PART- III

Chapter- 5.1

Detection of FLT3 alteration and AML1-ETO transcript in acute myeloid leukemia
5.1.1. Introduction
The molecular pathogenesis of acute myeloid leukemia (AML) is complex, but studies indicate that the multiple genetic defects converge on a set of biological properties of leukemic cells. The class III receptor tyrosine kinase FLT3 is an early acting factor which supports survival, proliferation and differentiation of primitive hematopoietic progenitor cells by signaling RAS/RAF/MAPK and PI-3 kinase cascades after ligand binding. The protein is expressed on blast cells of most AML and B-ALL. FLT3 mutations are one of the most frequent somatic alterations in AML, occurring in approximately 1/3 of patients. FLT3 mutations consist of two major types: One internal tandem duplication (ITD) mutations of 3-400 bp (in frame or contains foreign sequence/additions of extra nucleotides) that map to the juxtamembrane region (found in 23% of AML patients) and other is a point mutation that most frequently involves aspartic acid 835 of kinase domain (KD) (8-12% of AML patients) [Small D 2006, Schnittger S et al. 2002, Sheikhha MH et al. 2003, Thiede C et al. 2002, Yamamoto Y et al. 2001]. Details role of FLT3 mutations described in general introduction (chapter 1). The recurrent chromosomal translocations found in AML often involve transcription factors which are crucial for the myeloid cell differentiation. The reciprocal translocation t(8;21)(q22;q22) is one of the most common structural chromosomal aberrations in patients with AML [Bitter MA et al. 1987, Nucifora G et al. 1995, Rowley JD et al. 1990]. It is found in approximately 10-15% of patients with adult AML [Buonamici S et al. 2004, Berger R 1987, Campana D et al. 1995] and a strong association with about 40% of AML-M2 French-American-British subtypes [Peterson LF and Zhang DE 2004]. The t(8;21) creates an AML1-ETO fusion gene on the derivative chromosome 8, generating a chimeric AML1-ETO mRNA and the fusion protein that contains the DNA binding domain but lacks the transcriptional activation domain of AML1 and almost the full length of the ETO protein. These functions correlate with ability of AML1-ETO to inhibit the differentiation of myeloid cell lines [Lutterbach B et al. 1998, Gelmetti V et al. 1998, Wang J et al. 1998]. The presence of t(8;21) in the leukemic blast is associated with a high complete remission rate. However, the impact of this chromosomal aberration on long-term prognosis remains controversial [Bloomfeld CD et al. 1998, Fenaus P et al. 1990, Grimwade D et al. 1998, Heil G et al. 1997]. RT-PCR studies of patients in long-term remission have shown the persistence of AML1-ETO expression in cells of the peripheral blood (PB) and bone marrow (BM) [Kusec R et al. 1994, Nucifora G et al. 1993]. RT-PCR has also detected the fusion transcript in selected AML patients whose leukemic cells lack t(8;21) cytogenetically [Andrieu V et al. 1996, Maruyama F et al. 1994, Maseki N et al. 1993, Nucifora G et al. 1994, Zhang T et al. 1994]. So, the RT-PCR technique is more convenient to detect the transcript than cytogenetic method.
In the present work, the FLT3-ITD and FLT3-D835 mutations status have been detected in 63 de novo AML patient samples at diagnosis and possible correlation between FLT3 alteration and clinical parameters has been explored. Also, the bone marrow and/or peripheral blood samples of 54 AML patients (excluding AML-M3 subtype) have been investigated by RT-PCR in order to establish the frequency of AML-ETO rearrangement in AML of different subtypes of the FAB classification.

5.1.2. Materials and Methods

5.1.2.1. Patients

The patient group constituted of sixty-three prior untreated de novo Acute Myeloid Leukemia (AML) cases for FLT3 alteration study and 54 of AML patients excluding AML-M3 subtypes included in AML1-ETO detection. Characteristics of the patients have given in Table 3.1.1, chapter 3.1.

5.1.2.2. Genomic DNA and RNA isolation

Genomic DNA and total RNA were isolated from bone marrow/ blood described in general materials and methods chapter.

