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Full Length Research Paper

Deletion of derivative ABL, BCR or ABL-BCR fusion gene is associated with shorter disease free survival in CML patients

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Chronic myeloid leukemia (CML) is characterized by formation of the BCR/ABL fusion gene, usually as a consequence of the Philadelphia (Ph) translocation between chromosomes 9 and 22. However, deletions of the derivative 9 chromosome [der (9)] in 10 - 15% of CML patients with a standard Ph translocation as well as > 30% of CML patients with a variant Ph translocation may have verse prognosis. The study shed light on prognostic effect of submicroscopic deletions of the derivative chromosome 9 in CML in untreated patients and their follow up samples to correlate with survival. The study included blood and/or bone marrow (BM) samples of 65 untreated CML patients (PT) and 76 follow-up samples, classified as cytogenetic responders (CyR, n = 42), non cytogenetic responders (NCyR, n = 25) and partial cytogenetic responder (PCyR, n = 9). Karyotype analysis was performed on metaphases obtained through short term cultures of BM and blood. Detection of BCR–ABL fusion gene was performed using dual colour dual fusion (D-FISH) translocation probes. Data were analyzed using SPSS statistical software. CyR showed significantly elevated Hemoglobin (p = 0.0001) and decreased in total WBC (p = 0.0001) and Platelet counts (p = 0.0001) as compared to pretreatment levels. 61.5, 30.8 and 7.7% of the PT showed CyR, NCyR and PCyR respectively. Kaplan-Meier survival curve showed the patient with CyR, NCyR and PCyR as well as patients with different stage of the disease did not find difference in survival time. ABL-BCR deletion on derivative 9 was seen in 9.2% of PT, while ABL-BCR, ABL or BCR deletion on derivative 9 was found around 7.7% of PT. Patients with deletion of ABL-BCR on derivative 9 and deletion of ABL or BCR and/or ABL-BCR on derivative 9 have significantly reduced survival (log rank = 14.54; p = 0.001) than non deleted patients. Deletion in *ABL*, *BCR* or *ABL-BCR* on derivative 9 could predict over the survival of all CML patients.

Key words: CML, *BCR-ABL* fusion Gene, FISH, derivative 9 deletion.

INTRODUCTION

The Philadelphia (Ph) chromosome, resulting from the balanced translocation, t(9;22)(q34;q11.2), which fuses the 5' sequences of the BCR gene on chromosome 22 with 3' sequences of the ABL gene on chromosome 9, is the diagnostic hallmark of chronic myeloid leukaemia (CML).

This translocation generates two fusion genes, BCR–ABL on derivative chromosome 22q, known as the Ph chromosome and a reciprocal ABL–BCR fusion gene on derivative chromosome 9q. The technique of fluorescence *in situ* hybridisation (FISH), using probes that bind to specific sequences at the Ph translocation breakpoints, has been applied extensively at initial diagnosis of CML to investigate cases with failed cytogenetics, to detect cryptic BCR–ABL gene fusion and to decipher complex

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Abbreviations: AP, Accelerated phase; **BAC,** bacterial artificial chromosome; **BC,** blast crisis; **BCR-ABL fusion gene,** break point Cluster Region-Abelson fusion gene; **CML,** chronic myeloid leukemia; **CP,** chronic phase; **CyR,** cytogenetic responders; **D-FISH,** dual colour dual fusion fluorescence *in situ* hybridization; **FISH,** Fluorescence *in situ* hybridization; **NCyR,** non cytogenetic responders; **PCyR,** partial cytogenetic responder; **PT,** untreated patients.

Table 1. Patients and samples details.

Untreated CML patients (PT)	N = 65
Gender	
Male (%)	28 (43)
Female (%)	37 (57)
Age (Years)	
Mean	38
Median	37
Range	11 - 70
Diagnosis (%)	
Chronic Phase (CML-CP)	61 (93.8)
Accelerated Phase (CML-AP)	3 (4.7)
Blast Crisis (CML-BC)	1 (1.5)
Final response (%)	
Responded	40 (61.5)
Partially Responded	20 (30.8)
Not Responded	5 (7.7)
Follow-up in months	
Mean	24.86
Median	23.01
Range	10.82 - 45.57
Follow-up samples (%)	76
Cytogenetic Responder (CytR)	42 (55.3)
Non cytogenetic responder (NcyR)	25 (32.9)
Partial cytogenetic responder (PCyR)	9 (11.8)

Ph rearrangements (Wan et al., 20003).

