Donor Chimerism and Bcr-Abl Gene Status Following Non-Myeloablative Peripheral Blood Stem Cell Transplantation in Chronic Myeloid Leukaemia Patients in HUKM

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ABSTRACT

Objective: This study was done to determine the relationship between donor chimerism and the presence of bcr-abl gene in chronic myeloid leukaemia (CML) patients post-transplantation. Methods: The study population consisted of all CML patients who had undergone non-myeloablative peripheral blood stem cell transplant in Hospital Universiti Kebangsaan Malaysia (HUKM) during the study period. All patients had their bone marrow aspiration done at diagnosis and day 30, 60, 100, 130, 160 and 190 post-transplantation. The samples were analysed for bcr-abl transcript as well as chimerism status. Results: A total of nine cases underwent non-myeloablative peripheral blood stem cell transplant. All patients were transplanted during the chronic phase. One patient was found to show mixed chimerism at day 30 post-transplant coinciding with bcr-abl transcript disappearance. Six patients showed that full donor chimerism correlated with bcr-abl transcript disappearance. In one patient, chronic myeloid leukaemia transformed into acute myeloid leukaemia. Another patient had a graft failure. Conclusion: This observational cohort study showed that full chimerism is required for disappearance of bcr-abl transcript but one case showed disappearance of bcr-abl transcript at day 30 while full chimerism was not achieved.

Keywords: Chronic myeloid leukaemia, chimerism, bcr-abl

INTRODUCTION

Chronic myeloid leukaemia (CML) was first recognised as early as in 1845 when several cases presented with splenomegaly, anaemia and massive granulocytosis. It was the discovery of Philadelphia (Ph) chromosome in 1960 that led to a better understanding of the pathogenesis of the disease. It took another thirteen years before the Ph chromosome was shown to be generated by a specific translocation involving chromosomes 9 and 22. In the 1980s, the bcr-abl fusion oncogene was described, and found to be transcribed and translated into a functional protein. Finally, in 1990, the first definitive evidence of the ability of bcr-
abl protein to transform primary myeloid cells and induce a CML-like disease in mice was published.[1]

Conclusive evidence of CML relies on cytogenetic and molecular studies to detect the t(9; 22) (q34; q11) translocation. The Ph chromosome is an acquired cytogenetic abnormality that characterises all leukaemic cells in CML. However, it is not pathognomonic for CML as it is present in 25-50% of adult patients with acute lymphoblastic leukaemia and in 2% with acute myelogenous leukaemia.[3] It was the first chromosomal abnormality associated with a specific malignancy.[3]

In CML transplantation, three strategies have been developed that include the use of reduced intensity preparative regimens, the induction of tolerance between host and donor immune systems by creating a mixed chimaeric state after transplant and optimising graft versus leukaemic effect by increasing the speed and completeness of donor immune recovery. These approaches are collectively grouped under the term 'non-myeloablative stem cell transplant' or called as 'minitransplant'.

**METHODS**

*Patient Sample*

Bone marrow samples was collected with patients’ consent from the haematology wards of HUKM. Ethical committee approval was obtained from HUKM. Selection criterion was CML patients who had undergone minitransplant. An exclusion criterion was CML patients who only received medications. Nine patients had their bone marrow aspiration done at diagnosis, day 30 post-transplant, day 60 post-transplant, day 100 post-transplant, day 130 post-transplant and day 160 post-transplant. The samples were analysed for bcr-abl transcripts as well as chimerism status.

*Mononuclear Cell Extraction from Bone Marrow Aspiration*

Mononuclear cell isolation was performed by gradient density centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech Ab, Uppala, Sweden). Cells were washed to remove excess Ficoll-Hypaque. The cells were spun at 1000 rpm for 10 minutes to obtain cell pellet. The supernatant was decanted.

*Chimerism Assessment by Short Tandem Repeats*

DNA extraction

200 μl binding buffer and 40 μl proteinase K were added to 200 μl of the bone marrow specimen. The mixture was mixed immediately and incubated for 10 minutes at 72°C. 100 μl of isopropanol was added and the mixture was applied to a high pure filter tube and centrifuged for 1 minute at 8000 rpm. Then 500 μl of wash buffer was added and centrifuged for 1 minute at 8000 rpm. A new tube and 200 μl of elution buffer were added before centrifuging the mixture for 1 minute at 8000 rpm. The micro centrifuge tube then contained eluted nucleic acid.
DNA quantification
The purity and quantity of DNA were determined by spectrophotometer. The ratio of wavelength reading at 260:280 nm was between 1.8-2.0. The DNA concentration was estimated by measuring its absorbance at 260 nm.

DNA amplification
The master mix was prepared by using 21 µl of the reaction mix, 11 µl of the primer set and 1.0 µl of the Ampli Taq Gold (Applied Biosystems, Foster City, Canada). The mixture was vortexed for 5 seconds and 30 µl was pipetted into each PCR tube. 20 µl of DNA and positive control were added into the PCR tubes respectively. In the negative control tube, 20 µl of the TE buffer was added. All tubes were placed into the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, Canada) instrument.

Acrylamide gel electrophoresis
Acrylamide gel electrophoresis was done by using the ABI Prism 377 instrument. Once the gel temperature reached 51°C, 1.5 µl of DNA sample was injected using the flat tip into each of the sample loading areas.

