CHAPTER 6

RESULTS AND DISCUSSION

QUANTITATION OF MINIMAL RESIDUAL DISEASE IN ALL

6.1. RESULTS

A preliminary study on quantitation of MRD during treatment was done in 10 ALL cases (T-ALL= 6, precursor-B-ALL= 4) with 44 follow up samples using RQ-PCR. Bone marrow DNA from presentation and follow up samples were quantitated using the standard curve generated with RNaseP gene to correct for the quantity and quality (amplifiability). To determine the sensitivity of the PCR target, diagnostic DNA with 90-95% tumor cell involvement was serially diluted (50 ng to 5 pg leukemic cells) in 500ng of polyclonal control DNA that is equivalent to about $10^5$ cells (5-6 pg of DNA/cell). The serial dilution gives a final concentration of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$.

The dilution series of diagnostic DNA was subjected to RQ-PCR analysis together with 500ng DNA of follow-up samples and negative control (water and MNC DNA). The reverse primer and TaqMan probe sequence used for RQ-PCR analysis are presented in Table 2.5. The sequence of patient-specific forward primer or ASO is highlighted in Table 6.1. The lowest dilution of diagnostic DNA that gave a fluorescent signal, in the absence of a signal from a polyclonal control DNA, was defined as the sensitivity threshold of the PCR target. The standard curve established with the dilution series of diagnostic DNA was used to
define the amount of leukemia DNA in follow-up samples. The amplification plot and Standard curve of ASO-PCR is shown in Fig 6.1. Then the quantity of tumor cells in diagnostic DNA was set at 1.0 and the MRD quantities in follow-up samples were divided by the amplifiable quantity of DNA to obtain the normalized target value. Hence, MRD result is specified as the quantity of leukemic cells among total number of normal cells. MRD level during treatment in Case I to Case X is shown in Fig 6.2 to 6.11. In this study, the median follow up of patients is 18 months. For quantitation of MRD, TCRG clonal marker was used in 6 cases (T-ALL=4 and B-ALL= 2), TCRD was used in 2 cases and IgH was used in 2 cases.
<table>
<thead>
<tr>
<th>Patient Code</th>
<th>Germline V gene sequence</th>
<th>Junctional region sequence</th>
<th>Germline J gene sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Vγ9</td>
<td>ACTGTGCGCTTGTGGGGAGG</td>
<td>GCCCTA</td>
<td>ATAAGAAA (Jγ1)</td>
</tr>
<tr>
<td>2 Vγ10</td>
<td>ACT GTG CTG CGT CGT GG</td>
<td>GAT TAG GAG GCC G</td>
<td>TTATTATAAGAAACTCCTTTGGC (Jγ1)</td>
</tr>
<tr>
<td>3 Vδ1</td>
<td>TTTTGTGCTCTTTGGGAAA</td>
<td>GCACGCTGGGGGATT</td>
<td>ACACCGATAAACTCATCTJ (Jδ1)</td>
</tr>
<tr>
<td>4 Vδ1</td>
<td>TTTTGTGCTCTTT</td>
<td>ACTAGTTTTTTCTGGGGGTAC</td>
<td>ACACCGATAAACTCATCTJ (Jδ1)</td>
</tr>
<tr>
<td>5 Vγ9</td>
<td>ACTGTG CCT TGT</td>
<td>ATTGG</td>
<td>GAATTATTATAAGAAACTCCTTTGGC (Jγ1)</td>
</tr>
<tr>
<td>6 Vγ4</td>
<td>ACT GTG CCA CCT GGG</td>
<td>GAC CAA</td>
<td>ATA AGA AAC TC TTT (Jγ1)</td>
</tr>
<tr>
<td>7 Vγ1</td>
<td>ACTGTGCCACCTG</td>
<td>GGAGGGG</td>
<td>AAGAAACTC (Jγ1)</td>
</tr>
<tr>
<td>8 Vγ4</td>
<td>ACTGTGCCACCTGGATGGG</td>
<td>CCTTGCG</td>
<td>GAATTATTATAAGAAACTCCTTTGGC (Jγ1)</td>
</tr>
<tr>
<td>9 VH3</td>
<td>TGTGCCAGAGGA</td>
<td>TGGGGAAAAAGGGAGGGGTAGTGGCTATCGGAA</td>
<td>CCTCCTTTGAC (JH4)</td>
</tr>
<tr>
<td>10 VH3</td>
<td>TGTGCCGA</td>
<td>GTAGGGAGGGACCGATTTGACTGG</td>
<td>TTATACACTACTACGGTC (JH6)</td>
</tr>
</tbody>
</table>
Fig 6.1  MRD- RQ-PCR Amplification plot and Standard curve. Fig 6.1a shows the amplification plot for 10-fold dilution series of diagnostic DNA (50ng to 5pg of leukemic cells in 500ng of control DNA). Fig 6.1b shows the standard curve for 10-fold dilution series of diagnostic DNA
A case of **Common-ALL**

