Assessment of BCR /ABL variants and Survivin Gene Expression in Chronic Myeloid Leukemia Patients

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Abstract
Chronic Myeloid Leukemia (CML) is a condition where blood cells proliferate abnormally forming the Philadelphia chromosome (Ph). This chromosomal aberration arises from the fusion of segments from chromosomes 9 and 22 through a reciprocal translocation. The present study focused on detection of chromosomal abnormalities and the role of survivin expression in different stages of CML. This descriptive study was conducted over a period of 3 years which constitutes 55 diagnosed CML patients. Cytogenetic tests revealed 27 (49%) patients in chronic phase (CP) with the Ph chromosome including 1 case with additional Ph copy and also a unique case with three way translocations. In accelerated phase (AP), 7(13%) patients were Ph+ve and 1 case was with isochromosome 17q and extra Ph copy. In blast phase (BP), 5 (9.0%) cases were characterised by the presence of t(9;22) and 1 case showed abnormality like gain of X chromosome and Y chromosome loss.

Additionally, 9 (16%) cases were Ph-ve. FISH analysis showed varied fusion patterns, with 30(54%) cases having typical fusion,18 (33%) showing heterogeneous signal patterns. 7(13%) were categorized as normal hybridization pattern signals. In our current study, Kaplan–Meier analysis indicated higher survivin expression correlated with shorter survival and lower expression linked to longer survival in CML patients.

Keywords: BCR /ABL variant, Chronic myeloid leukemia, survivin gene.

Introduction

Chronic Myeloid Leukemia (CML) is a condition where myeloid cells abnormally proliferate and originate from bone marrow stem cells. This disease is marked by a characteristic genetic abnormality known as the Philadelphia (Ph) chromosome, resulting from the translocation (9;22) (q34; q11.2)[13]. This translocation leads to the fusion of the Abelson murine leukemia oncogene (ABL1) from chromosome 9q34 with the breakpoint cluster region (BCR) on chromosome 22q11, forming BCR/ABL1 gene fusion[14].

According to World Health Organization the disease advances through 3 defined phases namely chronic phase (CP) (presence of <10 % blasts), accelerated phase (AP), (10% to 19% blasts) and blast crisis phase (BP) (>20% blasts) in peripheral blood/bone marrow.

CML is commonly associated with the occurrence of Ph chromosome which is observed in approximately 90% of cases. This chromosomal abnormality is also referred as cytogenetic clonal evolution (CE), these non random instances serve as a significant indicator of disease progression and reduced life span[8]. Additional chromosomal aberrations become increasingly prevalent as the disease advances[2]. Structural chromosomal rearrangements such as translocations play a significant role in this process. Among CML patients, the most commonly occurring cytogenetic abnormalities by karyotyping include trisomy 8, trisomy 7, trisomy 19, isochromosome 17, gain of X chromosome, loss of Y chromosome, double Ph chromosome, three way translocation (3;9;22), 4 way translocation (9;12;19;22) and 5 way translocation (4;12;7;9;22)[3].

In CML Fluorescence *in situ* hybridization (FISH) confirms the occurrence of t (9;22) and depicts typical or atypical signal patterns. Different signaling patterns observed in FISH are 1R;1G;2F, 1R;1G;1F, 2R;1G;1F, 1R;2G;1F, 2R;2G;1F, 1R;2G;2F, 1R;1G;3F, 1G;3F, 2G;3F, 1G;4F, 1R;1G;4F and 1R;4F[21]. CML can be classified in to two distinct forms Typical and Atypical forms of CML. The typical form of CML is BCR-ABL positive and has the presence of Ph chromosome.

Atypical Chronic Myeloid Leukemia (aCML) is BCR-ABL negative whereas Ph chromosome is absent, characterized by poor survival[7]. In CML, the BCR-ABL constitutes the activity of tyrosine kinase and triggers various pathways that promote proliferation of cells. Consequently, BCR-ABL stimulates survivin expression at both the mRNA and protein levels, thereby suppressing the apoptotic mechanisms of CML cells[15]. Survivin, a protein of Inhibitor of Apoptosis (IAP) family, is involved in controlling the cell cycle and inhibits Apoptosis. Since survivin over expression is associated with CML progression, hence it is important to investigate about their underlying mechanism in CML[6]. The current study focuses to detect t (9;22) and the presence of additional chromosomal aberration across various stages of CML and explores their potential association with the Survivin gene expression.