5.1.2.3. Detection of FLT3 Internal Tandem Duplication (ITD)

Genomic DNA was amplified specifically from exon 11 to exon 12, including intron 11. The PCR mixture contained 500 ng of genomic DNA, 50 pmol of 11F and 12R primers [Kiyoi H et al. 1997], 0.2 mM of each deoxynucleotide triphosphate, 1X Taq assay buffer and 2.5 units of Taq polymerase (Bangalore Genei, India). Denaturing, annealing, and extension steps were performed at 94°C for 30 s, 56°C for 1 min and 72°C for 2 min, respectively, for 35 cycles on a GeneAmp PCR system 2400 (Perkin Elmer) including an initial 3 min denaturation step at 94°C and a final extension step at 72°C for 10 min. Amplification products were analyzed by 10% polyacrylamide gel electrophoresis and stained with ethidium bromide and photographed under UV transillumination (Figure 5.1.1A). Amplified band larger than 329bp was purified from polyacrylamide gel and sequenced by automated sequencer (ABI310 system, USA), chromatogram shown in Figure 5.1.1B.
Chapter 5.1
Detection of FLT3 alteration and AML1-ETO transcript in AML

5.1.2.4. Detection of FLT3-D835 mutation in kinase domain

The RFLP-mediated PCR assay was used to detect the mutations at codon 835 of FLT3 as described by Yamamoto Y et al. 2001 with minor modification [Shih LY et al. 2004]. I amplified exon 17 of the FLT3 gene by genomic PCR using the primers 17F and 17R, as above. 10 μl aliquot of amplified product was digested with 10 units of EcoRV (Fermentas) at 37°C overnight and then electrophoresed on 10% polyacrylamide gel (Figure 2A). If the amplified products showed the undigested band, it was cut out from the gel, purified, and directly sequenced on a DNA sequencer (Applied Biosystems, USA) using a BigDye terminator cycle sequencing kit (Applied Biosystems), chromatogram shown in Figure 2B.

Figure 5.1.2. Analysis of FLT3 D835 mutation (A) 10% polyacrylamide gel electrophoresis of PCR-RFLP. Mt showed an undigested band, arguing for a mutation and Lane Wt showed wild type D835, Lane M showed molecular weight marker. (B) Mutation was confirmed by direct sequencing of undigested band eluted from polyacrylamide gel.
5.1.2.5. Reverse transcription-polymerase chain reaction for detection of AML1-ETO transcript

Total RNA (400 ng - 1 μg) was reverse transcribed into cDNA using random hexamer and MuLV reverse transcriptase (Applied Biosystem, USA) in 20 μl reaction volume. Then PCR amplified in a reaction volume of 50 μl containing 15 μl of RT products, 8.5 mM MgCl₂, 200 μM of each dNTPs, 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystem, USA), 300 nmol each of primers were used [Krauter J et al. 1999]. The reaction conditions were 95°C for 10 min (for activation of AmpliTaq Gold polymerase) and then 40 cycles of 15 sec at 95°C followed by 60 sec at 59°C (annealing and extension). The positive controls were derived from RNA sample of AML1-ETO positive AML patients who has been confirmed by sequence analysis after cloning into a plasmid. The normal lymphocytes RNA were used as negative control, and no template control (NTC) was included in each experiment as the PCR-reagent mixed with water instead of cDNA. The PCR products were visualized directly on ethidium bromide stained 3% agarose gel (Figure 5.1.3). GAPDH gene was used as internal control.

5.1.2.6. Statistical analysis

The analysis was performed using the statistical software Statistica 7.0 version. P< 0.05 was considered statistically significant.

5.1.3. Results

5.1.3.1. FLT3 alteration

63 de novo AML patients were studied for FLT3-ITD and FLT3-D835 mutation. 7 (11%) FLT3-ITD and 7 (11%) FLT3-D835 mutation positive cases out of 63 AML samples were detected. The amplification ranged from 20-170 bp, and all patients showed the band corresponding to normal copy of the gene. FLT3 mutations in different types of FAB subtypes were shown in Table 5.1.2. No age differences in median age were found among the FLT3-ITD positive (33 years), FLT3-D835 positive (35 years) and those without either of these (34 years) patients. In this cohort of patients 5 were in pediatric group (age <15 years), and 2 of them [1 M2 and 1 M3]
were FLT3-ITD positive. None of the childhood AML cases had FLT3-D835 mutation. When stratified for WBC counts, it was found that FLT3-ITD positive patients had higher WBC counts than FLT3-ITD negative (P = 0.03) AML patients (Figure 5.1.4). FLT3-ITD mutation is detected in 6 males (14.6%) and 1 female (4.5%). FLT3-D835 mutation is detected in 4 females (19.4%) and 3 males (7.3%). Median value of survival of FLT3-ITD positive patients was not less than median value of survival of wild type FLT3 AML patients. No patient had both the mutations.