Age & Sex 19, F

Tc 1.08 lakh/mm$^3$

>90% blasts at diagnosis

Genotype: $\gamma _{H} L- \gamma _{1.3/2.3}$

Maximum Sensitivity 1L in $10^4N$

Reproducible sensitivity 1L in $10^4N$

**MRD at the end of**

- 20 days $1.1 \times 10^3$
- I1 (6 weeks) $1.3 \times 10^4$
- I2 (10 weeks) $5.3 \times 10^4$
- M1 (39 weeks) $3.7 \times 10^4$
- M3 (77 weeks) Negative

**Clinical Status:** Patient has completed treatment and in clinical and hematological remission.
A case of T-ALL.
Age & Sex 13,M
Tc 1.32 lakhs/mm$^3$
>90% blasts at diagnosis
Genotype $\text{Vg}^{\text{III}}$-$\text{Jg}^{1.3/2.3}$
Maximum Sensitivity 1L in $10^4$N
Reproducible sensitivity 1L in $10^4$N

MRD at the end of

<table>
<thead>
<tr>
<th>Stage</th>
<th>Weeks</th>
<th>MRD</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>3.8 in $10^3$</td>
</tr>
<tr>
<td>RI</td>
<td>15</td>
<td>1.2 in $10^3$</td>
</tr>
<tr>
<td>M1</td>
<td>42</td>
<td>2.8 in $10^4$</td>
</tr>
<tr>
<td>M2</td>
<td>64</td>
<td>3 in $10^4$</td>
</tr>
</tbody>
</table>

Clinical status: Patient is undergoing treatment and in clinical and hematological remission.
A case of T-ALL
Age & Sex 13,M
Tc 1.04 lakh/mm³
>90% blasts at diagnosis

Genotype Vδ1- Jδ1
Maximum Sensitivity 1L in 10³
Reproducible Sensitivity 1L in 10²

MRD at the end of

RI1 (13 weeks) 6.8 in 10²
M 1 (49 weeks) 6.0 in 10²
M5 (91 weeks) 4.0 in 10¹
End of M5 (94 weeks) 7.4 in 10¹

Clinical status: Patient had Bone marrow Relapse.
A case of T-ALL
Age & Sex 17, M
Tc 2.2 lakhs/mm³
>90% blasts at diagnosis
Genotype V81- J81
Maximum Sensitivity 1L in 10⁴N
Reproducible Sensitivity 1L in 10³N
MRD at the end of
20 days 5.6 in 10³
I 1 (5 weeks) 7.7 in 10⁴
I 2 (10 weeks) 1.6 in 10⁴
M2 (39 weeks) 1.6 in 10⁴
M3 (55 weeks) Negative

Clinical status: Patient has completed treatment and in clinical and hematological remission.
A case of T-ALL.
Age & Sex 4,M
Tc 4100/mm³
>90% blasts at diagnosis
Genotype Vγ11-Jγ1.3/2.3
Maximum Sensitivity 1L in 10³N
Reproducible Sensitivity 1L in 10²N

MRD at the end of

I 1 (6 weeks) 6.0 in 10²
I 2 (10 weeks) 1.2 in 10³
Consolidation (22 weeks) −Ve
M 1 (37 weeks) −Ve

Clinical Status: Patient had isolated CNS relapse, 4 months after completing treatment
A case of T-ALL
Age & Sex 17, M
Tc 63,000/mm³
> 90% blasts at diagnosis
Genotype Vγ1-Jγ1.3/2.3
Maximum Sensitivity 1L in 10⁵N
Reproducible Sensitivity 1L in 10⁴N
MRD at the end of

RI1 (17 weeks)  9.1 in 10⁴
M1 (37 weeks)  1.7 in 10⁶
M2 (52 weeks)  9 in 10⁶
M5 (104 weeks)  2.1 in 10⁴