Material and Methods

Fifty five clinically diagnosed CML patients were referred to the KSHEMA Centre for Genetic Services, KS Hegde Medical Academy, Mangaluru, Karnataka, India for

cytogenetic and FISH analysis. Written informed consent from all the patients was obtained after the approval from central ethics committee of the NITTE (Deemed to be University) (Ref: NU/CEC/2021/168). For karyotyping and FISH analysis, 2ml of bone marrow aspirate or peripheral venous blood was collected in Sodium Heparin vacutainer and 4ml of peripheral venous blood was collected in EDTA vacutainer for survivin gene analysis.

Chromosome analysis by Karyotyping: The appropriate sample was combined with 5ml of bone Marrow media (Gibco, USA), based on the white blood cell count. This mixture was then placed in a CO_2 incubator at 37°C for 24 to 48 hours. After the incubation period, the culture underwent treatment with 100µl of Karyo MAX colcemid (10 µg/mL, Gibco) for 20 minutes, after which sample underwent centrifugation at 2000rpm for 10 minutes. Supernatant was removed and the resulting cell pellet was treated with a hypotonic solution (0.075 M KCL) at 37°C for 20 minutes. Subsequently, the cell pellet was fixed using Carnoy's fixative (methanol and acetic acid in a ratio of 3:1). The fixed cell pellet was allowed to age overnight at 60°C after dropping on to the slides. Subsequently GTG banding was performed using 0.05% trypsin and 1% Giemsa stain.

Well-banded 20 spreads were captured using the Olympus BX53 bright field microscope (Tokyo, Japan). The captured metaphases were analyzed using GenASIs software (Applied Spectral Imaging, Edingen – Neckarhausen, Germany). The interpretation of results followed the guidelines outlined in the International System of Human Cytogenetic Nomenclature (ISCN 2013)[16].

FISH Analysis: The FISH analysis was carried out on the cell pellet for the probes targeting BCR-ABL1 t(9;22). Two drops of concentrated pellet fixed in Carnoy's fixative were dropped on to the marked area of the slide and kept on a hot plate at 45°C until the slide was dried. The slides were immersed in freshly prepared 2x SSC solution for 5 minutes. Following this, they were subjected to different alcohol gradients 70%, 85% and 100% each for 2 minutes, ensuring proper dehydration. Subsequently, the slides were left to air dry at room temperature. A 5µl FAST FISH probe was carefully applied to the designated hybridization area on the dried slides, securely sealed with glue. These prepared slides were then positioned in a Start Spin Thermobrite chamber. Here, the pellet and probe underwent co-denaturation at 88°C for 2 minutes,followed by an overnight hybridization process at 45°C.

Post Hybridisation: The slides underwent a series of steps to ensure optimal conditions for analysis. Initially, they were immersed in 2X SSC for 1 minute, followed by 0.4XSSC placed in a water bath at 68°C for 2minutes further followed by preheated deionized water for 1 minute at 37°C. Then 4µl of 4,6- diamino-2-phenylindole (DAPI) was added as a counterstain. The total number of interphase nuclei scored were 100. In order to effectively capture the diverse variations in FISH signals, more than 100 interphase cells are captured. FISH signals were observed using an Olympus BX53 Fluorescence microscope equipped with DAPI, fluorescein isothiocyanate (FITC) and sulforhodamine 101 acid chloride Texas Red filters. The metaphases and interphase nuclei were scored and the signals were captured using FISH View image acquisition (GENASIS, Applied Spectral Imaging) software (ISCN – 2013).

BCR-ABL dual probe (Wuhan Healthcare Biotechnology, China) utilizes fluorescent labels to detect specific sequences related to CML. In the absence of the BCR-ABL fusion, under fluorescent microscopy, two orange-red signals and two green signals (2R, 2G) typically appear, representing the normal hybridisation pattern.