Table 5.1.1. FAB classification of AML patients with FLT3-ITD and FLT3-D835 mutations

<table>
<thead>
<tr>
<th>FAB classification</th>
<th>Total</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
<th>Unclassified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number examined</td>
<td>63</td>
<td>36</td>
<td>7</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>FLT3-ITD</td>
<td>7 (11%)</td>
<td>2 (5.5%)</td>
<td>2 (28.5%)</td>
<td>1 (50%)</td>
<td>0</td>
<td>0</td>
<td>2 (16.6%)</td>
</tr>
<tr>
<td>FLT3-D835</td>
<td>7 (11%)</td>
<td>5 (13.9%)</td>
<td>1 (14.2%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (16.6%)</td>
</tr>
</tbody>
</table>

Figure 5.1.4. Mean WBC count in FLT3-ITD, FLT3-D835 mutations and wild type (WT) FLT3 AML patients.

5.1.3.2. AML1-ETO transcript detection

Seven (13%) of the 54 AML patients the AML-ETO transcript were detected. Characteristics of AML patients with and without AML1-ETO transcript has been provided in Table 5.1.2.
Table 5.1.2. Characteristics of patients with AML1-ETO transcripts positive and negative

<table>
<thead>
<tr>
<th></th>
<th>AML1-ETO positive (n = 7)</th>
<th>AML1-ETO negative (n = 47)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (Male/ Female)</td>
<td>4/3</td>
<td>31/16</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>28</td>
<td>34</td>
<td>NS</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>25.4± 10.5</td>
<td>38.5± 18.5</td>
<td></td>
</tr>
<tr>
<td>WBC (X10^9/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>17</td>
<td>32</td>
<td>NS</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>49.5± 54.4</td>
<td>71.6± 80.38</td>
<td></td>
</tr>
</tbody>
</table>

5.1.4. Discussion

FLT3 gene is overexpressed at RNA and protein level in AML [Kainz B et al. 2005]. Mutations in the juxtamembrane domain or kinase domain which activate the kinase constitutively are common in AML and are found in one-third of the cases. Of these, the internal tandem duplication of the juxtamembrane region or ITD is found in about 23% (986/4299) [Small D 2006] of the cases, with a little lower incidence in pediatric cases. However, this study registered a much lower (P = 0.03 with Yates correction) incidence of 11% of FLT3-ITD and all these patients had another wild type copy of the gene. Incidence of this mutation is usually low in pediatric patients and adult M2 subtype whereas increases in elderly patients and M3 subtype [Small D 2006]. Here the data does not show this age specific variation but the low incidence may be due to high number (36/63) of M2 subtype in this cohort of patients. Decreased survival in ITD positive patients has been observed in many studies, but not all. As seen in these study patients had low mutant to wild type ratio of band intensity (analyzed semi-quantitatively using Quantity One software of BioRad Gel documentation XR system (BioRad, USA) on PCR amplified product). They registered a very low ratio ranging from 0.07-1.15 (median 0.52, mean 0.47). Recently an observation published from India on FLT3-ITD in acute promyelocytic leukemia patients, found relatively lower (18.5%) incidence than other reports. FLT3-ITD conferred a poor prognosis to the positive patients [Hasan SK et al. 2007].

The point mutation at kinase domain is found in 11% of cases, which is not much different from that reported by others. The phenotype associated with ITD like higher WBC count is also observed in this cohort of patients (Figure 5.1.4). No patients had both the FLT3 mutations.
In this study AML1-ETO transcript is detected in 13% of patients which is in agreement with other studies. However, the frequency reported in M2 subtypes is around 40% [Peterson LF, Zhang DE 2004], while this study registered a much lower frequency of only 19.5%. No correlation was found between AML1-ETO transcript and age, sex, WBC counts.

It might be mentioned that the translocation t(12:21) producing TEL-AML1 fusion protein in pediatric acute lymphoblastic leukemia occurs at a frequency of 16-44% in western countries, but in Indian patients, the frequency ranged between 5-9% [Hill A et al. 2005]. The t(15:17) translocation of AML M3 patients occurs at a much higher frequency in patients of southern Europe relative to others [Deschler B et al. 2006]. Whether the population specific variations in occurrence of these chromosomal abnormalities in various leukemias stem from etiology of the disease or are precipitated by genetic variations remains to be investigated.

