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Molecular, Cytogenetic, and Hematological Monitoring and Response to Treatment for Chronic Myeloid Leukemia Patients

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ABSTRACT

Background: A genetic abnormality in chronic myeloid leukemia (CML) involves a translocation between the long arms of chromosomes 9 and 22, known as t(9;22)(q34;q11.2), resulting in the Philadelphia chromosome (Ph). This translocation fuses the Abelson (ABL1) gene on chromosome 9 with the breakpoint cluster region (BCR) gene on chromosome 22, creating the BCR-ABL1 fusion gene, which is a key feature of CML.
Objectives: To assess the prevalence and genetic anomalies of the Ph and BCR-ABL1 fusion gene in individuals diagnosed with CML. Besides, we aimed to investigate the relevance of CML corresponding with type of treatment, and the effectiveness of cytogenetics and molecular response with each other in addition to hematological response.
Materials and methods: Quantitative real-time polymerase chain reaction (qRT-PCR) for specific fusion genes and cytogenetic analysis for Ph chromosome was done for 55 previously diagnosed CML patients, who were attended at Anbar Cancer Center in Anbar Governorate, Iraq, between March 2023 and February 2024.
Results: In a study of 55 previously diagnosed CML patients, with a median age of 45. The commonest symptom was anemia (n = 50, 90.91%). The majority were male (61.82%). Hematological and molecular investigations revealed a significant improvement in the number of white blood cells (WBCs), the stability of red blood cells (RBCs), and hemoglobin (Hb) in the blood, accompanied by a reduction in the level of the BCR-ABL 1 gene according to the results of the qRT-PCR and a decline in the rate of abnormal cells shown by the karyotype.
Conclusion: Monitoring CML effectively involves hematological, molecular, and cytogenetic evaluations. qRT-PCR offers a more rigid analysis than karyotype. Complete blood count (CBC) parameters are crucial along with qRT-PCR for follow-up disease stages and guiding treatment decisions.
Keywords: Chronic myeloid leukemia; Breakpoint cluster region gene; Abelson 1 gene; Translocation; Philadelphia chromosome.

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INTRODUCTION

Myeloproliferative neoplasms (MPN) contain eight disorders, one of which is chronic myeloid leukemia (CML) which results from the exchange between breakpoint cluster region (BCR) gene and the Abelson (ABL) 1 gene [1]. When a cell in the bone marrow suffers damage in two chromosomes (9/22) this leads to form an abnormal gene called the Philadelphia. Furthermore, the accompanying fusion gene called BCR-ABL1 gene leads to the production of an abnormal protein, which leads to stimulates CML cells to raise and persist better than normal blood cells [2].

The BCR-ABL1 oncoprotein has uncontrolled tyrosine kinase activity which decontrols and decreases adherence of leukemia cells, cellular propagation and defends leukemic cells from apoptosis. On the other hand, the main treatment for CML is tyrosine kinase inhibitors (TKIs). These medicines

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work by stopping (switching off) the tyrosine kinase that the BCR-ABL1 gene makes [3, 4].

Imatinib (Gleevec) acts as a first-generation line for treating CML, while tasigna and bosutinib represent the second-generation line [5]. Further, the peripheral blood smear commonly demonstrates immature granulocytes, but in some patients with white blood cells (WBC) totals ($\leq 50\times10^9$/L) and even in some with higher counts, immature granulocytes may not be seen. Managing CML presents several challenges, delayed diagnosis, particularly in working-age adults, can result in advanced disease stages and higher morbidity. Treatment resistance is also a significant issue, with 20–30% of patients not responding as expected to TKIs. Additionally, diagnosing atypical forms of CML, such as lymphoid blastic crisis, can be difficult due to their resemblance to other hematological disorders [6].

The most sensitive test to measure the response of CML patients to treatment is a real-time polymerase chain reaction (RT-PCR) which can detect tiny amounts of the BCR-ALB1 gene even if other tests show no signs of CML in addition to cytogenetic techniques. The study highlights the importance of a comprehensive approach and a combination of hematological, molecular, and cytogenetic analyses in monitoring and follow-up of the CML patients. It highlights the need for personalized treatment and consistent monitoring to optimize disease management and increase patient therapeutic outcomes [7]. This study has been undertaken to detect the abnormalities in BCR-ABL1 fusion gene and the frequency of Ph chromosome. Also, to determine CML relevance with treatment type, hematological response and the effectiveness of cytogenetic and molecular response with each other.