However, in CML during reciprocal translocation, the BCR gene from chromosome 22 fuses with ABL gene from chromosome 9. This fusion event results in development of hybrid BCR-ABL gene. As a result, probes bind producing a distinct signal pattern under fluorescence microscopy. Specifically, the probes recombine to create a yellow fusion signal, characterized by one yellow signal (representing the fusion of BCR and ABL), one red signal (representing the ABL gene) and one green signal (representing the BCR gene), denoted as (1F, 1R, 1G).

RNA isolation: Isolation of RNA was carried out from peripheral venous blood samples by RNA Iso Plus kit from Takara Japan following the manufacturer's guidelines. RNA was quantified by nano-drop spectrophotometer (Eppendorf).

Synthesis of Complementary DNA (cDNA): The synthesis of cDNA was performed by Primescript 1st strand kit Cat-RR037A, Takara, using the provided instructions in the kit. This mixture underwent incubation at 37°C for 15 minutes and then at 85°C for 5 minutes in a thermocycler. cDNA was stored at -20°C until use.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR): SYBR Premix Ex Taq II was used according to manufacturer's guidelines, Takara.The PCR master mix contained SYBR Premix, cDNA template, ROX reference dye (50x), each of gene-specific forward and reverse primers and molecular grade water. Quant Studio 3 (Applied Biosystems, Thermo Fisher Scientific, USA) was used with thermocycler conditions:10 minutes of initial denaturation at 95°C were preceded by step of denaturation for 30 seconds at 94 °C (40 cycles), annealing at 55.5°C for 60 seconds, 72°C elongation for 90 seconds. 10 minutes at 72°C was the last extension. GAPDH served as the housekeeping gene and target gene expression levels were stated as fold expression. To calculate relative mRNA expression levels, $RQ=2-\Delta\Delta Ct$ method was used. Gene names and corresponding primer sequences in the study are detailed in table 1. The triplicate samples ensured reliability[10].

Table 1
Genes with primer sequences, size and references

S.N.	Gene	Sequence (5'-3')	Size (bp)
1	GAPDH[11]	F: GTCAGCCGCATCTTCTTTTG	100
		R: GCGCCCAATACGACCAAATC	
2	Survivin[4]	F: ACCAGGTGAGAAGTGAGGGA	305
		R: AACAGTAGAGGAGCCAGGGA	

Table 2
Variations in FISH signal pattern

	CP	AP	BP
COMPLEX (N=18)	2F,1R,1G/1F,1R,1G/2R,2G (N=1)	1F,1G,1R/1F,1R,2G/2R 2G(N=1)	1F,1R,2G/2F,1R,1G (N=1)
	2F,1R,1G/1F,1R,1G/1F,2R,2G (N=1)	1F,1R,1G/2R,2G (N=1)	3F,1R,1G/2F,1R,1G (N=1)
	2F,1R,1G/ 1F,1R,2G,/1F,2R,2G/ 1F,1R,1G/ 2R,3G1F,2R,1G/ 2R,2G(N=1)	1F,3R,1G/1F,1R, 1G(N=1)	2F,1R,1G/1F,1R,1G /2R,2G (N=1)
	1F,2R,1G / 1F,1R,1G (N=1)	3F,1R,1G/2F,1R, 1G(N=1)	
	1F,2R,2G/2F,1R,1G (N=2)		
	1F,1R,1G / 2R,2G (N=2)		
	2F+1R+1G/1F,1R,1G/ 1F,2R,1G (N=1)		
	2F,1R,1G /1F,2R,2G (N=1)		
	2F,1R,1G / 1F,1R,1G (N=1)		
TYPICAL (N=30)	2F,1R,1G(N=19)	2F,1R,1G(N=4)	2F,1R,1G (N=2)
	1F,1R,1G(N=4)	1F,1R,1G(N=1)	

Statistical analysis: Chromosome and FISH analysis data were expressed in frequency and percentages. The cut-off points for expression of survivin were confirmed by Receiver Operating Characteristic (ROC) analysis. Subsequently, these values were applied in Kaplan-Meier analysis to assess Overall Survival (OS). Comparisons were made using the log-rank test. Statistical significance was defined by $P < 0.05$. Analysis of data was performed using SPSS version 16.0 USA.