The quantification of BCR-ABL positive cells is achievable through interphase FISH and hence facilitates the monitoring of disease response to treatment.

A dual colour dual fusion (D-FISH) BCR–ABL probe system has been developed (Dewald et al., 1998; Buño et al., 1998), which are large probes designed to span the translocation breakpoints and are labelled with different fluorochromes. In addition to one signal each from the normal BCR and ABL genes, two fusion signals are created in a Ph positive cell: a BCR–ABL fusion signal on derivative chromosome 22q and an ABL–BCR fusion signal on derivative chromosome 9q. Variant signal patterns are encountered in three way Ph translocations and the addition of an extra Ph chromosome when investigated by the D-FISH system (Dewald et al., 2000). It has been reported that *ABL* deletion on derivative 9 was associated with poor prognosis while *BCR* deletion did not affect survival in CML (Sinclair et al., 2000; Gonza´lez et al., 2001). However, role of *ABL-BCR* fusion gene is not well documented (Huntly et al., 2002).

Therefore, the present study aimed to evaluate prognostic effect of sub-microscopic deletions of the derivative chromosome 9 in untreated CML patients and their follow up samples. We investigated the frequencies of ABL, BCR or ABL-BCR deletion on the derivative chromosome 9 using D-FISH and analyzed the association of deletion on derivative 9 with disease free survival in CML patients.

MATERIALS AND METHODS

Subjects

The study included 65 samples from untreated CML patients (PT) from the medical oncology department of The Gujarat Cancer and Research Institute. Among them 28 were male and 37 female.

Follow-up samples

The patients were evaluated at various intervals after initiation of therapy. Blood and/or bone marrow (BM) samples were collected at the time of diagnosis and during follow-up visit of these patients. Seventy six follow-up samples from these patients, classified as cytogenetic responders (CcyR, n = 42), non cytogenetic responders (NCyR, n = 25) and partial cytogenetic responder (PCyR, n = 9) were also collected. Imatinib (Gleevec) was given to all the patients as per the protocol. Clinical details of the patients are given in Table 1. Clinical, radiological and other necessary examinations like molecular cytogenetics were performed during the follow-up study. Patients were monthly followed-up for first 3 months after initiation of the treatment and then after every 3 months based on heamatological and cytogenetic response. As the patients were not regular for follow-up, follow-up number and samples were not identical in all the patients. Clinical status of the patients during/after therphy was evaluated by the clinicians treating them by international criteria of heamatological and cytological parameters.

Study ethics

The study design and patients consent to participate in the study was ethically approved by hospital based ethical committee of The Gujarat Cancer and Research Institute.

Methods

Conventional cytogenetic analysis

Cytogenetic analysis was performed on Giemsa banded (G banded) metaphases obtained through short term cultures of bone marrow and/or peripheral blood cells using standardized protocols. Karyotypes were reported in minimum of 30 metaphases as per ISCN 2005 (Shaffer and Tommerup, 2005). Metaphase cells were captured and analyzed using automated karyotyping system consisting of Axioplan universal epifluorescence microscope (Carl Zeiss) and IKAROS software (Metasystems, Germany).

Fluorescence in situ hybridization (FISH)

Detection of BCR–ABL fusion gene was performed using BCR/ABL dual colour dual fusion (D-FISH) translocation probe (Vysis, Downers Grove, Illinois, USA), according to the manufacturer's instructions. This probe mixture contained directly labelled Spectrum Orange^{TM} probe that spanned the ABL locus at 9q34 ("O"

Table 2. Comparison of haematological parameters between untreated samples and their paired three groups of follow-up samples.