MATERIALS AND METHODS

This prospective cross-sectional descriptive study included 55 previously diagnosed CML patients who were admitted at Anbar Cancer Center in Anbar Governorate, Iraq between March 2023 and February 2024. All patients were followed-up using hematological, molecular, and cytogenetic analyses for three periods (> 3, > 6, and > 9 months) following treatment with 1st generation drug, imatinib.

The research achieved compliance with the ethical principles of the Helsinki Declaration, 1979. The study protocol received approval from the Ethical Committee of the University of Anbar on February 23, 2023 (Reference number 140). Informed consent was obtained from all patients participating in the study. The patient can leave the study at any time.

The inclusion criteria included patients with a confirmed diagnosis of the chronic phase of CML based on hematological, molecular, and cytogenetic evaluations. They must be 18 years of age or older and should indicate the Ph or BCR-ABL1 gene. Hematological parameters must show elevated WBCs, as well as abnormal red blood cells (RBCs), and hemoglobin (Hb) levels indicative of anemia associated with the disease. Furthermore, patients should present common symptoms such as weight loss, abdominal pain, night sweats, or hair loss, which support the clinical diagnosis of CML. Patients were excluded if they had a diagnosis other than CML or secondary malignancies. Individuals younger than 18 years or those who decline to participate were also excluded. Furthermore, patients in any phase of CML other than the chronic phase, those who lacked complete baseline or follow-up laboratory data, were non-compliant with TKI treatment or did not have scheduled follow-up assessments at three periods (> 3, > 6, and > 9 months) were excluded from the study.

Hematological parameters including WBCs, RBCs, and Hb along with molecular assays including quantitative RT-PCR (qRT-PCR) for specific fusion genes and cytogenetic examination (Karyotype) for abnormalities. Moreover, peripheral blood samples were divided into two parts; the heparinized blood tube was used for culturing in cytogenetic analysis and Ethylenediaminetetraacetic acid (EDTA) treated blood tube was used for ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) extraction for molecular findings and these methods is explained as follow:

Cytological technique (Karyotyping)

During the examination, 20 to 50 metaphases per patient were analyzed. The International System for Human Cytogenetic Nomenclature (ISCN) guidelines were followed for reporting chromosomal abnormalities. Heparinized peripheral blood samples were cultured with Roswell Park Memorial Institute (RPMI)-1640 media in 1,500 µl sterilized cylinders, with 100 μl of colchicine added after 72 hours. Cells were then incubated for 30 minutes, centrifuged, and fixed with a methanol-acetic acid solution. A hypotonic solution of 0.075 M KCl was applied, and four drops of the cell suspension were placed on slides, which were allowed to dry at a 45° angle. Chromosomes were stained with Sorenson's buffer. Meta Class Karyotyping was used to analyze chromosomal structures and metaphases [8, 9].

Molecular technique (qRT-PCR)

The procedure was started by isolating RNA from EDTA-treated peripheral blood PB samples. Subsequently, RNA undergoes reverse transcription to generate cDNA through the action of reverse transcriptase. The reagents and conditions required for the steps, including a specific temperature profile for optimal reverse transcription which provided by the BCR-ABL M-bcr Real-TM Quant kit [10].

Hematological assay

It remains a basis in the monitoring of CML patients, providing valuable information about peripheral blood cell counts, differential leukocyte counts, and morphological changes in blood cells. A complete blood count (CBC) examination was analyzed for blood using a hematology analyzer. CBC analysis includes (WBC 10^9/L), (Hb g/L) and (RBC 10^9/L) [11].

Statistical analysis

The analysis of information from patients with CML was conducted using a version of Microsoft Office and data were studied using the statistical package for the social sciences (SPSS) statistical software version 29 (IBM SPSS Corp., Armonk, New York). The data analysis for this paper was generated using the Real Statistics Resource Pack software (Release 8.9.1), copyright (2013-2023) Charles Zaiontz. The Kruskal-Wallis H test (sometimes also called the "one-way ANOVA on ranks") is used as a rank-based nonparametric test that can be used to determine if there are statistically significant differences between two or more groups of an independent variable on a continuous or ordinal dependent variable. Further, Dunn's test is the appropriate procedure following a Kruskal–Wallis's test which works by adjusting the level of

statistical significance to account for the number of pairs of means being compared. This is necessary because the more pairs of means that are compared, the greater the chance that a difference between means will be found simply by chance. The statistically significant difference has been considered using a P-value of less than 0.05 as a statistical threshold.