Results

Cytogenetic analysis for 55 CML was undertaken to detect the presence of Ph +ve and Ph -ve cells. Among these patients, 34(62%) were males and 21(38%) were females. The distribution of CML according to disease phase was as follows: the CP were 40 (72%), AP were 9(16%) and 6 (12%) were in BP respectively.

In CP, 27(49%) patients exhibited the presence of Ph +ve and 1 patient displayed an additional Ph copy of the chromosome derivative 22 and another exhibited three way translocation involving 9,22 and 21. In the AP,7(12.7%) were Ph +ve out of which 1 case showed i(17q) with additional Ph copy of the chromosome. In the BP,5(9.0%) cases exhibited t(9;22) while one patient revealed chromosome anomaly involving gain of X and missing of Y chromosome. Furthermore, one case displayed random missing of chromosomes. In addition to these cases, 9 (16.36%) were identified as Ph -ve atypical CML representing a distinct subset. Moreover 7(13%) were categorized as culture failure cases.

FISH analysis was carried out for all 55 samples, out of which, 30(54%) had typical signal fusion and 18(33%) cases had diverse variations in the signal patterns and are listed as in table 2.

The survivin gene expression for 55 CML patients is shown in different phases of CML. In the CP, survivin gene expression levels were measured at 57.70 ± 12.26 whereas in the AP and BP, the levels increased significantly to 130.5 ± 34.03 and 158.7 ± 52.88 respectively. In comparison, controls exhibited a expression level of 4.02 ± 1.8 (N=20). Survivin gene expression was more expressed in phases as the disease progressed to the later stages. The expression is high in cases compared to controls. Statistical significance was found between control and all stages of CML ($P<0.0001$) (Fig. 3A).

Based on the chromosomal abnormalities, the survivin gene expression in typical form of CML was 70.80± 13.16, while in atypical forms of CML, it was notably higher at 81.52 ± 29.53. This indicates that survivin gene expression is elevated in atypical forms compared to typical forms of CML (Fig. 3B).

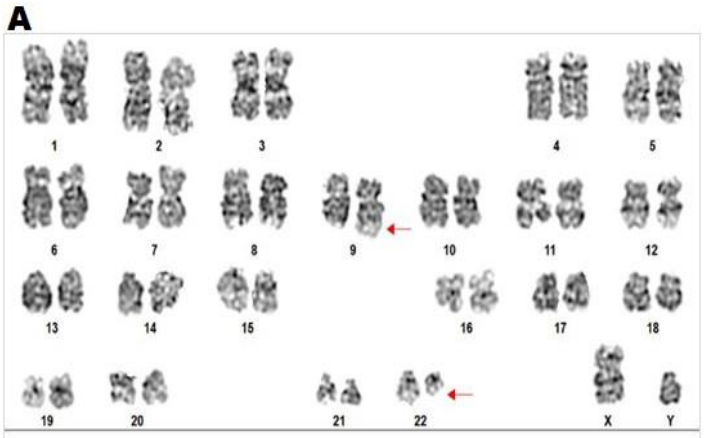

Fig. 1A: 46, XY, t (9;22) (q34;q11.2);

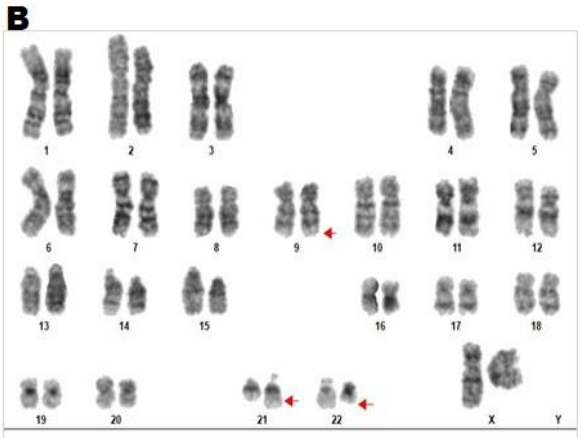

Fig. 1B: 46,XX t (9;22;21)

