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ORIGINAL ARTICLE

FISH versus real-time quantitative PCR for monitoring of minimal residual disease in chronic myeloid leukaemia patients on tyrosine kinase inhibitor therapy

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Abstract

Introduction: BCR-ABL fusion gene, the oncogenic driver of CML, results from a translocation between short arms of chromosome 9 and 22. Monitoring of CML patients during treatment is essential, not only for tailoring the treatment but also to detect early relapse to enable timely intervention. Commonly used methods for detection of residual disease are conventional karyotyping, FISH and molecular methods. In this study, we compared FISH with QRT-PCR for detection of residual disease in CML. *Materials and Methods:* CML patients on tyrosine kinase inhibitor (TKI) therapy and on regular follow up at University Kebangsaan Malaysia Medical Center (UKMMC) were selected. A comparative study was conducted between FISH and QRT-PCR for *BCR-ABL* transcripts at diagnosis and during follow-up. *Results:* There was good correlation between FISH and QRT-PCR for *BCR-ABL*. At 6th month of follow-up post diagnosis, FISH had a sensitivity of 83.3% and specificity of 65.2% (k >0.339, p<0.033). At 12th month, the sensitivity of FISH was 83% and the specificity was 59.1% (k >0.286, p <0.065). Similarly, at the 24th month, FISH had a sensitivity of 100% and specificity of 68.8% (k >0.642, p<0.000). *Discussion:* Early achievement of major molecular response (MMR) and complete cytogenetic remission (CCyR) were reliable predictors of long-term maintenance of molecular remission.

Keywords: QRT-PCR, chronic myeloid leukaemia, FISH, BCR-ABL, MMR

INTRODUCTION

CML is a myeloprolifrative neoplasm characterised by a balanced genetic translocation, t(9;22)(q34;q11.2), involving a fusion of the Abelson gene (*ABL*) from chromosome 9q34 with the breakpoint cluster region (*BCR*) gene on chromosome 22q11.2.[1] The molecular consequence of this translocation is the generation of a *BCR-ABL* fusion gene, which in turn translates into oncogenic *BCR-ABL* tyrosine kinase.[2] In CML, *BCR-ABL* is found in all myeloid lineages and in some lymphoid and endothelial cells.[3] This oncoprotein is the main driving force in CML(4–6). CML can be diagnosed from peripheral blood or bone marrow sample using the conventional karyotyping to identify Philadelphia (Ph) chromosome, molecular cytogenetics also known as fluorescence in-situ hybridisation (FISH) analysis for detection of *BCR-ABL* or by molecular methods that includes real-time qualitative polymerase chain reaction (RT-PCR) or quantitative real-time polymerase chain reaction (QRT-PCR) for *BCR-ABL* transcripts.[4-6] Bone marrow karyotype analysis is the only method that allows for the evaluation of all chromosomes and is recommended at diagnosis to identify the presence of additional cytogenetic abnormalities of prognostic significance.[7,8] Bone marrow morphologic analysis is also necessary to identify the disease phase.[7] Patients who are diagnosed at accelerated phase (AP) have inferior responses to all tyrosine kinase inhibitors TKIs compared to chronic phase (CP) patients, while patients at blast phase are treated as acute leukaemia.[9,10]

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Tyrosine kinase inhibitors (TKI) are the novel drugs that target the *BCR-ABL* oncogenic tyrosine kinase protein and are used to treat CML patients in chronic phase.[4,11] Patients on TKI therapy are monitored at regular intervals with the aim to adjust the dose, change the type of TKI and early detection of non-responders, accelerated phase (AP) or blast phase (BP).[8,12] The therapeutic targets in CML patients on TKI therapy are complete hematologic remission (CHR), complete cytogenetic remission (CCyR) and major molecular remission (MMR).[13-15] Studies have shown that *BCR-ABL* transcript levels decline continuously in patients with CML chronic phase (CP) on TKI therapy for more than five years and approximately half of all first-line treated patients have stable undetectable *BCR-ABL* using strict sensitivity criterias.[15,16]

Majority of the studies on CML treatment with tyrosine kinase inhibitors (TKIs) focus on efficacy, particularly the molecular response and outcome.[5] Recent studies implement the highly sensitive and specific QRT-PCR techniques in the assessment of the disease in terms of MMR, which is now harmonised across the world through the international system (IS) for harmonisation of QRT-PCR for *BCR-ABL*.[17-20] The IS relies upon two specific concepts, the standardised baseline, or IS 100%, which is, by definition, the median pre-treatment level of *BCR-ABL* RNA in early chronic phase CML (as defined in IRIS imatinib trial), and MMR is a 3-log (1,000-fold) drop from the baseline value.[20-21] A level of IS 1-2% roughly corresponds to the threshold for CCyR that precedes MMR while following MMR, a "complete molecular remission" was defined as undetectable *BCR-ABL* transcripts, that is, below the sensitivity of the assay.[18] However, the desirable test sensitivity is the achievement of MMR.[5,21]