In summary, frequency of incidence of FLT-ITD is lower in this group, and is not age dependent. AML1-ETO transcript was detected low frequency in M2 subtype. As FLT3 based therapeutic approaches are being considered, this finding should be followed up in more patient groups from India.
Chapter- 5.2

Characterization of

BCR-ABL transcript in chronic

myeloid leukemia
Chapter 5.2 Characterization of BCR-ABL transcript in chronic myeloid leukemia

5.2.1. Introduction

Chronic myeloid leukemia (CML) is probably the most extensively studied human malignancy. 90-95% of CML patients harbor the Philadelphia (Ph) chromosome, a shortened chromosome 22 resulting from a reciprocal translocation t(9;22)(q34;q11) between the long arms of chromosome 9 and 22, fusing ABL protooncogene on chromosome 9 with the BCR gene on chromosome 22. Product of the chimeric mRNA transcribed from the hybrid BCR-ABL fusion gene plays a causal role in pathogenesis of the disease. Ph chromosome is not exclusive to CML and is found in 15-30% of adult and 2-5% of childhood acute lymphoblastic leukemia (ALL), in <3% of patients with acute myeloid leukemia (AML) and occasionally in lymphoma and myeloma patients. The gene is leukemia specific and can therefore be used as a sensitive marker of the disease and its progression. 99% of CML patients have breakpoints that result in a fusion mRNA in which either BCR exon 13 (b2) or exon 14 (b3) (M-bcr) is fused to ABL exon a2 to form the b2a2 or b3a2 transcripts which get translated into an oncoprotein p210BCR-ABL. In 60-80% of patients with Ph-positive ALL the breakpoint occurs in the first intron of the BCR gene, in a region referred to as the minor breakpoint cluster region (m-bcr) producing the shorter isotype p190BCR-ABL from the e1a2 type mRNA. A third breakpoint μ-bcr connects exon 19 of BCR with ABL exon a2 giving rise to the e19a2 transcript corresponding to the p230 fusion protein, which has been associated with a mild CML phenotype [Saglio G et al. 1990, Pane F et al. 1996]. Pane F et al proposed classifying these later cases as neutrophilic chronic myeloid leukemia (CML-N), a rare disease characterized by moderate and persistent neutrophilia without precursors in the peripheral smear, absent or normal splenomegaly and a benign clinical course, with a lower white blood cell (WBC) count with minimal basophile, a milder anemia. So far e19a2 transcript has been observed in five patients with neutrophilic chronic myeloid leukemia, seven patients with CML in chronic phase, four with CML rapidly evolving to the blastic phase and in two with acute myeloid leukemia [Saglio G et al. 1990, Pane F et al. 1996, Mittre H et al. 1997, Bernasconi P et al. 2001, Haskovec C et al. 1998, Martinez-Lopez J et al. 2002]. Occasionally other breakpoints yielding b2a3, b3a3, e1a3, e6a2, or e2a2 transcript have been detected [Deininger MW et al. 2000]. Cytogentic analysis used to be the standard technique for monitoring treatment response. However, BCR-ABL transcripts can also be detected by reverse transcription polymerase chain reaction (RT-PCR) technique, which enables molecular characterization of the exact breakpoint and detection of residual leukemic cells at the level of one cell in a background of 10^5-10^6 normal cells [Lion T et al. 1994, Branford S et al. 1999, Amabile M et al. 2001], and is currently used in many countries along with the quantitative PCR technique.
A. Primary BCR transcript

B. Normal RNA transcript

C. RNA splicing using cryptic branchpoint

D. Alternative RNA splicing

Figure 5.2.1: Mechanism of alternative splicing in the M-bcr region of the BCR gene involving polymorphism at the invariant A of the putative branchpoint (shown by underline) of intron 13. (A) A region of the primary sequences is represented. The large double nucleotides correspond to the polymorphic sites in exon 13 and intron 13 (g.108654 T>C; 109368A>G) [shown by arrow]. The intronic polymorphism occurs within a region which is the best match to the poorly conserved branchpoint consensus sequence of YNYTRAY, where Y = T or C, N = any nucleotide and R = G or A. In RNA splicing, the invariant A at position six of the branchpoint is critical for formation of splice intermediate, the lariat structure. (B) Represent normal splicing in the presence of exon 13 T and intron 13 A genotype. (C) The predominant transcript is the normally spliced BCR transcript; the replacement of A with G at the branchpoint activates a cryptic branch point near by possibly at the dashed underlined site, which is utilized in RNA splicing. (D) Cryptic branchpoint are less efficiently used and utilizing the branchpoint of intron 14 alternative splices a proportion of the RNA [Modified from Branford S et al. 2002].