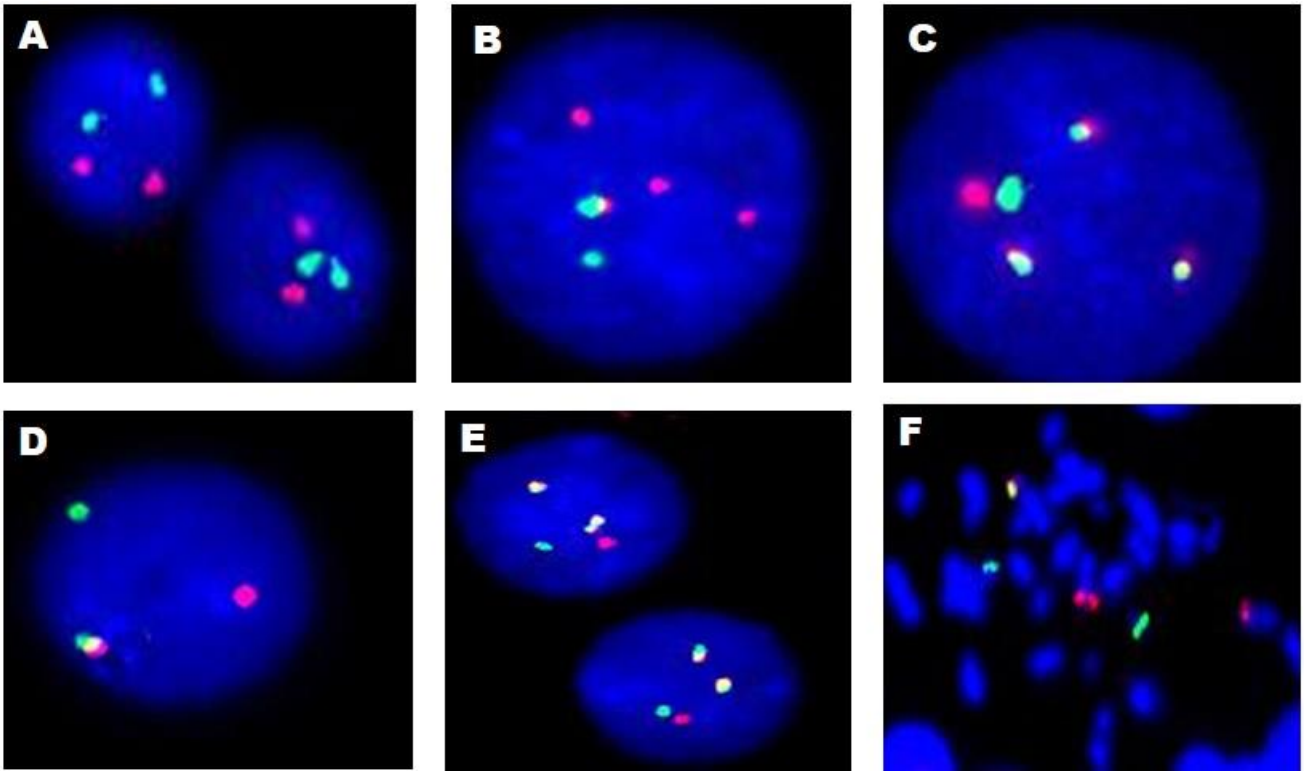

Fig. 2: A. FISH performed on Interphase cells 2R; 2G signals. B. 1F; 3R; 1G Signals. C. 3F; 1R; 1G signals. D. 1F; 1R; 1G signals. E. 2F; 1R; 1G signals F. 1F; 2R; 2G signals.