Failing to achieve such milestones are associated with an increased risk of worse long-term outcomes, such as loss of response, disease progression, or death.[22,23] With ongoing treatment, patients not in CCyR face a decreasing probability of ever achieving an MMR and increasing risk of disease progression.[23,24] Available data, therefore, support treatment recommendations based on achieving defined levels of response within a specified duration of treatment.[25,26] Three TKIs (Imatinib, Nilotinib, Dasatinib) are recommended first-line.[27-29] Similarly, the novel agents; Bosutinib and Ponatinib are available second-line.[10,18-20] Allogeneic transplantation remains an important therapeutic option especially for young patients with CML-CP who have failed to achieve MMR with at least 2 TKIs, and for all patients in advanced phase disease.[30-32]

The aim of the present study was to compare FISH analysis with QRT-PCR for detection of BCR-ABL at diagnosis and then 6th, 12th and 24th months after diagnosis of CML patients treated with Imatinib or Nilotinib. We also aimed to determine the role of "time to MMR" and "time to CCyR" as predictors of long-term maintenance of molecular remission.

MATERIALS AND METHODS

Sample selection

This was a retrospective comparative study with prospective follow-up, which was carried out at UKM Medical Centre Kuala Lumpur between January 2006 and September 2017. Thirty-nine CML patients, who were diagnosed to have *BCR-ABL* fusion gene by FISH and QRT-PCR and had regular follow-up at 6th, 12th and 24th month post diagnosis, were selected. The study was approved by the Ethical Committee, University Kebangsaan Malaysia (UKM) Medical Center (Ethical approval code: UKM FPR.4/244/FF-2016-045). All CML patients were started and continued on Imatinib therapy. At diagnosis, bone marrow aspirate samples and during follow-up peripheral blood samples were collected in EDTA (3ml) tubes for routine morphologic assessment and molecular analysis. For FISH analysis the bone marrow aspirate samples were collected in the in-house transport medium (consisting of Roswell Park Memorial Institute (RPMI) medium, heparin and foetal calf serum) while peripheral blood samples were collected in lithium heparin (4 ml) tubes.

Fluorescence In-Situ Hybridisation (FISH)

Peripheral blood or bone marrow aspirate samples were sent to Cytogenetics laboratory and were processed following standard procedure. FISH was performed using LSI *BCR/ABL* dual colour, dual fusion translocation probe from Vysis, according to the manufacturer's instruction. Cells with translocation t (9;22) exhibited a two green/orange fusion signal in addition to one separate green signal and one separate orange signal, whereas normal cells showed a two orange and two green signal patterns. At least 200 cells (metaphases and nuclei were analysed in each case, a value of <5% positive cells were considered as background signal, not significant to make a diagnosis of CML.[33]

Quantitative Reverse Transcription-Polymerase Chain Reaction (QRT-PCR)

RNA was extracted from EDTA blood samples of CML patients using RNA extraction kit (TRIzol, RNA-STAT; Invitrogen, Carlsbad, CA, USA). The blood and bone marrow aspirate samples were treated with RNAase free DNAase (Qiagen, Valencia, CA, USA). A rapid 3-hour, two–step standardised QRT-PCR was performed using LightCyler Technology (Roche Applied sciences; Indianapolis, IN, USA), where the level of fusion gene expression is normalised against standard ABL1 to compensate for variations in the quality of RNA and the efficiency of the reverse transcription reaction. The QRT-PCR ratio was obtained by dividing the amount of *BCR-ABL* signal by the amount of control gene ABL1 (*BCR-ABL/ABL*). Forward primers located in the BCR exon e1 and b2 were combined with the reverse primer in the ABL exon a4. The combined primer set for BCR-ABL can detect the e13a2 and e14a2 (210 kda) fusion transcripts, which cover >95% of the described chimeric *BCR-ABL* fusion gene product. In CML as a positive control, 100pg of mRNA of the cell line K562 was used per test (Roche Applied Sciences). Negative result was defined as absence of a positive fluorescence signal beyond background in the LightCycler quantification, and positive result was defined as the detection of a specific fluorescence signal. The standardisation of the assay and quality control were performed as recommended by Roche.