Co-expression of both the b2a2 and b3a2 transcripts by alternative splicing is detected in minority of patients (1.4 - 11%). Co-expression has been linked to two polymorphisms in the patient’s DNA, one a thymine (T) to cytosine (C) polymorphism in the eighth position before the end of exon 13 of BCR gene, altering the third nucleotide of the relevant codon and other, an adenine (A) to guanine (G) polymorphism within intron 13 occurring upstream of the 3’ splice site. The exonic
change does not alter the amino acid sequence. The BCR intronic polymorphism however occurs at the invariant A of a sequence that is the best match to the poorly conserved branchpoint. It has been proposed that the change results in reduced efficiency of splicing of intron 13 of BCR and BCR-ABL allele [Meissner RV et al. 1998, Saussele S et al. 2000, Branford S et al. 2002] and use of the acceptor site at the end of intron 14, leading to co-expression of both the transcripts (Figure 5.2.1).


In this work an attempt has been made to establish the frequency of different type of BCR-ABL transcripts in CML patients of eastern India, in order to obtain more information on BCR-ABL chimeric mRNA type and its possible association with different clinical features observed during the course of CML at chronic phase and to find possible correlation between the polymorphisms at exon 13 and intron 13 with co-expression of b2a2 and b3a2 transcript.

5.2.2. Materials and Methods

5.2.2.1. Patients
This study includes 141 patients (96 male, 45 female) who were under treatment in the hematology department at two medical centres in Kolkata from 2001 to 2006. The diagnosis of CML was established on the basis of cytochemical analysis of bone marrow aspirates.

5.2.2.2. Sample collection and isolation of genomic DNA and RNA
Genomic DNA and total RNA were isolated from whole blood/bone marrow samples as described earlier.

5.2.2.3. Characterisation of junctional breakpoint by RT-PCR
cDNA synthesis was performed in a total volume of 20 μl with 400 ng to 1μg of RNA and other reagents according to the manufacturer’s protocol (TaqMan Reverse Transcription Reagent Kit, Applied Biosystem, USA). PCR was performed in a reaction volume of 50 μl containing 10 μl of RT products, 5.5 mM MgCl₂, 200 μM of each dNTPs, 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystem, USA), 15 pmol primer concentration, sequence were as described earlier and primer location shown in Figure 2A. The positive controls were derived from RNA sample of b2a2 and e1a2 positive CML patients who has been confirmed by sequence analysis, and K562 cell line RNA was used as control for b3a2. The negative control was normal
lymphocyte RNA. The PCR products were visualized directly on ethidium bromide stained 3% agarose gel. ABL gene was used as internal control (Figure 5.2.2 B, C, D, E and F).

5.2.2.4. Genotyping of BCR exon 13 polymorphism
The T to C polymorphism of BCR exon 13 at eighth position before the junctional region of BCR-ABL transcript was detected by PCR, using allele specific primer BCR (M), BCR (N) and common reverse primer BCR (R). The allele specific primers amplified a 158 bp fragment when the corresponding allele was present. The 25 μl PCR reaction mixture contained 300 ng genomic DNA, 1X Buffer (MBI Fermentus, Lithuania), 1.5 mM MgCl₂, 5 pmol of each primer, 200 μM dNTPs and 1.25 U Taq polymerase (MBI Fermentus, Lithuania). After denaturation for 5 minutes at 95°C, PCR was performed 30 cycles for 45 seconds at 95°C, 45 seconds at 60°C and 1 minute at 72°C. The last elongation step was extended to 5 minutes at 72°C. A 323 bp fragment of β-globin cluster amplified by primers BG F and BG R was used as internal control (Figure 5.2.3A). The fragments were resolved by 2% agarose gel electrophoresis. The protocol was validated using positive and negative controls which have been genotyped by direct sequence analysis.

5.2.2.5. Genotyping of BCR intron 13 polymorphism
The A>G polymorphism destroys a Sau3Al restriction site which was used for typing it. A 101 bp DNA fragment amplified using forward primer IntBCR13 and reverse primer ExBCR14 was digested with 10 U of Sau3Al and the products were visualised by polyacrylamide gel electrophoresis (Figure 5.2.3B).

5.2.2.6. Sequencing reaction
The BCR-ABL junction sequence, as well as the intron 13 and exon 13 polymorphisms were initially determined by sequencing relevant products using Thermosequenase cycle sequencing kit version 2.0 (Amersham, USA). Once the RT-PCR and PCR technique had been validated, the consecutive patients were classified on the basis of the PCR product size (Figure 5.2.2 G, H, I).
Chapter 5.2 Characterization of BCR-ABL transcript in chronic myeloid leukemia

Figure 5.2.2: Detection of BCR-ABL transcripts by RT-PCR using exon specific primers (ethidium bromide stained 3% agarose gel).

Panel (A) Schematic representation of BCR and ABL genes and primer location shown by arrow head in different color.

Panel (B) RT-PCR product of b3a2 transcript. K562 cell line used as positive control; Lane 1 and 3, b3a2 positive patients, Lane 2, negative control used mRNA from normal peripheral blood; lane M molecular weight marker; lane B, no template control.