Paired t test		Haemoglobin (gm/dl) Mean (S.D.)	Leukocyte count (counts/cumm.) Mean (S.D.)	Platlets count (counts/cumm.) Mean (S.D.)
PT vs. CR	PT	10.2444 (1.78)	131022.22 (127638.64)	495363.63 (261726.58)
	CR	11.7333 (1.18)	6672.22 (1924.82)	244333.33 (75636.33)
significance	p =	0.0001	0.0001	0.0001
PT vs. PR	PT	10.45 (1.848)	186914.28 (134101.01)	488000.0 (240806.28)
	PR	10.8 (2.65)	6228.57 (1943.98)	190142.86 (81462.52)
significance	p =	0.696	0.012	0.033
PT vs. NR	PT	9.94 (1.89)	134576.12 (121281.19)	425500.0 (303006.01)
	NR	10.35 (2.03)	15454.17 (31851.5)	383954.54 (451010.98)
significance	p=	0.466	0.0001	0.732

denotes Orange labelled ABL gene) and directly labelled Spectrum Green^{TM} probe that spanned the BCR locus at 22q11.2 ("G" denotes Green labelled BCR gene). 400 interphase/metaphase nuclei were analyzed for the presence of fusion signals. The OGFF pattern is the typical pattern for CML and indicates no gross sub-microscopic deletions. ("F" denotes yellow Fusion signal of orange and green probe indicative of BCR-ABL fusion on derivative 22 and ABL-BCR fusion on derivative 9). Atypical patterns of D-FISH include OGGF, OOGF and OGF which are indicative of deletion of ABL, BCR and ABL-BCR respectively on derivative 9. Image acquisition was performed either on automated Olympus epifluorescence microscope and Cytovision software (version 3.7, Applied Imaging System) or Carl Zeiss with ISIS software (Metasystems, Germany).

Treatment response criteria

Treatment response in follow-up samples was evaluated based on bone marrow pathology/morphology report and cytogenetic analysis for Philadelphia chromosome. The CcyR was defined as disappearance of signs and symptoms of disease, including palpable splenomegaly, normalization of peripheral blood counts and differentials (white blood cell [WBC] count < 10×10^9/L; no peripheral blasts or promyelocytes; < 5% myelocytes -metamyelocytes; platelets < 450×10^9/L) as well as absence of Ph chromosome in conventional cytogenetics and/or absence of BCR-ABL fusion gene by FISH analysis, with BM morphology report as remission. The complete cytogenetic response was confirmed by qualitative BCR-ABL PCR outside the institute (Data not shown). Minor cytogenetic response having Ph and/or BCR-ABL fusion 35 to 90% with no morphology change/relapse in BM report was considered as NCyR. PCyR was defined as presence of Ph positive (1 to 34%) cells by conventional cytogenetic and/or presence of mixed clone of BCR-ABL fusion gene positive by FISH aided with BM pathology report being controlled CML activity.

Statistical analysis

Data were statistically analyzed using the SPSS statistical software (version 15.0; SPSS, Inc., Chicago, IL, USA). Students paired't' test was performed to compare haematological parameters like haemoglobin, total white blood cells (WBC) and platelet counts between untreated and their follow-up samples. Kaplan-Meier survival curves were plotted using log rank test to compare disease free survival between patients with deletion of ABL or BCR and/or ABL-BCR on derivative 9 with patients with non deleted derivative 9. Statistical significance was considered when 'p' values were less then 0.05.

RESULTS

Variations in haematological parameters between untreated samples and follow-up samples Paired t test was performed between untreated samples and their paired follow-up samples for haemoglobin, leukocyte and platelet counts (Table 2). CcyR showed significantly elevated Hemoglobin (p = 0.0001) and decreased in leukocyte (p=0.0001) and Platelet counts (p = 0.0001) as compared to their paired PT levels. PcyR samples showed significantly lower leukocyte and platelet counts as compared to their paired PT levels (p = 0.012 and p = 0.033 respectively). NcyR samples showed significantly elevated leukocyte counts (p = 0.0001) as compared to their paired PT levels.