Statistical Analysis

Cross-table statistics were used for the calculation of sensitivity and specificity of FISH analysis in comparison to QRT-PCR for *BCR-ABL* transcripts at diagnosis and then 6th month, 12th month and 24th month post diagnosis. Cohen's Kappa agreement (k) with probability value (*p*-value) was calculated between FISH analysis and QRT-PCR at 6th month, 12th month and 24th month post diagnosis. All the analyses were performed using Statistical Package for Social Sciences (SPSS) software. Bar charts were plotted using Microsoft Excel.

Standard receiver operating characteristics (ROC) curves were plotted under the non-parametric assumption, considering time (in months) to achieving CCyR and MMR as the test variables and sustained molecular remission beyond 48 months as a state variable. Area under the curves for time (in months) to CCyR and MMR were calculated using non-parametric method based on construction of trapezoids under the curves to approximate the area, considering asymptotic significance (*p*-value) <0.05.

RESULTS

Out of the 39 selected patients, 36 were diagnosed in CP and three were diagnosed in accelerated phase. Molecular analysis using QRT-PCR and RT-PCR identified *BCR-ABL* major fragment in all of the patients. For the selected 39 patients, a total of 113 comparative studies were carried out between FISH and QRT-PCR for *BCR-ABL* at diagnosis, 6th months, 12th months and 24th months post diagnosis, as shown in Table 1 and 2. For FISH analysis, positive and negative results were defined by the presence or absence of fluorescent signals for *BCR-ABL* fusion, respectively. The results of FISH analysis were compared with the molecular targets, as defined by MMR (<0.05 copies of BCR-ABL transcripts by QRT-PCR for *BCR-ABL*). At diagnosis, 29 comparative studies were carried out which showed 100% concordance between FISH analysis and QRT-PCR for *BCR-ABL*. At 6th month after diagnosis 29 comparisons were done between FISH and QRT-PCR for *BCR-ABL* which revealed a sensitivity of 83.3% and specificity of 65.2% with good agreement (k >0.339) between FISH and QRT-PCR (p <0.03). At 12th Month after diagnosis, 28 comparisons were done between FISH analysis and QRT-PCR for BCR-ABL that showed a sensitivity of 83% and specificity of 59.1% with moderate agreement (k >0.24, p <0.03). At 24th month after diagnosis 27 comparisons were done between FISH analysis vs QRT-PCR for *BCR-ABL* that showed a sensitivity of 100% and specificity of 68.8% having good agreement (k >0.698, p <0.001).

As shown in Fig. 1, the early achievement of molecular target i.e MMR was a reliable predictor of long-term maintenance of molecular remission at or beyond 48 months. Early achievement of cytogenetic target i.e. CCyR was less satisfactory in predicting the long-term maintenance of molecular remission, as shown in Fig. 2. This was further supported using ROC curves, which demonstrated that early achievement of MMR (defined by QRT-PCR for *BCR-ABL* ≤0.05 copies) was an excellent predictor of maintenance of molecular remission MR beyond 48 months (defined by QRT-PCR for *BCR-ABL* ≤0.05 copies or undetectable *BCR-ABL* by RT-PCR) with an area under the curve = 0.923 (p <0.001), followed by the time to achieve CCyR (defined

TABLE 1: Sample demography and description of MR (molecular remission), MMR (major molecular response), RT-PCR (real-time qualitative PCR for *BCR-ABL)*, QRT-PCR IS% (*BCR-ABL* transcripts by real-time quantitative PCR in international scale percentage) and FISH (fluorescence in-situ hybridisation for *BCR-ABL)*.

