(6-(4-(2-hydroxyethyl)-piperazin-1-yl)-2-methylpyrimidin-4-ylamino) thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. J Med Chem 47:6658–6661
11. Weisberg E, Manley PW, Breitenstein W et al (2005) Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. Cancer Cell 7:129–141
12. Network CGAR (2013) Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med 368:2059–2074
13. Papaemmanuil E, Gerstung M, Malcovati L et al (2013) Clinical and biological implications of driver mutations in myelodysplastic syndromes. Blood J Am Soc Hematol 122:3616–3627
14. Walter MJ, Shen D, Ding L et al (2012) Clonal architecture of secondary acute myeloid leukemia. N Engl J Med 366:1090–1098
15. Sakaguchi H, Okuno Y, Muramatsu H et al (2013) Exome sequencing identifies secondary mutations of SETBP1 and JAK3 in juvenile myelomonocytic leukemia. Nat Genet 45:937–941
16. Lindsley RC, Mar BG, Mazzola E et al (2015) Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. Blood J Am Soc Hematol 125:1367–1376
17. Piazza R, Valletta S, Winkelmann N et al (2013) Recurrent SETBP1 mutations in atypical chronic myeloid leukemia. Nat Genet 45:18–24
18. Yoshida K, Sanada M, Shiraishi Y et al (2011) Frequent pathway mutations of splicing machinery in myelodysplasia. Nature 478:64–69
19. Maxson JE, Gotlib J, Pollyea DA et al (2013) Oncogenic CSF3R mutations in chronic neutrophilic leukemia and atypical CML. N Engl J Med 368:1781–1790
20. Hochhaus A, Baccarani M, Silver RT et al (2020) European LeukemiaNet 2020 recommendations for treating chronic myeloid leukemia. Leukemia 34:966–984
21. Patel AB, O'Hare T, Deininger MW (2017) Mechanisms of resistance to ABL kinase inhibition in chronic myeloid leukemia and the development of next generation ABL kinase inhibitors. Hematol Clin 31:589–612
22. Jabbour E, Kantarjian H, Jones D et al (2006) Frequency and clinical significance of BCR-ABL mutations in patients with chronic myeloid leukemia treated with imatinib mesylate. Leukemia 20:1767–1773
23. Soverini S, Bavaro L, De Benedittis C et al (2020) Prospective assessment of NGS-detectable mutations in CML patients with nonoptimal response: the NEXT-in-CML study. Blood 135:534–541
24. Gorre ME, Mohammed M, Ellwood K et al (2001) Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science (80-) 293:876–880
25. Hochhaus A, Kreil S, Corbin AS et al (2002) Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. Leukemia 16:2190–2196
26. Von Bubnoff N, Schneller F, Peschel C, Duyster J (2002) BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukaemia to STI571: a prospective study. Lancet 359:487–491
27. Roumiantsev S, Shah NP, Gorre ME et al (2002) Clinical resistance to the kinase inhibitor STI-571 in chronic myeloid leukemia by mutation of Tyr-253 in the Abl kinase domain P-loop. Proc Natl Acad Sci 99:10700–10705
28. Shah NP, Nicoll JM, Nagar B et al (2002) Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. Cancer Cell 2:117–125
29. Hughes TP, Saglio G, Quintás-Cardama A et al (2015) BCR-ABL1 mutation development during first-line treatment with dasatinib or imatinib for chronic myeloid leukemia in chronic phase. Leukemia 29:1832–1838
30. Branford S, Yeung DT, Parker WT et al (2014) Prognosis for patients with CML and> 10% BCR-ABL1 after 3 months of imatinib depends on the rate of BCR-ABL1 decline. Blood J Am Soc Hematol 124:511–518
31. Zabriskie MS, Eide CA, Tantravahi SK et al (2014) BCR-ABL1 compound mutations combining key kinase domain positions confer clinical resistance to ponatinib in Ph chromosome-positive leukemia. Cancer Cell 26:428–442