Conventional cytogenetic and FISH

Table 3 shows frequency of different clone in karyotype and FISH at PT. Seventy two percentage of PT showed t (9; 22) at the time of diagnosis, while 24.6% of PT showed non informative karyotype results due to no metaphase or poor metaphase preparations. One patient showed normal karyotype with no t(9; 22), which was confirmed for BCR-ABL negative by FISH. Only one patient showed variant translocation having t (9; 22)?add9q.

Table 3. Karyotype and FSIH results.

Karyotype	(PT = 65) (%)
t (9:22)	47 (72.31)
Normal (N)	01 (1.54)
Non Informative (NI)	16 (24.61)
Variant translocations other than t(9:22)	01 (1.54)
FISH for BCR-ABL DCDF	**(PT=65)**
Negative (OOGG) (no fusion of BCR-ABL)	01 (1.54)
Positive (OGFF) (no deletion of derivative 9)	36 (55.39)
Positive (OGF) (Deletion of derivative 9)	06 (9.23)
Positive mix clone (OGFF + OGF)	17 (26.15)
Variant mix clone (OGF + OOGF + OGGF) (Deletion on derivative 9 for ABL/BCR/ABL-BCR)	05 (7.69)

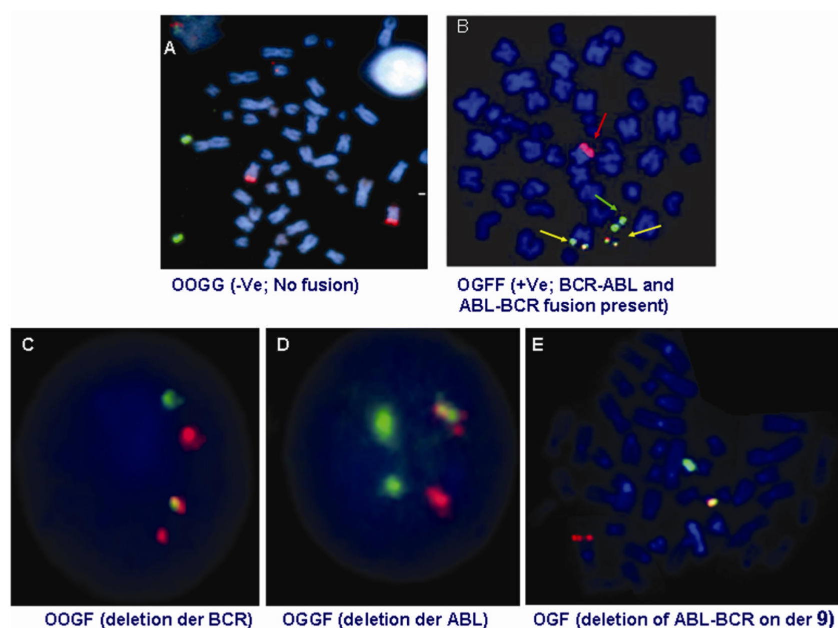

Figure 1. Representative FISH signal pattern using DCDF BCR-ABL FISH probe.

Figures 1A - E shows representative FISH signal pattern observed in the study. Sample with no fusion of BCR-ABL showed OOGG signal pattern (Figure 1A), samples with BCR-ABL fusion with no deletion in derivative 9 showed OGFF signal pattern (Figure 1b). Deletion in BCR, ABL and ABL-BCR on derivative 9 was seen as OOGF, OGGF and OGF respectively (Figure 1C, D and E respectively). Fifty-five percent of untreated patients showed presence of BCR-ABL fusion gene with no deletion of ABL-BCR on derivative 9 chromosome (Table 3). Deletion of ABL-BCR on derivative 9 was seen in 9.23% of PT while 26.15% of PT showed mixed clone for non-deleted and deleted ABL-BCR on derivative 9 showing signal pattern of OGF+OGFF. Mix clone for deletion of ABL or BCR or ABL-BCR (OGF + OOGF + OGGF) was seen in 7.69% of PT.