No	Age and sex	Status, treatment	Year	Molecular status at >48 months	FISH%	QRT-PCR IS%	RT-PCR
1	82, Male	Diagnosis	2007	MR	97	1.4	
		3 months	-		81		Detected
		6 months	-		0	0.052	
		12 months	-		0	0.049	
		24 months	-		0	0.02	
		Follow up	2017		0		Not detected
2	77, Male	Diagnosis	2012	MR	NA	166.93	
		3 months	-		10.5		Detected
		6 months	-		0	NA	
		12 months	-		0	0.387	
		24 months	-		0	0.045	
		Follow up	2017		0		Not detected
3	32, Male	Diagnosis	2008	MR	80.5	67.9	
		3 months	-		57.5		Detected
		6 months	-		0	43.61	
		12 months	-		0	32.75	
		24 months	-		0	0.008	
		Follow up	2017		0		Not detected
4	31, Male	Diagnosis	2011	No MR	76	189.5	
		3 months	-		0		Detected
		6 months	-		0	0.91	
		12 months	-		0	0.92	
		24 months	-		0	0.92	
		Follow up	2017		0		Detected
5	21, Female	Diagnosis	2013	No MR	78	83.48	
		3 months	-		80		Detected
		6 months	-		0	0.183	
		12 months	-		0	0.02	
		24 months	-		0	0.282	
		Follow up	2017		0		Detected
6	24, Male	Diagnosis	2011	MR	55.5	27.48	
		3 months	-	Post Allogenic	31		Detected
		6 months	-	PBSCT	0	0.26	
		12 months	-		60	0.607	
		24 months	-		87.2	0.936	
		Follow up	2017		NA		Not detected
7	22, Female	Diagnosis	2010	No MR	91	6.61	
		3 months	-		57		Detected
		6 months	-		21	1.25	
		12 months	-		12	0.58	
		24 months	-		3	0.222	
		Follow up	2017		NA		Detected
8	22, Male	Diagnosis	2007	MR	84	94.32	
		3 months	-		72	23.6	Detected
		6 months	-		75	0.99	
		12 months	-		49	3.3	
		24 months	-		0	0.027	
		Follow up	2017		0		Not detected
9	40, Female	Diagnosis	2007	No MR	48.6	48	
		3 months	-		77	31	Detected
		6 months	-		62	45	
		12 months	-		65	18.54	
		24 months	-		41	37.37	
		Follow up	2017		NA		Detected

No	Age and sex	Status, treatment	Year	Molecular status at >48 months	FISH%	QRT-PCR IS%	RT-PCR
10	57, Male	Diagnosis	2007	No MR	100	20.7	
		3 months	-		100	12.3	Detected
		6 months	-		84.5	84.5	
		12 months	-		92.1	17.22	
		24 months	-		25.6	29.29	
		Follow up	2017		NA		Detected
11	49, Male	Diagnosis	2008	No MR	90	95.6	
		3 months	-		99	22.18	Detected
		6 months	-		69.3	8.7	
		12 months	-		71.5	73.1	
		24 months	-		70	72.13	
		Follow up	2017		NA		Detected
12	56, male	Diagnosis	2008	MR		48.57	
		3 months	-		2.5		Detected
		6 months	-		0	0.04	
		12 months	-		0	0.02	
		24 months	-		0	0.002	
		Follow up	2017		0	0.0015	Not detected
13	64, male	Diagnosis	2008	Defaulted	62.5	96	
		3 months	-	follow-up	42		Detected
		6 months	-		13	51.4	
		12 months	-		43	NA	
		24 months	-		55	NA	
		Follow up	NA				NA
14	64, Male	Diagnosis	2008	No MR	67	54.8	
		3 months	-		63.5		Detected
		6 months	-		55	23.6	
		12 months	-		53	15.17	
		24 months	-		62	NA	
		Follow up	2017		NA		Detected
15	59, Female	Diagnosis	2006	No MR	2	42	
		3 months	-		6		Detected
		6 months	-		2	35	
		12 months	-		2	10.9	
		24 months	-		13.3	13.4	
		Follow up	2017		0		Detected
16	62, Female	Diagnosis	2007	Defaulted	82	52	
		3 months	-	Follow-up	NA		Detected
		6 months	-		35	4.42	
		12 months	-		46	16	
		24 months	-		51	26.64	
		Follow up	NA		NA		NA
17	58, Male	Diagnosis	2009	MR	95	52.6	
		3 months			6.5	16.8	Detected
		6 months			0	1.3	
		12 months			0	4.46	
		24 months			0	21.56	
		Follow up			0	0.03	Not detected
18	NA, Female	Diagnosis	2009	Defaulted	98	NA	
		3 months	-	Follow-up	61	1.92	Detected
		6 months	-		74	41.6	
		12 months	-		65	109	
		24 months	-		91	15.9	
		Follow up	NA		NA		NA
19	NA, Male	Diagnosis	20010	Defaulted	97	60	
		3 months	-	Follow-up	92		Detected
		6 months	-		0	112	
		12 months	-		100	0.83	
		24 months	-		NA	7.2	
		Follow up	NA		NA		NA