Panel (C) for b2a2 transcript. Lane 1 and 3, b2a2 positive; lane 2, 4; b2a2 negative patients sample; lane 5, negative control same as above.

Panel (D) RT-PCR product of ABL gene which is used as control to check RNA input and efficiency of reverse transcription.

Panel (E) for ABL and e1a2 transcript. Lane 1 to 3 for ABL gene; lane 4, e1a2 positive patients.

Figure legend continued to next page
Figure 5.2.2:
(F) RT-PCR product of the BCR-ABL fusion gene. Lane 1 and 3, e19a2 transcript from bone marrow cells of two patients by using BOF-B3A2 and BOR-ABL primers. Lane 2, molecular weight marker. Lane 4, b3a2 transcript from K562 cell line using same primers.
(G) Sequence analysis of the RT-PCR product of b2a2 transcript after clone into a vector using T/A cloning kit from one of the patient studied. Fusion position has shown by arrow with name of the genes.
(H) Sequence analysis of e1a2 transcript. Fusion position has shown on the side of the autoradiogram.
(I) Autoradiogram of e19a2 transcript sequence. Fusion position has shown on the side.

Figure 5.2.3: (A) Detection of BCR exon 13 polymorphism using allele specific PCR.
Lane M, 50-bp ladder marker; Lanes 1-3 and B, mutation specific primer; Lanes 4-6 and B, normal specific primer. Lanes 1 and 4, normal; Lanes 2 and 5, polymorphism heterozygote; Lanes 3 and 6, polymorphism homozygote; Lanes B, no template control. PCR product size shown on the side.
(B) BCR exon 13 polymorphism (T>C) confirmed by direct sequencing the PCR product; polymorphism shown by arrow.
(C) BCR intron 13 polymorphism (G>A) detected by RFLP-PCR. Lanes 1and 2, homozygous (GG); Lane 3, heterozygous (AG) and Lane M. 50bp molecular weight marker. Digested product size shown on the side.
(D) Sequence analysis of BCR intron 13 of PCR product. Panel (a) homozygous normal (AA), panel (b) homozygous polymorphic (GG) and panel (c) heterozygous (AG) at intron 13 branchpoint.

Chapter 5.2
Characterization of BCR-ABL transcript in chronic myeloid leukemia
Chapter 5.2 Characterization of BCR-ABL transcript in chronic myeloid leukemia

5.2.3. Results

Of the 141 CML patients 130 (92%) patients were positive for one or more of the 4 junctional types tested. The b2a2 variant of BCR-ABL transcript was detected in 37 (28.5%) patients, b3a2 in 83 (63.85%) patients, both b2a2 and b3a2 transcripts were detected in 6 (4.6%) patients. Two (1.54%) patients expressed e1a2 transcript and two (1.54%) had e19a2 transcript.

One of the e1a2 expressing patients, a 58 years old male had very short period of chronic phase and progressed to blast crisis and died. Other, male aged 35 years, was treated with hydroxyurea, and progressed towards blast crisis stage in about 5 years. He was then treated with imatinib mesylate and is well maintained for the last one year.

The e19a2 expressing two patients are described here. One is a 38-year-old male diagnosed in 1997 as a classical CML and treated by hydroxyurea. On January 2004 his peripheral blood findings were: Hb 6.6 g/dl, MCV 112 FL, WBC 3.6X10^9/l, with differential counts Neutrophils 44%, Lymphocytes 33%, Monocytes 0%, Eosinophils 05%, Basophils 07%, myeloid blast 1%, promyelocytes 1%, myelocytes 2%, metamyelocytes 5% and platelet 736X10^9/l. In February 2004 his basophile count in peripheral blood increased to 14% and bone marrow smear examination revealed 16% blast cells, indicative of accelerated phase of CML. On examination it was observed that he had mild anemia, no hemorrhage, liver was palpable 6 cm below coastal margin and splenomegaly was absent but he had fever, night sweat and weight loss of five week duration. He had generalized lymph nodal enlargement and largest lymph node in his left axilla was palpable. Patient was started on imatinib mesylate treatment as CML at accelerated phase. The patient died in May 2004.