RESULTS

A total of 55 previously diagnosed CML patients, the age ranged from 18 to 72 years, with a median age of 45 years. The highest age group affected was 36-53 years (n = 22, 40%)). There were 34 (61.82%) males with a male-to-female ratio of 1.62:1. The majority of patients were from rural areas (n = 33, 60%). The most common feature was anemia (n = 50, 90.91%), as shown in Table 1.

The H-ties values in Table 2 are slightly higher than the H-statistics but are relatively close (e.g., qRT-PCR H-ties = 40.061, H-stat = 39.978). This indicates that the correction for tied ranks has been properly applied and does not significantly alter the results. The close alignment between H-statistics and H-ties suggests that tied ranks did not substantially affect the statistical outcomes, confirming the robustness of the findings.

Dunn's test results indicate significant differences (P-value < 0.05) in molecular and cytogenetic measures between ($>$ 3 vs. $>$ 6 and $>$ 3 vs. $>$ 9 months) for qRT-PCR, WBCs, RBCs, and Hb (Table 3).

The karyotype profiles in CML patients evolve over time, with increased proportions of both normal karyotypes and Ph presence was represented in Figure 1.

In the current study, (47.27%) of patients achieved CMR as in Figure 2, indicating undetectable BCR-ABL levels. (10.91%) reached MLMR, while (41.81%) reached MMR. (78.18%) patients attained n-Stage, and (21.82%) achieved n/Ph+ stage.

Table 1. Demographic and clinical characteristics of 55 previously diagnosed chronic myeloid leukemia Patients.

Variables	Frequency	Percent
Age (years)		
18–35	20	36.36
36–53	22	40
54–71	11	20
>72	2	3.64
Sex		
Female	21	38.18%
Male	34	61.82%
Residence		
Urban	22	40
Rural	33	60
Sign and symptom		
Night sweating	27	49.1
Hair loss	7	12.73
Weight loss	42	76.36
Abdominal pain	29	52.73
Anemia	50	90.91
Liver enlargement	33	60
Spleen enlargement	25	45.46
Jaundice	20	36.36%
Treatment		
Bostinib	6	10.91%
Gleevec	48	87.27%
Tasigna	1	1.82%

DISCUSSION

CML represents MPN which alters about 1-2 cases per 100,000 adults from healthy persons into weak persons. Also, it appeared in approximately 15% of diagnosed cases of leukemia in adults according to the American Cancer Society [12]. The age distribution showed that the majority of the patients were between 36 and 53 years old, with a median age of 45 years. This was consistent with previous studies which have found that CML was more common in middle-aged adults [13, 14]. The gender distribution revealed a higher percentage of males (61.82%). This aligns with other studies [15, 16]. Furthermore, our finding is almost similar to the study of Asmaa et al. which revealed a male to female ratio of 1.3:1 [17]. Another study reported a male predominance among CML [18]. This could be due to hematological neoplasms are usually more common in males compared to females owing to genetic and hormonal differences [19].

Regular monitoring of hematological parameters is important for the optimal management of CML patients [20]. The number of WBCs ranges from 20,000/L and may exceed 500,000/L in most studies. In the context of hematological parameter reflection, the anemia percentage of (90.91%) agrees with recent studies conducted that most patients have normochromic/normocytic anemia at the time of diagnosis, whereas these studies reported that Hb levels were normal or even high [21], which mean the presence of leukemic cells can interfere with the bone marrow's ability to produce healthy RBCs, resulting in normocytic/normochromic anemia despite normal Hb levels [22].

Large Z-statistics in Table 3 for comparisons between $>$ 3 and $>$ 9 months suggest substantial changes in the parameters over this period, indicating a strong treatment effect and disease elimination. While, smaller Z-statistics for other comparisons imply that the differences are less pronounced between those time points. In addition, the similar Standard Error values across parameters indicate consistent reliability of the statistical estimates, ensuring that the comparisons made are accurate. Besides that, the consistency of R-Critical values confirms that the Z-statistics are evaluated against a stable threshold, reinforcing the significance of the differences observed. WBC showed significant reductions from qRT-PCR after 3 months to both 6 and 9 months, indicating improved response or treatment effect over time. This result was in agreement with a recent study from Iraq [23], which revealed that as a result of treatment of CML patients after three months with Gleevec, the mean of WBCs count reduced to 6,700/μL from 18,800/μL.