No	Age and sex	Status, treatment	Year	Molecular status at >48 months	FISH%	QRT-PCR IS%	RT-PCR
20	70, Male	Diagnosis	2007	MR	7	12.5	
		3 months			0		Detected
		6 months			0	2.9	
		12 months			0	1.83	
		24 months			0	0	
		Follow up			0	0.042	Not detected
21	NA, Female	Diagnosis	2007	Deceased	75	59.71	
		3 months	-		NA		Detected
		6 months	-		NA	0.26	
		12 months	-		NA	NA	
		24 months	-		81	NA	
		Follow up	NA		NA		NA
22	NA, Male	Diagnosis	2006	Deceased	93	4.1	
		3 months			70		Detected
		6 months			NA	13.8	
		12 months			6.5	57.5	
		24 months			0	87.5	
		Follow up			NA		NA
23	NA, Female	Diagnosis	2007	Defaulted	15	62.28	
		3 months	-		0		Detected
		6 months	-		0	0	
		12 months	-		0	0.31	
		24 months	-		0	0.03	
		Follow up	NA		NA		NA
24	74, Female	Diagnosis	2009	No MR	NA	30%	
		3 months	-		NA		Detected
		6 months	-		10	5.4	
		12 months	-		NA	28.9	
		24 months	-		NA	10.3	
		Follow up	2017		NA	0.189	Not detected
25	57, Female	Diagnosis	2013	MR	93	31.4	
		3 months	-		65		Detected
		6 months	-		0	5.328	
		12 months	-		0	NA	
		24 months	-		0	NA	
		Follow up	2017		0		Not detected
26	66, Female	Diagnosis	2012	No MR	80	77.67	
		3 months	-		50		Not detected
		6 months	-		70	78.43	
		12 months	-		82		
		24 months	-		13		
		Follow up	2017		NA		Detected
27	54, Female	Diagnosis	2007	Defaulted	86	14.7	
		3 months	-	Follow-up	53	29.47	Detected
		6 months	-		53	30.27	
		12 months	-		45	NA	
		24 months	-		63	NA	
		Follow up	NA		NA		NA
28	39, Male	Diagnosis	2008	No MR	80	NA	
		3 months	-		45		Detected
		6 months	-		23	NA	
		12 months	-		0	5.4	
		24 months	-		0	3.1	
		Follow up	2017		0		Detected
29	NA, Female	Diagnosis	2008	Deceased	NA	141.8	
		3 months	-		61		Detected
		6 months	-		36	150	
		12 months	-		0	NA	
		24 months	-		0	NA	
		Follow up	NA		NA		NA

No	Age and sex	Status, treatment	Year	Molecular status at >48 months	FISH%	QRT-PCR IS%	RT-PCR
30	53, Female	Diagnosis	2009	No MR	75.5	26.7	
		3 months	-		50		Detected
		6 months	-		85	0.85	
		12 months	-		42.5	0.39	
		24 months	-		65	16.3	
		Follow up	2017		NA	38	Detected
31	64, Female	Diagnosis	2008	MR	96	40	
		3 months	-		45		Detected
		6 months	-		0	26	
		12 months	-		0	NA	
		24 months	-		0	NA	
		Follow up	2017		0		Not detected
32	28, Female	Diagnosis	2011	MR	63	90	
		3 months	-		43	NA	Detected
		6 months	-		35	NA	
		12 months	-		11	NA	
		24 months	-		0	0	
		Follow up	2017		0		Not detected
33	77, Male	Diagnosis	2012	MR	NA	167	
		3 months	-		11		Detected
		6 months	-		0	NA	
		12 months	-		0	0.38	
		24 months	-		0	0.04	
		Follow up	2017		0		Not detected
34	70, Female	Diagnosis	2010		42	121	
		3 months	-		40		Detected
		6 months	-		0	NA	
		12 months	-		0	NA	
		24 months	-		0	0.002	
		Follow up	2016		0		Not detected
35	47, Female	Diagnosis	2009	No MR	NA	NA	
		3 months	-		72		Detected
		6 months	-		92	NA	
		12 months	-		85	73	
		24 months	-		82	32	
		Follow up	2016		0		Detected
36	NA, Male	Diagnosis	2008	Deceased	100	NA	
		3 months	-		87		Detected
		6 months	-		83	NA	
		12 months	-		69	NA	
		24 months	-		73	19.1	
		Follow up	NA		NA		Detected
37	49, Male	Diagnosis	2007	MR	100	NA	
		3 months			NA		Detected
		6 months			NA	NA	
		12 months			NA	NA	
		24 months			11	3.43	
		Follow up			0		Not detected
38	NA, Female	Diagnosis	2010	Deceased	66	59	
		3 months	-		57		Detected
		6 months	-		NA	NA	
		12 months	-		NA	NA	
		24 months	-		40	NA	
		Follow up	NA		NA		NA
39	60, Male	Diagnosis	2014	MMR	98	NA	
		3 months			NA		Detected
		6 months			NA	0.001	
		12 months			0	NA	
		24 months			0	NA	
		Follow up			0		Not detected