The second patient was a 19-year-old male having high WBC count, chronic anemia, hepatosplenomegaly. He first reported with low-grade fever since last five months, breathlessness, fatigue, hepatosplenomegaly, chronic anemia and priapism in February 2004 and peripheral blood findings were: Hb 11.4 g/dl, MCV 62 FL, WBC 587X10^9/l with differential counts Neutrophils 40 %, Lymphocytes 0%, Monocytes 0%, Eosinophils 05%, Basophils 06%, myeloblast 1%, myelocytes 27%, metamyelocytes 21% and platelet 541X10^9/l. His bone marrow aspirate examination was hypercellular with bone smear showing 4% blast and this patient was started on hydroxyurea treatment 2000 mg/day and later1500 mg/day as a typical CML patient at chronic phase as per institutional protocol and is well maintained to date.

The clinical data, i.e. sex, age, white blood cell (WBC) count and platelet count at diagnosis before the start of chemotherapy was compared among patients with b2a2 and b3a2 junctional breakpoints (Table 5.2.1).

Co-expression of b2a2 and b3a2 transcripts has been correlated with two polymorphisms, one at intron 13 and other at exon 13. In the present study, typing of the 6 patients co-expressing both the transcripts showed that all of them harbored the
polymorphism at the intron, but not the one at the exon. Specifically, 4 co-expressing patients had GG, and 2 GA genotype at the intron. At the exon, 2 were TT, 3 were TC and 1 was of CC genotype (Table 5.2.2).

Table 5.2.1. Clinical parameters at diagnosis of CML patients in chronic phase (CP) expressing the b2a2 or b3a2 type of the BCR-ABL rearranged mRNA.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>b2a2</th>
<th>b3a2</th>
<th>b2a2</th>
<th>b3a2</th>
<th>b2a2</th>
<th>b3a2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female</td>
<td>n = 17</td>
<td>n = 31</td>
<td>n = 8</td>
<td>n = 11</td>
<td>n = 9</td>
<td>n = 20</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>Mean±SEM 34.2±3.2</td>
<td>38.5±2.7</td>
<td>40.7±4.9</td>
<td>44.3±2.5</td>
<td>29.2±3.8</td>
<td>35.2±3.8</td>
</tr>
<tr>
<td>Median (range)</td>
<td>30.5 (21-61)</td>
<td>40 (6-71)</td>
<td>34 (30-61)</td>
<td>46 (32-57)</td>
<td>27 (21-58)</td>
<td>37.5 (6-71)</td>
</tr>
<tr>
<td>WBC x10^9/l</td>
<td>Mean±SEM 161±34.6</td>
<td>162.3±38.0</td>
<td>160.3±35.0</td>
<td>162.3±38.0</td>
<td>160.3±35.0</td>
<td>162.3±38.0</td>
</tr>
<tr>
<td>Median (range)</td>
<td>162 (12.5-448)</td>
<td>250 (59-2262)</td>
<td>250 (59-2262)</td>
<td>250 (145-1007)</td>
<td>250 (59-2262)</td>
<td>250 (59-2262)</td>
</tr>
<tr>
<td>Platelets x10^9/l</td>
<td>Mean±SEM 419±107.3</td>
<td>378.2±77.7</td>
<td>537±248.5</td>
<td>307±72.7</td>
<td>338±60</td>
<td>437±122</td>
</tr>
<tr>
<td>Median (range)</td>
<td>373 (57-2245)</td>
<td>250 (59-2262)</td>
<td>250 (59-2262)</td>
<td>250 (145-1007)</td>
<td>250 (59-2262)</td>
<td>250 (59-2262)</td>
</tr>
</tbody>
</table>

Table 5.2.2. Six patients expressed both b2a2 and b3a2 type of BCR-ABL transcripts.

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Sex (M/F)</th>
<th>Age (yrs)</th>
<th>WBC x10^9/l</th>
<th>Platelets x10^9/l</th>
<th>BCR exon 13 polymorphism (T&gt;C)</th>
<th>BCR intron 13 polymorphism (A&gt;G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>3</td>
<td>50</td>
<td>250</td>
<td>TT</td>
<td>GG</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>28</td>
<td>35</td>
<td>198</td>
<td>CC</td>
<td>GG</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>58</td>
<td>50</td>
<td>150</td>
<td>TC</td>
<td>AG</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>45</td>
<td>157</td>
<td>250</td>
<td>TC</td>
<td>GG</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>6</td>
<td>172</td>
<td>598</td>
<td>TT</td>
<td>AG</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>53</td>
<td>337</td>
<td>650</td>
<td>TC</td>
<td>GG</td>
</tr>
</tbody>
</table>
Chapter 5.2  Characterization of BCR-ABL transcript in chronic myeloid leukemia

Some essential thrombocythemia (ET) patients are Ph+ and they mostly express the b3a2 transcript [Martiat P et al. 1989, Cervantes F et al. 1993, Morris CM et al. 1991] and only a few the b2a2 transcript. One of our b2a2 expressing patients had essential thrombocythemia with several features resembling CML. Clinical data of the patient are: Age 30 years, Sex male, Hb 7.7 g/dl, RBC 3.3x10^6/ c mm, WBC 12.5X10^9/l, differential count Neutrophils 83%, Lymphocytes 6%, Monocytes 2%, Eosinophils 1%, Basophils 8%, Platelets 2245X10^9/l, MCV 74.5 fl, MCH 22.6 pg, MCHC 30.3 g/dl, reticulocytes 1%. In bone marrow megakaryocytes increased in number, both active and inactive forms were seen.