32. Machova Polakova K, Kulvait V, Benesova A et al (2015) Next-generation deep sequencing improves detection of BCR-ABL1 kinase domain mutations emerging under tyrosine kinase inhibitor treatment of chronic myeloid leukemia patients in chronic phase. J Cancer Res Clin Oncol 141:887–899
33. Branford S, Yeung DT, Prime JA et al (2012) BCR-ABL1 doubling times more reliably assess the dynamics of CML relapse compared with the BCR-ABL1 fold rise: implications for monitoring and management. Blood J Am Soc Hematol 119:4264–4271
34. Khorashad JS, Kelley TW, Szankasi P et al (2013) BCR-ABL1 compound mutations in tyrosine kinase inhibitor–resistant CML: frequency and clonal relationships. Blood J Am Soc Hematol 121:489–498
35. Grossmann V, Kohlmann A, Zenger M et al (2011) A deep-sequencing study of chronic myeloid leukemia patients in blast crisis (BC-CML) detects mutations in 76.9% of cases. Leukemia 25:557–560
36. Menezes J, Salgado RN, Acquadro F et al (2013) ASXL1, TP53 and IKZF3 mutations are present in the chronic phase and blast crisis of chronic myeloid leukemia. Blood Cancer J 3:e157–e157
37. Makishima H, Jankowska AM, McDevitt MA et al (2011) CBL, CBLB, TET2, ASXL1, and IDH1/2 mutations and additional chromosomal aberrations constitute molecular events in chronic myelogenous leukemia. Blood J Am Soc Hematol 117:e198–e206
38. Jelinek J, Gharibyan V, Estecio MRH et al (2011) Aberrant DNA methylation is associated with disease progression, resistance to imatinib and shortened survival in chronic myelogenous leukemia. PLoS ONE 6:e22110
39. Uehara E, Takeuchi S, Yang Y et al (2012) Aberrant methylation in promoter-associated CpG islands of multiple genes in chronic myelogenous leukemia blast crisis. Oncol Lett 3:190–192
40. Schmidt M, Rinke J, Schäfer V et al (2014) Molecular-defined clonal evolution in patients with chronic myeloid leukemia independent of the BCR-ABL status. Leukemia 28:2292–2299

41. Shlush LI, Minden MD (2015) Preleukemia: the normal side of cancer. Curr Opin Hematol 22:77–84
42. Haznedaroğlu İC, Kuzu I, İlhan O (2020) WHO 2016 definition of chronic myeloid leukemia and tyrosine kinase inhibitors. Turk J Hematol 37:42
43. Adnan-Awad S, Kankainen M, Mustjoki S (2021) Mutational landscape of chronic myeloid leukemia: More than a single oncogene leukemia. Leuk Lymphoma 62:2064–2078
44. Ochi Y, Yoshida K, Huang Y-J et al (2021) Clonal evolution and clinical implications of genetic abnormalities in blastic transformation of chronic myeloid leukaemia. Nat Commun 12:2833
45. Kim T, Tyndel MS, Kim HJ et al (2017) Spectrum of somatic mutation dynamics in chronic myeloid leukemia following tyrosine kinase inhibitor therapy. Blood J Am Soc Hematol 129:38–47
46. Marnell CS, Bick A, Natarajan P (2021) Clonal hematopoiesis of indeterminate potential (CHIP): linking somatic mutations, hematopoiesis, chronic inflammation and cardiovascular disease. J Mol Cell Cardiol 161:98–105
47. Xie M, Lu C, Wang J et al (2014) Age-related mutations associated with clonal hematopoietic expansion and malignancies. Nat Med 20:1472–1478
48. Park TS, Choi JR, Yoon SH et al (2008) Acute promyelocytic leukemia relapsing as secondary acute myelogenous leukemia with translocation t (3; 21)(q26; q22) and RUNX1–MDS1–EVI1 fusion transcript. Cancer Genet Cytogenet 187:61–73
49. Senyuk V, Sinha KK, Li D et al (2007) Repression of RUNX1 activity by EVI1: a new role of EVI1 in leukemogenesis. Cancer Res 67:5658–5666