Frequency of FISH variant signals in t(9;22)

As mentioned in Table 3, 72.31% of untreated samples showed t(9;22) by conventional cytogenetic. When D-FISH was performed in these patients, they showed wide range of FISH signal pattern indicating deletion of ABL, BCR or ABL-BCR on derivative 9 chromosome which was not possible to diagnose by conventional cyto-genetic. Table 4 shows frequency of patients showing response to therapy and their D-FISH signal pattern. Sixty percentages of patients having deletion of ABL-BCR on derivative 9 showed no response to therapy

Table 4. FISH signal pattern analysis in patients with t(9;22) by conventional cytogenetics (karyotyping).

FISH signal pattern	Final response		
	Response (%)	No response (%)	Partial response
Der 9 non deleted (OGFF) (n=28)	20 (71.43)	06 (21.43)	02 (7.14)
Der 9 deleted (OGF) (n=05)	02 (40)	03 (60)	-
Mix (OGF/OGFF) (n=11)	07 (63.64)	04 (36.36)	-
Other variants (OGGF/OOGF/OGF/OGFF) (n=03)	02 (66.66)	-	01 (33.33)

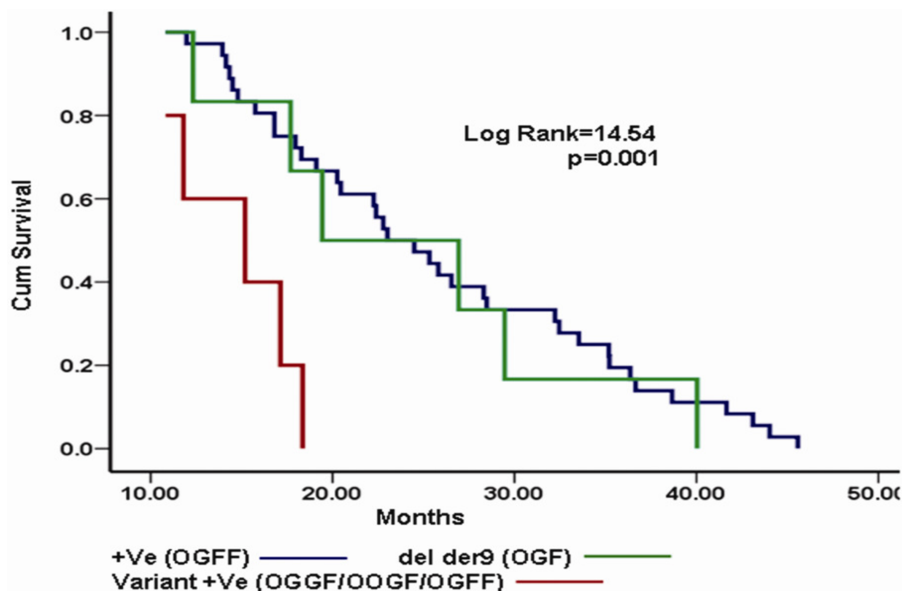

Figure 2. Kaplan-Meier survival analysis between derivative 9 deletion and non deletion.

while 40% showed response. While 71.43% of patients with non deleted ABL-BCR (OGFF) showed response and 21.43% showed no response to therapy.

Kaplan-Meier survival analysis

Kaplan-Meier disease free survival curve showed patients with deletion of ABL-BCR on derivative 9 and deletion of ABL or BCR and/or ABL-BCR on derivative 9 have significantly reduced survival (log rank = 14.54; p = 0.001) than non deleted patients (Figure 2).

DISCUSSISON

The present study aimed to evaluate prognostic effect of sub-microscopic deletions of the derivative chromosome 9 in CML in untreated patients and their follow up samples to correlate with disease free survival. The submicroscopic deletion can not be detected by conventional cytogenetic method using karyotype analysis. D-FISH *BCR/ABL* probe used in the present study is useful to reveal the locations of 3' *ABL* and 5'*BCR* as well as 5'*ABL* and 3'*BCR* on metaphase chromosomes making this technique capable of detecting minimal residual disease in CML (Wan et al., 20003; Qiu et al., 2009). However, it is widely accepted that the clinical, prognostic and haematological features of CML patients with complex variant translocations are not different from those with the classical t(9; 22) translocation because it is accepted that the key pathological event is the formation of the BCR/ABL fusion gene (Johansson et al., 2002). In the present study only one patient showed complex variant translocation at the time of diagnosis showing t (9; 22)? add9, however at the end of 21 months, the patient showed complete response to therapy.