NA- Not available

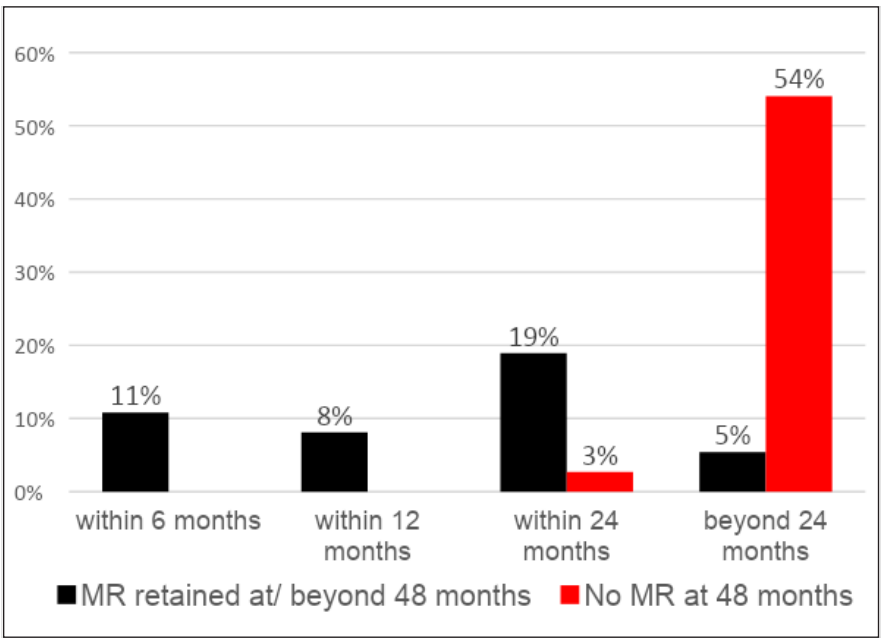

FIG. 1: Time (in months) to achieve major molecular response (MMR) as a predictor of long-term maintenance of molecular remission (MR) at and/or beyond 48 months after diagnosis.

by undetectable *BCR-ABL* by FISH analysis) with an area of 0.744 under the curve ($p < 0.013$), shown in Tables 3 and Fig. 3.

DISCUSSION

Molecular methods including molecular cytogenetics or FISH analysis, the qualitative RT-PCR and the QRT-PCR quantitation of *BCR-ABL* are direct markers of disease burden in CML patients.[34] FISH, RT-PCR and QRT-PCR for *BCR-ABL* at regular points in time have always been important components in the management of CML patients.[34] Many

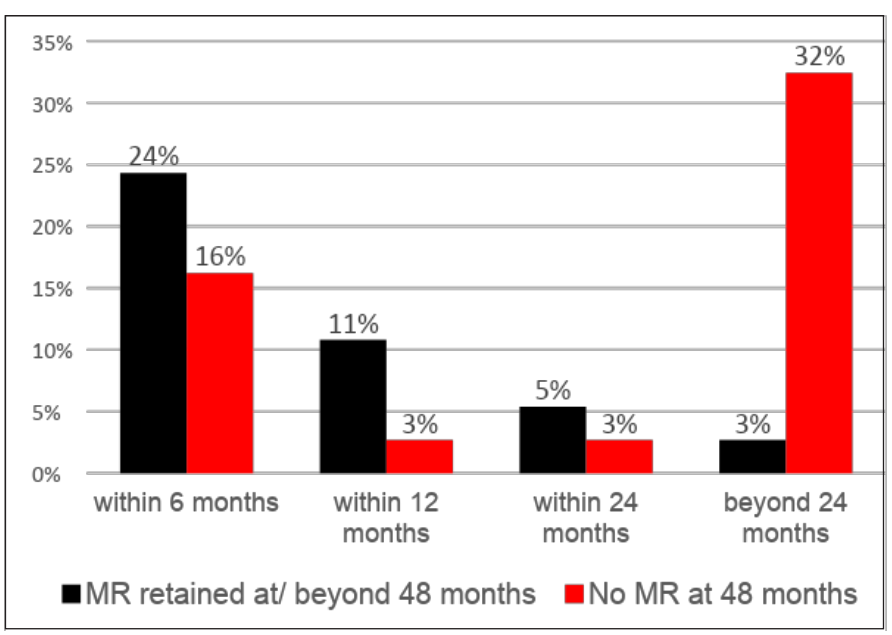

FIG. 2: Time (in months) to achieve complete cytogenetic remission (CCyR) as a predictor of long-term maintenance of molecular remission (MR) at and/or beyond 48 months after diagnosis.