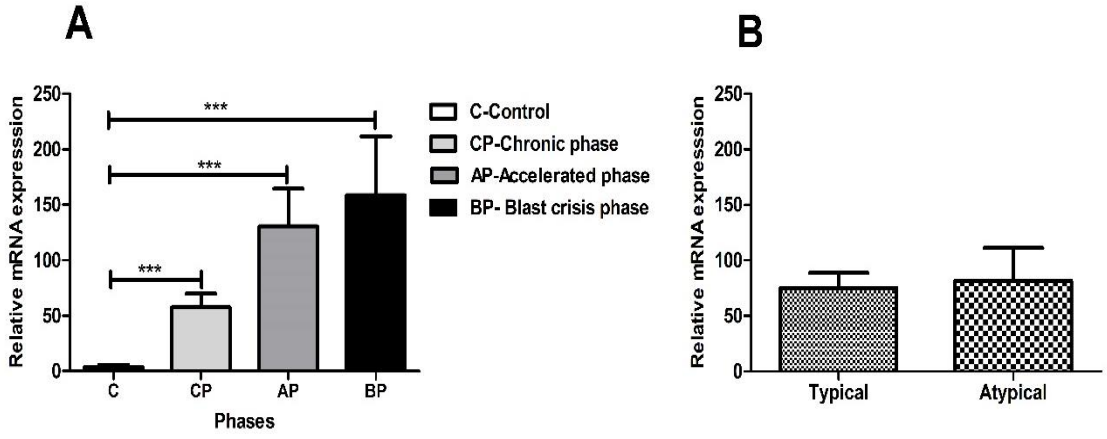

Fig. 3: A. Survivin levels in different stages; B. Survivin gene expression in typical and atypical pattern based on chromosomal abnormalities

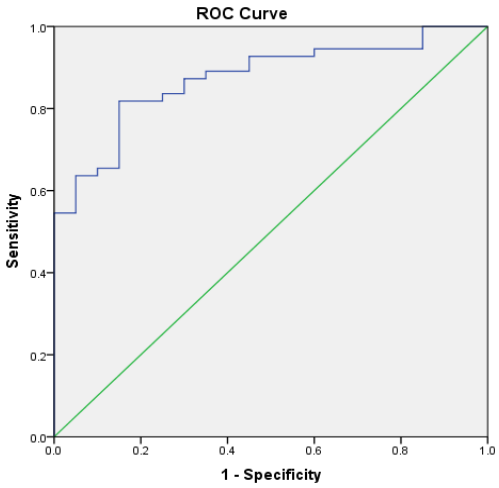

Fig. 4: Receiver Operating Curve (ROC) for the survivin Cq Values obtained from RT-qPCR

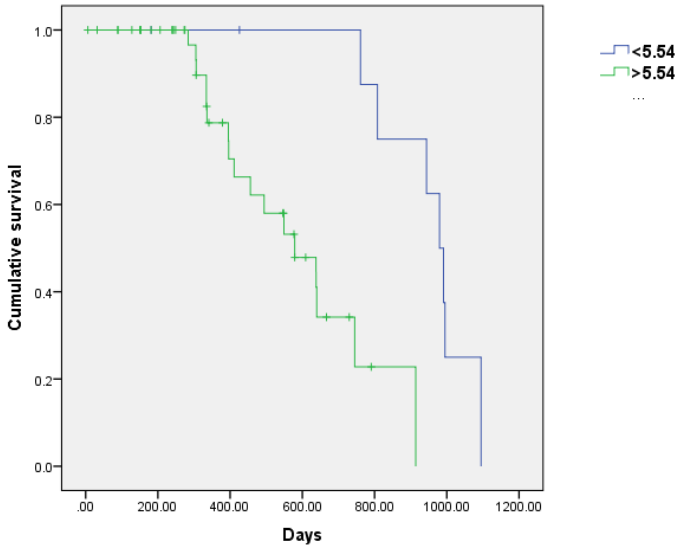

Fig. 5: Kaplan Meir Overall survival (OS) for the survivin Cq Values obtained from RT-qPCR

The obtained cut-off value was highly statistically significant (AUC=0.87; P<0.0001) based on ROC analysis using GAPDH and relative expression of the survivin gene obtained from RT-qPCR. A cut-value of 5.54 was determined, dividing patients into higher and lower expression groups. Among the 55 patients, survivin gene expression was found to be highly expressed in 45 (81.8%) patients and lower expression in 10 (18.1%) patients (Fig 4).

Kaplan-Meier analysis was conducted to explore the association between survivin expression and survival of patients. The curves of survival indicated that cases with high survivin expression experienced shorter survival days whereas those with low survivin expression had longer survival days which was statistical significant (P<0.0001) (Fig. 5).