Clinical status: Patient is undergoing treatment and in clinical and hematological remission.
A case of Common-ALL
Age & Sex 24, M
Tc 2700/mm³
>90% blasts at diagnosis
Genotype Vγ1-Jγ1.3/2.3
Maximum Sensitivity 1L in $10^4$N
Reproducible Sensitivity 1L in $10^3$N
MRD at the end of
I2 (10 weeks)  2.4 in $10^2$
RI1 (16 weeks)  1.8 in $10^2$
Consol (24 weeks)  6.3 in $10^4$
M3 (59 weeks)  9.7 in $10^4$
M4-2nd phase (64 weeks)  5.1 in $10^4$
End of M4 (79 weeks)  2.3 in $10^4$

Clinical status: Patient has completed treatment and is in clinical and hematological remission.
Fig. 6.9. MRD IN CASE VIII

A case of T-ALL
Age & Sex 13, M
Tc 2.39 l/mm³
74% blasts at diagnosis
Genotype Vγ1-Jγ1.3/2.3
Maximum Sensitivity 1L in $10^5$N
Reproducible Sensitivity 1L in $10^5$N
MRD at the end of

- **Consolidation** (22 weeks) 4.4 in $10^2$
- M1 (36 weeks) 2.1 in $10^6$
- M4 (83 weeks) 1.4 in $10^5$
- M5 (98 weeks) Negative

**Clinical status:** Patient has completed treatment and in clinical and hematological remission.
Fig. 6.10. MRD IN CASE IX

A case of **Common-ALL**
Age & Sex 13,M
>70% blasts at 1st relapse
Tc 5400/mm³
**Genotype: VH3-JH**
Maximum Sensitivity 1 in 10⁵
Reproducible sensitivity 1 in 10⁵

**Clinical status:** This is a case of relapse ALL, re-treated with BFM protocol. RQ-PCR was studied using the DNA at 1st relapse as standard. RQ-PCR results at the end of 20 days, Induction, consolidation and repeat induction phases were negative, possibly due to disappearance of the clonal marker. Now the patient is undergoing treatment and in clinical and hematological remission.
A case of **Common-ALL**

Age & Sex 4,M

Tc 2300/mm³

>90% blasts at diagnosis

Genotype: **VH3-JH**

Maximum Sensitivity 1 in $10^5$

Reproducible sensitivity 1 in $10^4$

**MRD at the end of**

<table>
<thead>
<tr>
<th>Interval</th>
<th>MRD</th>
</tr>
</thead>
<tbody>
<tr>
<td>I1 (6 weeks)</td>
<td>1.1 in $10^3$</td>
</tr>
<tr>
<td>I2 (11 weeks)</td>
<td>1 in $10^5$</td>
</tr>
<tr>
<td>M1 (45 weeks)</td>
<td>3.2 in $10^5$</td>
</tr>
<tr>
<td>M3 (70 weeks)</td>
<td>RQ-PCR –ve</td>
</tr>
</tbody>
</table>

**Clinical Status**: Patient is undergoing treatment and in clinical and hematological remission.
Table 6.2: Gene rearrangements shown in ALL patients and sensitivity of detection in the MRD study