Moreover, Hb showed significant improvements from qRT-PCR after 3 months to 6 and 9 months, with no significant change between qRT-PCR after 6 months and qRT-PCR at 9 months, this result was in agreement with the study by Goh et al. [24] which suggested that between 6 and 9 months, levels stabilize, suggesting that although initial treatment is effective, further monitoring may not lead to significant changes. Further, the substantial differences in qRT-PCR detected by all tests suggest that the treatment periods have a substantial impact on qRT-PCR levels. Dunn's and D test's pairwise analysis reveals the exact nature of these differences, which

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Table 2. Kruskal-Wallis Test Evaluation Parameters.

Parameters	qRT-PCR			WBCs			RBCs			HB		
	> 3	> 6	> 9	> 3	> 6	> 9	> 3	> 6	> 9	> 3	> 6	> 9
Median	0.12	0.053	0.0025	13.2	7.9	7.6	3.72	4.4	4.6	11.3	12.4	12.6
Rank sum	6252.5	4332.5	3110	5960	4218	3517	3194	4670.5	5830.5	3537.5	4658.5	5499
Count	55	55	55	55	55	55	55	55	55	55	55	55
D.F	2			2			2			2		
P-value	Less than 0.005			Less than 0.005			Less than 0.005			Less than 0.005		
Significances	yes			yes			yes			yes		

Table 3. DUNN's and D test performance components. qRT-PCR = Quantitative real time polymerase chain reaction, WBCs = White blood cells, RBCs = Red blood cells, and Hb =Hemoglobin.

Groups (per month)	DUNN's test for qRT-PCR			DUNN's test for WBCs			DUNN's test for RBCs			DUNN's test for Hb		
	Rank Sum	Size	Rank Mean	Rank Sum	Size	Rank Mean	Rank Sum	Size	Rank Mean	Rank Sum	Size	Rank Mean
> 3	6252	55	113.68	5960	55	108.36	3194	55	58.07	3537	55	64.32
> 6	4332	55	78.77	4218	55	76.69	4670	55	84.92	4658	55	84.70
> 9	3110	55	56.55	3517	55	63.95	5830	55	106.01	5499	55	99.98
Z-Stat	1.959			1.959			1.959			1.959		
Groups (per month)	D test for qRT-PCR			D test for WBCs			D test for RBCs			D test for Hb		
	Rank Mean	Z-Stat	P-Value	Rank Mean	Z-Stat	P-Value	Rank Mean	Z-Stat	P-Value	Rank Mean	Z-Stat	P-Value
> 3 *vs.* > 6	34.91	3.836	0.0001	31.67	3.477	0.001	26.85	2.950	0.003	20.38	2.239	0.025
> 3 *vs.* > 9	57.14	6.278	0.0001	44.42	4.876	0.0001	47.94	5.268	0.0001	35.66	3.917	0.0001
> 6 *vs.* > 9	22.23	2.442	0.015	12.75	1.399	0.162	21.09	2.318	0.021	15.29	1.678	0.093
Std. error	9.100			9.108			9.099			9.104		
R-Crit	17.837			17.852			17.834			17.843		

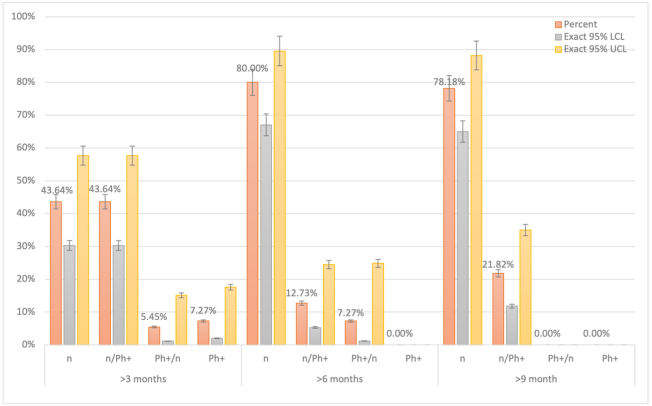

Figure 1. Cytogenetic monitoring (Karyotype). Statistical analysis based on lower confidence interval (LCL) and upper confidence interval (UCL).