50. Tokita K, Maki K, Mitani K (2007) RUNX1/EVI1, which blocks myeloid differentiation, inhibits CCAAT–enhancer binding protein α function. Cancer Sci 98:1752–1757
51. Kellaway SG, Keane P, Kennett E, Bonifer C (2021) RUNX1-EVI1 disrupts lineage determination and the cell cycle by interfering with RUNX1 and EVI1 driven gene regulatory networks. Haematologica 106:1569
52. Kiehlmeier S, Rafiee M-R, Bakr A et al (2021) Identification of therapeutic targets of the hijacked super-enhancer complex in EVI1-rearranged leukemia. Leukemia 35:3127–3138
53. Tang Z, Wang W, Yang S et al (2023) 3q26.2/MECOM rearrangements by Pericentric Inv (3): diagnostic challenges and clinicopathologic features. Cancers (Basel) 15:458
54. Arber DA (2019) The 2016 WHO classification of acute myeloid leukemia: what the practicing clinician needs to know. In: Seminars in hematology. Elsevier, pp 90–95
55. Kidoguchi K, Kojima K, Yokoo M, Kimura S (2019) BCR-ABL1- and CBFB-MYH11-positive chronic myeloid leukemia presenting with primary blast crisis and marrow fibrosis. Ann Hematol 98:2461–2462
56. Shoumariyeh K, Hussung S, Niemöller C et al (2020) Blastic transformation of BCR-ABL1 positive chronic myeloid leukaemia through acquisition of CBFB-MYH11 and mutant KIT. Br J Haematol 190:e339–e343
57. Khoury JD, Solary E, Abla O et al (2022) The 5th edition of the World Health Organization classification of haematolymphoid tumours: myeloid and histiocytic/dendritic neoplasms. Leukemia 36:1703–1719
58. Bidikian A, Kantarjian H, Jabbour E et al (2022) Prognostic impact of ASXL1 mutations in chronic phase chronic myeloid leukemia. Blood Cancer J 12:144
59. Milholland B, Auton A, Suh Y, Vijg J (2015) Age-related somatic mutations in the cancer genome. Oncotarget 6:24627
60. Von Bubnoff N, Peschel C, Duyster J (2003) Resistance of Philadelphia-chromosome positive leukemia towards the kinase inhibitor imatinib (STI571, Glivec): a targeted oncoprotein strikes back. Leukemia 17:829–838
61. Soverini S, Colarossi S, Gnani A et al (2006) Contribution of ABL kinase domain mutations to imatinib resistance in different subsets of Philadelphia-positive patients: by the GIMEMA Working Party on Chronic Myeloid Leukemia. Clin Cancer Res 12:7374–7379
62. Soverini S, Abruzzese E, Bocchia M et al (2019) Next-generation sequencing for BCR-ABL1 kinase domain mutation testing in patients with chronic myeloid leukemia: a position paper. J Hematol Oncol 12:1–11
63. Soverini S, Martinelli G, Rosti G et al (2005) ABL mutations in late chronic phase chronic myeloid leukemia patients with up-front cytogenetic resistance to imatinib are associated with a greater likelihood of progression to blast crisis and shorter survival: a study by the GIMEMA Working Party on Chr. J Clin Oncol 23:4100–4109
64. Hamed NA, Elhalawani NA, Kassem HS et al (2020) The prognostic significance of TET2 single nucleotide polymorphism in Egyptian chronic myeloid leukemia. Mediterr J Hematol Infect Dis. https://doi.org/10.4084/MJHID.2020.004
65. Branford S, Wang P, Yeung DT et al (2018) Integrative genomic analysis reveals cancer-associated mutations at diagnosis of CML in patients with high-risk disease. Blood J Am Soc Hematol 132:948–961
66. Roche-Lestienne C, Marceau A, Labis E et al (2011) Mutation analysis of TET2, IDH1, IDH2 and ASXL1 in chronic myeloid leukemia. Leukemia 25:1661–1664
67. Branford S, Kim DDH, Apperley JF et al (2019) Laying the foundation for genomically-based risk assessment in chronic myeloid leukemia. Leukemia 33:1835–1850