<table>
<thead>
<tr>
<th>Patient and Lineage</th>
<th>Gene rearrangement</th>
<th>Junctional region (nt)</th>
<th>Maximum sensitivity</th>
<th>Reproducible sensitivity</th>
<th>Non-specific amplification of control DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (B) VγII-Jγ1.3/2.3</td>
<td>6</td>
<td>1 in 10⁴</td>
<td></td>
<td>1 in 10⁴</td>
<td>40.4</td>
</tr>
<tr>
<td>2 (T) VγIII-Jγ1.3/2.3</td>
<td>13</td>
<td>1 in 10⁴</td>
<td>1 in 10⁴</td>
<td>1 in 10⁴</td>
<td>NIL</td>
</tr>
<tr>
<td>3 (T) Vδ1-Jδ1</td>
<td>15</td>
<td>1 in 10³</td>
<td>1 in 10²</td>
<td>1 in 10³</td>
<td>NIL</td>
</tr>
<tr>
<td>4 (T) Vδ1-Jδ1</td>
<td>21</td>
<td>1 in 10⁴</td>
<td></td>
<td>1 in 10³</td>
<td>39.3</td>
</tr>
<tr>
<td>5 (T) VγII-Jγ1.3/2.3</td>
<td>5</td>
<td>1 in 10³</td>
<td></td>
<td>1 in 10²</td>
<td>NIL</td>
</tr>
<tr>
<td>6 (T) VγI-Jγ1.3/2.3</td>
<td>6</td>
<td>1 in 10⁵</td>
<td></td>
<td>1 in 10⁴</td>
<td>43.3</td>
</tr>
<tr>
<td>7 (B) VγI-Jγ1.3/2.3</td>
<td>7</td>
<td>1 in 10⁴</td>
<td></td>
<td>1 in 10³</td>
<td>39.4</td>
</tr>
<tr>
<td>8 (T) VγI-Jγ1.3/2.3</td>
<td>7</td>
<td>1 in 10⁵</td>
<td></td>
<td>1 in 10⁵</td>
<td>40.0</td>
</tr>
<tr>
<td>9 (B) VH3-JH4</td>
<td>32</td>
<td>1 in 10⁵</td>
<td></td>
<td>1 in 10⁵</td>
<td>NIL</td>
</tr>
<tr>
<td>10 (B) VH3-JH6</td>
<td>25</td>
<td>1 in 10⁵</td>
<td></td>
<td>1 in 10⁴</td>
<td>NIL</td>
</tr>
</tbody>
</table>
In 4 cases (Cases VI, VIII, IX and X), a maximal sensitivity of detecting one leukemic cell in $10^5$ normal cells was detected and in 4 more cases (Cases I, II, IV and VII) a maximal sensitivity of detecting 1 in $10^4$ was reached. A reproducible sensitivity of detecting 1 in $10^5$ and 1 in $10^4$ was reached in 2 cases and 4 cases respectively (Table 6.2). The amount of bone marrow blasts at presentation was >90% in all but one case (Case VIII). In Case VIII due to 74% blasts at diagnostic DNA, the quantity of tumor cells in diagnostic DNA was set at 1.35 (100/74) instead of considering it as one and the MRD quantities in follow-up samples were compared to 1.35. In Case V, the junctional region contained only 5nt and the maximum sensitivity and reproducible sensitivity were 1 in $10^3$ and 1 in $10^2$ respectively. TCRD target was used for the present MRD study in two cases only. The reproducible sensitivity in Case III was reduced due to the poor quality of diagnostic DNA. In Case IV, a maximal sensitivity of 1 in $10^4$ was reached. In 2 cases of B-ALL, $\text{IgH} (\text{V_H-D_H-J_H})$ was used as a clonal marker. That reached a reproducible sensitivity of detecting 1 in $10^5$ in case IX and a reproducible sensitivity of 1 in $10^4$ in Case X.

6.2. DISCUSSION

This preliminary study was aimed to quantitate the MRD in follow up samples of ALL using RQ-PCR with rearranged TCR and IgH as PCR targets. MRD was studied in 10 cases during treatment with MCP 841 protocol. RQ-PCR can detect the amplification at an exponential phase and can precisely define the initial amount of target DNA. RQ-PCR is a sensitive technique, which can detect
one tumor cell amidst one million normal cells. The sensitivity of RQ-PCR also depends on the PCR target. Quantitation of MRD using chromosomal translocations as PCR target, generally reaches a sensitivity of $10^{-5}$ to $10^{-6}$ (Van der Velden et al., 2003b). The sensitivity of MRD-PCR analysis of junctional region is dependent on the type of rearrangement specified and also on the ‘background’ of normal lymphoid cells with comparable TCR or Ig rearrangements. MRD-PCR analysis of short $V_\gamma$-$J_\gamma$ junctional region is generally less sensitive ($10^{-3}$ to $10^{-5}$) than long $V_\delta1$-$J_\delta1$ junctional region ($10^{-4}$ to $10^{-6}$) due to the abundance of polyclonal $V_\gamma$-$J_\gamma$ joinings in normal T-cells (Sczepanski et al., 2002a). In our study, amplification of control DNA decreased the sensitivity in 5 of 10 cases. Van der velden et al. in their MRD study using TCRG target obtained a reproducible sensitivity of $\leq1\times10^{-4}$ in 10 of 15 (67%) T-ALL patients and 4 of 19 (21%) precursor B-ALL patients (Van der Velden et al., 2002b). In Case X, though a reproducible sensitivity of 1 in $10^5$ was reached, the follow-up samples were not amplified, possibly due to continuing or secondary Ig gene rearrangements (clonal evolution) of the target. Such false-negative result can be prevented by using two TCR or Ig targets per patient (Van Dongen et al., 1998).