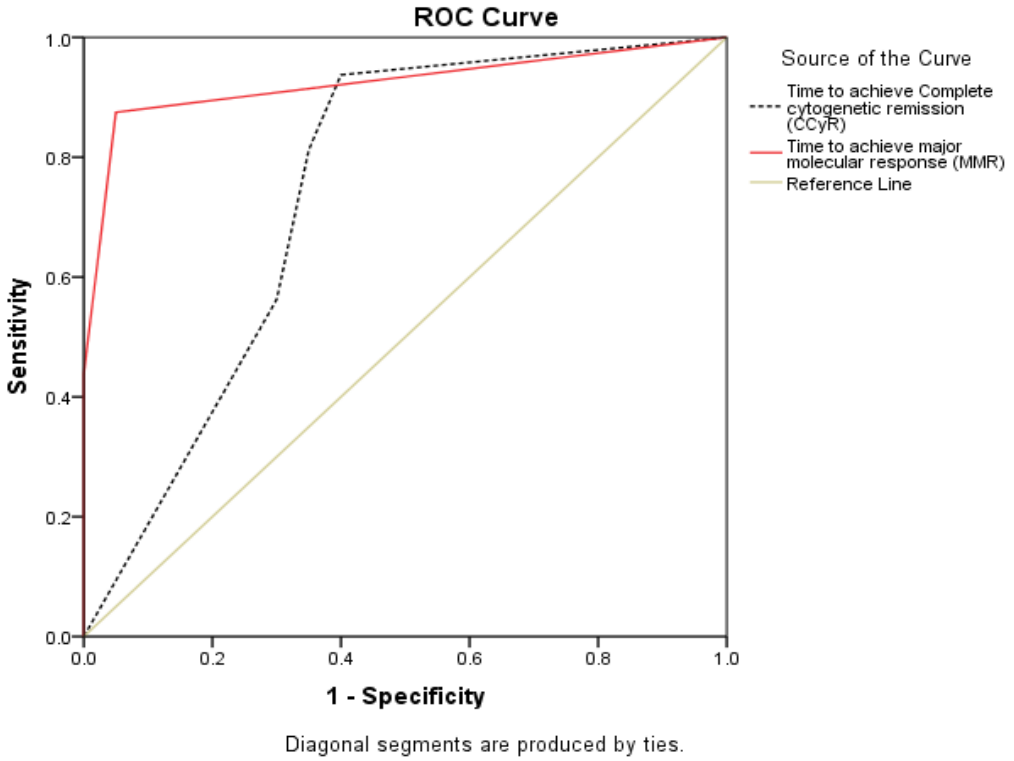

FIG. 3: ROC curves for time (in months) to achieve CCyR and MMR as predictors of sustained molecular remission (MR) at and/or beyond 48 months as independent variable.

TABLE 2: Comparison of sensitivity and specificity between FISH and QRT-PCR for *BCR ABL* at diagnosis and then 6th, 12th and 24th month post diagnosis.

Disease timeline	Number of comparison s between FISH and QRT-PCR	Sensitivity of FISH	Specificity of FISH	Cohen's Kappa agreement (*k*)	Statistical significance (p-value) of the correlation
At diagnosis	29	100%	100%	-	-
6th month	29	83.3%	65.2%	> 0.34	< 0.03
12th month	28	83%	59.1%	> 0.286	< 0.065
24th month	27	100%	68.8%	> 0.642	< 0.001
	(Total 113)				

TABLE 3: ROC curves for time (in months) to major molecular response (MMR) and complete cytogenetic remission (CCyR) as predictors of sustained molecular remission (MR) beyond 48 months.

Parameter	Area under the curve (null hypothesis: true area = 0.5)	Asymptotic significance (*p* value)
Time to MMR (in Months)	0.923	0.000
Time to CCyR (in months)	0.744	0.013

studies compared different methods for the detection of residual disease in CML patients that include conventional cytogenetics, FISH analysis, RT-PCR and QRT-PCR. An early study compared conventional cytogenetics with FISH analysis to detect minimal residual disease (MRD) and emphasised on the need for sequential cytogenetic and molecular analysis in the management of patients with CML and for the evaluation of MRD in patients managed for CML.[35]