Discussion

CML diagnosis primarily relies on the occurence of t(9;22). In our study had 39(71%) displayed this standard translocation. This translocation leads to development of Ph chromosome noted in 90% of CML patients, resulting in the BCR-ABL fusion gene, as elucidated by Vaidya et al[17]. Regarding the distribution of chromosomal abnormalities across disease phases, in the CP, 27 (49%) patients showed the presence of the Ph chromosome, with one patient exhibiting an additional copy of the derivative 22 chromosome.

Notably, Vinhas et al[18] reported a case with an additional Ph chromosome alongside trisomy 8 and derivative 22, a finding consistent with our study. Moreover, one case in our study demonstrated a three-way translocation involving chromosomes 9, 22 and 21. In the AP, 7(13%) were Ph +ve, out of which 1 case showed i(17q) with additional Ph copyof the chromosome.

The presence of i(17q) is characteristic feature of AP. This was consistent with existing literature. It was also stated that the disease may evolve into BP which is associated with the

emergence of new genetic alterations such as trisomy 8, an double Ph chromosome, i(17q) and trisomy 19. While involvement of i(17q) in the advancement of CML has been documented, its precise contribution to BP development remains uncertain. In the BP, 5(9.0%) cases were characterized by the presence of t(9;22) while one patient revealed chromosome anomaly involving gain of X and missing of Y chromosome. Notably Wang et al[19] reported the missing of Y chromosome which is a good prognosis which was similar to the present study.

Furthermore, one case displayed random missing of chromosomes. Wafa et al[20] reported minor percentage of CML patients showing missing of chromosomes such as 7, 17 and 19 which was inconsistent with the present study.

The missing of chromosome 7 was similar to the current study. In addition to these cases, 9(16%) were identified as Ph –ve, representing a distinct subset. Moreover 7(13%) were categorized as culture failure cases. Additionally, FISH analysis carried out for all 55 samples, out of which, 30(54%) had typical signal fusion and 18(33%) cases had diverse variations in the signal patterns. And also atypical patterns were observed in this study such as 1F, 2R, 2G, 1F, 1R 2G, 3F, 1R, 1G with variations.

Zang et al[21] reported similar patterns of BCR-ABL1 signals presenting complexities and diversityin BCR-ABL1 signal patterns, aligning closely with the findings of our study.The signal patterns 2R, 3G and 1F, 3R, 1G were identified in the current study. Our study focused to elucidate the survivin gene expression in CML patients across different phases of CML in comparison to control group. Notably, expression of survivin was significantly higher in cases. When it was compared with controls, statistical significance was observed between the control group and all stages of CML .According to Conte et al[6] survivin gene expression is markedly elevated in a majority of CML patients across all disease phases, contrasting with low expression levels observed in healthy controls. Notably, survivin levels were significantly increased in the AP and BP. This was consistent with the existing literature.

Based on the chromosomal abnormalities the survivin gene expression was elevated in atypical forms compared to typical forms of CML. Badran et al[5] reported expression of survivin in 12 CML patients. Among 6 patients in CP, none showed detectable survivin transcripts. In contrast, all 4 Ph +ve patients in BP (100%) exhibited significant survivin expression levels. In contrast, survivin expression was notably reduced in 2 aCML patients during BP.

In our study, the analysis of overall survival revealed that higher expression of the survivin gene corresponded to lower survival rates while lower expression was associated with higher overall survival rates. This is similar with the study of Mori et al[12] who demonstrated Kaplan-Meier survival curves for acute leukemia patients, revealing that those with survivin expression had notably lower leukemia-free survival rates at 35 months compared to those without survivin expression.

Conclusion

Chromosomal translocations play a crucial part in the pathophysiology of CML. In addition to the well-known Ph, CML has also been linked to additional chromosomal abnormalities. These translocations are beneficial in designing an effective therapy in the future. Combining methods like karyotyping and FISH allows researchers to better understand the genetic changes in CML patients, to identify new translocations and variant fusion signal pattern may be linked to different stages of the disease to improve diagnostic and prognostic results.

The detection of survivin in CML samples could serve as a novel biological marker for assessing clinical outcomes and treatment responses. Upon validation at a larger scale, it could emerge as a significant candidate for developing innovative therapeutic strategies. Hence, assessing survivin gene expression across various phases of CML may elucidate the impact of the progression of the disease and treatment efficacy.

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(Received 13th July 2024, accepted 20th August 2024)