5.2.4. Discussion
The significance of the different types of transcript, and whether they have any correlation with the age at diagnosis, sex, race, clinical parameters and outcome of the disease have been analysed in many studies.

The distribution of transcript type has been studied in the European, and some other populations [Martínez-Mancilla M et al. 2002, Eisenberg A et al. 1988, Lee MS et al. 1989], the ratio of b2a2 to b3a2 transcripts being roughly of the order of 40:60. However, one study on Ecuadorian population registered a very different ratio of 95:5 [Paz-y-Mino C et al. 2002]. This data, which is probably the first such report on Indian population is consistent with the studies on Caucasian population and does not show any large deviation.

It has been reported that the sub-group of patients who express b2a2 mRNA in chronic phase CML had a high WBC count at the time of diagnosis and were younger with short chronic phase duration [Martínez-Mancilla M et al. 2002]. In this study, b2a2 carrying patients were also relatively younger with WBC count on the higher side. However, statistical significance could not be reached due to small sample size and the time elapsed since the start of the study is too short to distinguish chronic phase duration between the two groups.

Two different groups reported that patients with 3' M-bcr breakpoint [Inokuchi K et al. 1991, Ardem JC et al. 1993] or with b3a2 transcripts [Inokuchi K et al. 1991] had significantly higher number of platelets. However, a third series [Opalka B et al. 1992a,b] showed no correlation between the type of BCR-ABL transcript and platelet counts. A randomized CML trial in United Kingdom also failed to detect a correlation between either genomic or RNA findings and platelet numbers [Shepherd P et al. 1995]. Our data also showed no correlation between the transcript type and platelet counts at diagnosis. However, when the UK series was divided into two groups according to the WBC counts, it was observed that in patients with <100X10^9 WBC/l, b3a2 transcript carriers had significantly higher platelet counts. Such a correlation
was not observed (Table 5.2.1) in this study even when the patients were sub-grouped according to WBC count.

Recent experimental findings concerning in vivo leukemogenic activity of p230 protein compared to the other BCR-ABL forms are not consistent. One proposed that the e19a2 rearrangement is indicative of good prognosis [Li S et al. 1999], and others found that p230 induced a myeloproliferative disease with much longer latency compared with that induced by p185 and p210bcR-ABL [Quackanbush RC et al. 2000, Inokuchi K et al. 2003], and thrombocytosis [Quackanbush RC et al. 2000]. CML-N is characterized by a more benign course compared with typical CML, with lower WBC count with minimal basophile, a milder anemia, less prominent splenomegaly [Pane F et al. 1996]. In contrast here the first case, patient was typical CML at accelerated phase, with increase of basophile in blood, increase of blast cell in bone marrow and lower WBC counts. The second case was a typical CML at chronic phase with significant basophile, marked hepatosplenomegaly and high WBC counts.

Co-expression of b2a2 and b3a2 transcript has been linked to two polymorphisms, T>C at exon 13 and A>G at intron 13. Analysis of the six patients co-expressing both transcripts in this study showed that the exonic polymorphism is not obligatory for co-expression. However, the intronic polymorphism was detected in all these six patients, at least in heterozygous state. Considering that the BCR intronic polymorphism occurs at the invariant A of the splice branchpoint, it is likely that the polymorphism results in reduced efficiency of intron 13 splicing and alternative transcription of the normal BCR and BCR-ABL alleles as suggested by other authors [Branford S et al. 2002].

In summary, this work is the first description of distribution of different BCR-ABL fusion transcripts in CML patients of Indian origin and their clinical parameters. Two more cases of typical CML in accelerated and chronic phase with e19a2 reported here, we opine that there is more evidence in support of the recent viewpoint that perhaps this new transcript is not associated to a particular type of myeloproliferative syndrome. Lack of the exon 13 polymorphism in some co-expressing patients of this study shows that the polymorphism does not have any role in dual expression of b2a2 and b3a2 transcripts and the intron 13 polymorphism at the putative branchpoint is necessary and sufficient for dual expression.