In the late 90's, QRT-PCR was studied to determine residual disease in patients with chronic myeloid leukaemia and demonstrated high sensitivity and accuracy.[36] In 1999, for the first time rapid and reliable QRT-PCR method was developed which demonstrated to be sensitive, reproducible, and enabled to detect and early change in *BCR-ABL* transcript levels in serial blood samples.[37] In a study including 21 CML and two *BCR-ABL* positive acute lymphoblastic leukaemia patients, FISH and QRT-PCR analyses were compared at 77 random points in time which showed good comparability.[33] Another study evaluated FISH and QRT-PCR in 24 CML patients treated with imatinib demonstrated good correlation between FISH and QRT-PCR, concluding that FISH and multiplex RT-PCR can be used to complement QRT-PCR.[38] Quantitation of the *BCR-ABL* transcripts using QRT-PCR especially detects and quantifies the disease burden with high sensitivity and specificity, making it the gold standard molecular method for this purpose.[34] A comprehensive comparison of FISH, RT-PCR and QRT-PCR was done for monitoring *BCR-ABL* oncogene after haematopoetic stem cell transplant and demonstrated good correlation between the three methods with QRT-PCR being the most reliable method for this purpose.[39] FISH for *BCR-ABL* has been a complementary analysis done along with other molecular methods in detecting BCR-ABL fusion gene.[14,40] It should be noted that FISH analysis is able to detect subpopulation of cells with rare minor breakpoint *BCR-ABL* translocations that are not amplified by molecular methods.[33,41]

With the availability of more potent new generations TKI regimens, a timely switch of treatment can be considered if there is a failure to achieve molecular targets i.e MMR at a given point in time or when a genetic evolution is detected.[7,42] The new generation multi-kinase inhibitors such as nilotinib, dasatinib and ponatinib as well as stem cell transplantation, represent alternative treatment options to the first line TKI therapy, that again requires accurate and sensitive monitoring of disease in the form of MMR by QRT-PCR.[43,44]

The present study demonstrated moderate to high sensitivity and specificity of FISH analysis when compared to the gold-standard method QRT-PCR for *BCR-ABL*. FISH analysis demonstrated moderate comparability of statistical significance to QRT-PCR for detection of residual disease. Some earlier comparative studies between FISH analysis and QRT-PCR had cases where FISH was positive for BCR-ABL that was missed by QRT-PCR and RT-PCR for *BCR-ABL*.[33,38] In this study, using strict protocols, larger sample size, including only CML patients and sampling at fixed points in time during the management milestones of our CML patients, we demonstrated that QRT-PCR was the most reliable modality for detection of *BCR-ABL*.

Numerous studies have demonstrated the association of favourable long-term outcome with early achievement of MMR.[45] One such previous comparative study, where the patients were monitored at different points in time until 12 months demonstrated that early molecular response was associated with a cumulative survival probability.[42] Recently, it was demonstrated that achievement and maintenance of MMR was associated with restoration of immune responses along with reduction in immune suppressor and CML driving mechanisms, ultimately determining the long term maintenance of remission in CML patient on TKI therapy.[46] One trial demonstrated that few CML patients who achieved stable molecular remission can safely cease their therapy without relapsing treatment-free remission (TFR).[47] But proper criteria to define the best strategies to achieve TFR need to be established for safe and appropriate TKI cessation.

In this study, we monitored the patients using QRT-PCR as well as FISH analysis for *BCR-ABL* at 6th month, 12th month and 24 months after initial diagnosis and then on yearly basis. We also monitored the long-term maintenance of molecular remission beyond 48 months using RT-PCR and/or QRT-PCR for *BCR-ABL*, while patients were continued on TKI therapy. The results of the present study demonstrated that early achievement of molecular and cytogenetic targets were reliable indicators of long-term maintenance of molecular remission beyond 48 months. In this regard, the time to achieve MMR was shown to be the most reliable parameter

to detect long term maintenance of molecular remission. Therefore, it is obvious that early achievement of MMR and CCyR are reliable therapeutic targets for CML patients on TKI therapy.

CONCLUSIONS

QRT-PCR was the most reliable and therefore the current gold-standard method to monitor residual disease in CML patients. In our study FISH analysis was demonstrated to be a reliable investigation that should be considered with QRT-PCR for *BCR-ABL*. It was also demonstrated that time to achievement of molecular and cytogenetic targets i.e. MMR and CCyR respectively, were reliable indicators to detect the long-term outcome of the disease.

LIMITATION

The limitation of this study was that for a few of the patients QRT-PCR and FISH analysis did not have any yield, most likely due to insufficiency/ deterioration of the sample. Those samples were excluded from the analysis.

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