For each patient, pre treatment and follow-up haematological evaluation was also carried out to confirm haematological response. We found that the standard haematological response criteria in responders, non responder and partial responders (Table 2). However, there was no difference in leukocyte and platelet counts were seen between derivative 9 deleted and non deleted

patients (data not shown). Lee et al. (2006) have shown that patients with ABL deletion showed significantly higher leukocyte count then deletion in ABL-BCR together. However, other clinical and haematological parameters were similar in deleted and non deleted group (Lee et al., 2006). FISH plays a complementary role in providing information in which cytogenetic studies are inadequate because of poor metaphase yield (Tefferi et al., 2005; Qiu et al., 2009). In this study, 16 (24.61%) patients at the time of diagnosis showed non informative metaphase. In these patients FISH showed not only positive results but also be useful to pin point deletion on derivative 9. Out of these 16 patients, 8 showed mix clone for deletion in ABL-BCR and non deleted derivative 9. Similarly, patients with t(9;22) at the time of diagnosis also showed different deletion status in derivative 9 by D-FISH (Table 4). Majority of patients with non deleted derivative 9 showed response as compared to deletion in ABL, BCR or ABL-BCR on derivative 9.

However, the association of poor prognosis and poor response to therapeutic modalities for patients harbouring the deletion of 9q34 is controversial. Several studies have suggested that 9q34 deletions confer an adverse prognosis and a poor response to therapeutic modalities (Li et al., 2008; Wu et al., 2007; Kreil et al., 2007; Gorusu et al., 2007; Cohen et al., 2001; Huntly et al., 2001; Storlazzi et al., 2002; Huntly et al., 2003) whereas others did not observe this association (Dong et al., 2008; Yoong et al., 2005; Quintas-Cardama et al., 2005).

We have observed significantly reduced disease free survival in patients with deletion in ABL, BCR or ABL-BCR on derivative 9 as compared to non deleted derivative 9. The survival time was shorter in those patients with deletions, regardless of the deletion patterns. These findings suggest that the gene segments around the breakpoint are deleted in heterogeneous patterns when the translocation occurs. In addition, the genes located near the breakpoint may play a role in the delayed tumor progression and a loss of this sequence may reduce the survival time.

Conclusion

In this study deletion on derivative 9 was heterogeneous involving either *ABL* or *BCR*, or *ABL-BCR*. Similar finding were observed by Lee et al. (2006) and Wu et al. (2006). This raised the question which region of the derivative 9 is important for disease prognosis. Many tumor-related genes are located near the translocation breakpoints (Huntly et al., 2001). It has been reported that p21rac acts on cell growth and proliferation associated with *RAS*, which moves along with 3'*BCR* region on derivative 9 during translocation. GTPase-activating protein binds with p21rac and inhibits its activity. Therefore, a loss of this region can induce abnormal cell growth and proliferation (Diekmann et al., 1991). If these genes are deleted during *BCR-ABL* gene rearrangement and the residue allele is injured by “two hit” events, the tumor suppressor functions are destroyed and the disease can progress. Similarly Argininosuccinate Synthetase (*ASS*) gene located on 9q34 region adjacent to *ABL* and Immunoglobulin light chain (*IGL1*) gene located near *BCR* gene on chromosome 22 might be candidate genes to analyze along with deletion in *ABL-BCR* on derivative 9 (Storlazzi et al., 2002). Therefore, an array of adjacent genes at the breakpoint and fusion regions needs to be analyzed using Bacterial Artificial Chromosome (BAC) FISH clones. We are currently working on the BACs for different chromosome 9 and 22 breakpoint and fusion regions in cases with deletion in derivative 9.

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