In our study, the follow-up samples were obtained at different time points and at the end of I1 phase, in 4 cases (T-ALL=2 and B-ALL= 2) MRD was $<4\times10^{-3}$ and in one case of T-ALL MRD was $6\times10^{-2}$. In 5 cases (B-ALL=3 and T-ALL= 2) after I2 phase the MRD was $<6\times10^{-3}$ in 4 cases (Cases I, IV, XI and V) and $2.4\times10^{-2}$ in one case (case VII). At the end of repeat Induction phase (RI1),
the MRD was $<2 \times 10^{-3}$ in two T-ALL cases (Cases II and VI) and $<7 \times 10^{-2}$ in two cases (Cases III and VII). Several studies have verified that absence of residual disease after remission induction is associated with good prognosis (Cave et al., 1994; Brisco et al., 1994; Steenbergen et al., 1995c). Brisco et al in their study showed that patients with residual leukemia $>1 \times 10^{-3}$ at the end of induction had a poor clinical outcome (Brisco et al., 1994). In the present study, 4 of 6 T-ALL cases WBC count at diagnosis was $>1$ lakh/mm$^3$ and in 3 of 4 cases of B-ALL, WBC count at diagnosis was $<5500$/mm$^3$. The percentage of bone marrow blasts at diagnosis was $>90\%$ in 8 cases. However, the MRD level after treatment has no correlation with age, WBC counts, blasts and immunophenotype at diagnosis. Some studies also showed that prognostic value based on MRD is independent of other clinically relevant risk-factors including age, blast count at diagnosis, immunophenotype at diagnosis, presence of chromosome aberrations, response to prednisone and classical clinical risk group assignment (Jacquy et al., 1997; Cave et al., 1998; Van Dongen et al., 1998).

Case I has responded to treatment well and the leukemia load has reduced from 90$\%$ lymphoblasts to $3.7 \times 10^{-4}$ at the end of M1 and the MRD was not detectable at the end of M3. In a case of T-ALL (Case III), the tumor load was $6.8 \times 10^{-2}$ at the end of RL1, hinting that the patient had not responded to treatment and after M2 the tumor load remained at $6 \times 10^{-2}$, later at M5 phase the patient had a hematological bone marrow relapse. Though the patient was in clinical and hematological remission, Real-time PCR had detected the residual disease at an
early time-point but the disease emerged as clinically evident at end of M5 phase only. In Case V, at the end of I2 phase the MRD was $1.2 \times 10^{-3}$. In this case, RQ-PCR was negative at the end of the consolidation phase and maintenance I phase. However the patient had isolated CNS relapse, 4 months after completion of treatment. Both the relapsed cases in this study were of T-ALL.

In Case VI, the MRD level was drastically increased from $9 \times 10^{-6}$ at the end of M2 phase to $2.1 \times 10^{-4}$, after M5 phase and the patient is in clinical and hematological remission. In Case VII, a B-ALL, at the end of RI1 the MRD was $1.8 \times 10^{-2}$ which was reduced to $6.3 \times 10^{-4}$ at the end of consolidation phase then reduced to $2.3 \times 10^{-4}$ at the end of M4 and the case is in clinical and hematological remission. In Case VIII, MRD at the end of consolidation phase was $4.4 \times 10^{-2}$ and after M1 phase it was noticeably reduced to $2.1 \times 10^{-6}$. Case IX though reached a reproducible sensitivity of $1 \times 10^{-5}$, the follow-up samples were not amplified possibly due to clonal evolution. The International BFM Study Group identified three different MRD-based risk groups according to the kinetics of tumor reduction during the first three months of therapy (end of induction and consolidation phase): a low-risk group defined by MRD negativity at time-points one and two, comprised 43% of patients and had a 3-year relapse rate of only 2%. In contrast, the high-risk group (15% of patients) with MRD levels $\geq 10^{-3}$ at both time-points relapsed in 75% of cases; the intermediate risk group, accounting for 43% of patients had a relapse rate of 23% (Van Dongen et al., 1998). In this study, MRD value at last follow-up of maintenance phase (M1 to
M6) revealed MRD-negative in five cases (Cases I, IV, V, VIII and X) and showed MRD-positive in four cases (Cases II, III, VI and VII).

Our preliminary study reiterates that MRD level after treatment has no correlation with age, WBC counts, blasts and immunophenotype at diagnosis. Due to the limited number of cases with different time-points of follow-up it is difficult to risk-stratify the patients. Hence, a larger number of cases with longer follow-up is needed to risk-stratify the patients based on the quantity of MRD.