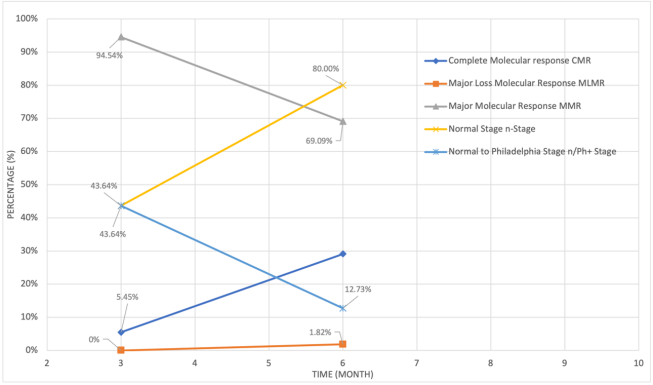

Figure 2. Molecular response state over follow-up periods.

could reflect changes in gene expression over treatment time. The lack of major difference between WBC counts after 6 and 9 months might indicate stabilization of WBC counts in the later stages of treatment. Also, RBC noteworthy pairwise differences across all comparisons suggest that treatment considerably affects RBC levels.

The elimination of Ph+ cases by > 6 months and > 9 months and Ph+/n cases by > 9 months as in Figure 1 indicates a high efficacy of TKI therapy in achieving major or complete cytogenetic responses. This is a positive indicator of treatment success and suggests that the TKIs is effectively

targeting and reducing the Ph+ cells. The exact 95% confidence intervals (LCL and UCL) provide a range within which the true proportion of each karyotype category lies. The intervals indicate variability in the data but also reinforce the trend of decreasing Philadelphia chromosome positivity over time, the 95% confidence intervals (LCL and UCL) provide a statistical framework indicating the reliability of observed trends in Ph+ cell reduction, reinforcing the effectiveness of TKIs over time according to the previous study [25].

As a consequence of following up on the patients' response to treatment for the qRT-PCR and karyotype analyses in Figure 2, it was found that monitoring of the BCR-ABL1 gene by qRT-PCR revealed itself as a method to further quantify residual disease in patients already in n-stage by karyotype analysis. Karyotype analysis may not detect all abnormal cells because it relies on examining cells during metaphase, a stage of cell division that is less frequent. Cells with low division rates might not be captured, leading to missed abnormalities. Other studies confirm that it is possible that there is a remnant of the BCR-ABL1 level even though the patient has reached the n-stage [18, 26]. Also, the current study showed higher treatment responses and decreased disease outcomes with Gleevec where 87.27% of total patients and those who did not respond to imatinib, as indicated in Figure 2, were transferred to second generation line of 10.91% for Bostinib and only 1.82% for Tasigna. A Parallel result reported that Gleevec treatment can stimulate a n-stage in a high percentage of CML patients [27, 28]. Other studies such as Oehler's study [29] have shown that a high proportion of patients who fail imatinib may benefit from switching to second-generation TKIs, with response rates to bosutinib and Tasigna often exceeding those observed in this study.

The study was constrained by several limitations: Spatial limitations restricted sample collection to Anbar Governorate, temporal limitations pertained to a one-year duration, and diagnostic limitations arose from the exclusive use of the hematological, karyotype, and qRT-PCR methods for patient follow-up.

CONCLUSION

Molecular analysis via qRT-PCR is more rigid than cytogenetic analysis via karyotype, especially during treatment when karyotype may miss abnormal cells due to low cell division (in metaphase). Observation of the BCR-ABL1 gene by qRT-PCR revealed itself as a method to further quantify residual disease in patients already in n-stage by karyotype analysis. Other studies confirm that it is possible that there is a remnant of the BCR-ABL1 level even though the patient has reached the n-stage. Hematological tests are also essential for diagnosing and confirming disease stages by evaluating WBCs, RBCs, and Hb levels. These comprehensive tests guide treatment decisions, including dosage adjustments and therapy changes for resistant cases.

ETHICAL DECLARATIONS

Acknowledgments

None.

Ethics Approval and Consent to Participate

The study protocol received approval from the Ethical Approval Committee of the University of Anbar on February 23, 2023 (Reference number 140). Informed consent was obtained from all patients participating in the study.

Consent for Publication

Not applicable (no individual personal data included).

Availability of Data and Material

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that there is no conflict of interest.

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Authors' Contributions

Al-Ouqaili MTS was responsible for conceptualizing, supervising, and conducting a formal analysis. Bahar NAHA had a role in the methodology and writing. Talib NM played a crucial role in the clinical investigation, providing clinical data, and specimen collection. All authors read and approved the final version of the manuscript.

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