Bcr-abl Status Assessment by RT-PCR
RNA was extracted according to the manufacturer’s instructions as described before. Following that, the RNA was converted to cDNA. 8 µl of cDNA sample and 1 µl of Taq Polymerase were mixed with the PCR mix which contained 2.4 µl of PCR buffer, 0.9 µl of MgCl₂, 0.48 µl of dTPs, 0.112 µl of CA3, 0.106 µl of C5e, 0.100 µl of B2B, 0.088 µl of BCR-C and 11.812 µl of distilled water. The mixture was amplified using thermal cycle 480 and the product was run onto agarose gel for electrophoresis. The band was visualised using ultraviolet light.

RESULTS

Clinical Features
A total of nine cases of CML underwent non-myeloablative peripheral blood stem cell transplant in Hospital Universiti Kebangsaan Malaysia during the study period (Table 1). All patients presented and were transplanted during the chronic phase. Eight patients were transplanted within one year of diagnosis of CML. However, one patient (CML 9) was transplanted 4 years after he had been diagnosed to have CML. All except one were males. The median age was 32 years (range 15-49 years). Four were Malays while five were Chinese.

All patients had high total white cell count at presentation, with the lowest being 16.0x10⁹/l and the highest 231.0x10⁹/l. CML Patient 6 had the biggest spleen size at
Table 1. Patients characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>Ethnic group</th>
<th>Total white blood cell</th>
<th>Spleen</th>
<th>Liver</th>
<th>Bcr-abl at diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML 1</td>
<td>23</td>
<td>Male</td>
<td>Malay</td>
<td>60.0 x 10^9/L</td>
<td>3cm</td>
<td>4cm</td>
<td>Positive</td>
</tr>
<tr>
<td>CML 2</td>
<td>28</td>
<td>Male</td>
<td>Chinese</td>
<td>231.0 x 10^9/L</td>
<td>15cm</td>
<td>6cm</td>
<td>Positive</td>
</tr>
<tr>
<td>CML 3</td>
<td>37</td>
<td>Male</td>
<td>Chinese</td>
<td>36.6 x 10^9/L</td>
<td>2cm</td>
<td>nil</td>
<td>Positive</td>
</tr>
<tr>
<td>CML 4</td>
<td>49</td>
<td>Male</td>
<td>Malay</td>
<td>45.6 x 10^9/L</td>
<td>7cm</td>
<td>1cm</td>
<td>Positive</td>
</tr>
<tr>
<td>CML 5</td>
<td>17</td>
<td>Male</td>
<td>Malay</td>
<td>63.2 x 10^9/L</td>
<td>12cm</td>
<td>3cm</td>
<td>Positive</td>
</tr>
<tr>
<td>CML 6</td>
<td>35</td>
<td>Female</td>
<td>Malay</td>
<td>150.0 x 10^9/L</td>
<td>16cm</td>
<td>6cm</td>
<td>Positive</td>
</tr>
<tr>
<td>CML 7</td>
<td>40</td>
<td>Male</td>
<td>Chinese</td>
<td>23.4 x 10^9/L</td>
<td>5cm</td>
<td>2cm</td>
<td>Positive</td>
</tr>
<tr>
<td>CML 8</td>
<td>15</td>
<td>Male</td>
<td>Chinese</td>
<td>16.0 x 10^9/L</td>
<td>3cm</td>
<td>1cm</td>
<td>Positive</td>
</tr>
<tr>
<td>CML 9</td>
<td>49</td>
<td>Male</td>
<td>Chinese</td>
<td>47.5 x 10^9/L</td>
<td>4cm</td>
<td>nil</td>
<td>Positive</td>
</tr>
</tbody>
</table>

presentation and she also had a hepatomegaly of 6 cm. All patients exhibited bcr-abl gene transcripts positivity pre-transplant.

Chimerism Status and bcr-abl Analysis

All patients had bcr-abl analysis done pre-transplant and all were found to be positive. Both bcr-abl and chimerism study were performed on day 30, 60, 100, 130, 160 and day 190 post-transplantation.

CML Patient 1 attained mixed chimerism at day 60 post-transplant, however bcr-abl still showed positivity. He only attained complete chimerism at day-130 post-transplant and simultaneous disappearance of the bcr-abl transcripts. The same findings were observed for CML Patient 2. In the case of CML Patient 3, he attained complete chimerism and disappearance of the bcr-abl transcripts at day 100 post transplant. In CML Patient 4, after day-100 post transplant, analysis still showed no chimerism; he received donor lymphocyte infusion. Subsequently, only at day 160 post transplant did he achieve complete chimerism together with the disappearance of the bcr-abl transcripts. He also did not experience any graft versus host disease (GVHD) which may delay bcr-abl transcripts clearance. Whereas CML Patient 5 showed graft failure signs at day 60 post transplant and subsequently received donor lymphocyte infusion. Further, at day 100 post transplant, he did not achieve any chimerism status. Only at day 130 post transplant, did he show complete chimerism as well as disappearance of the bcr-abl transcripts. CML Patient 6 achieved transient mixed chimerism at day 30 post transplant, but reverted to no chimerism status for the subsequent analysis. She received donor lymphocyte infusion two times, at day 120 and day 180 post-transplant; however, her leukaemic cells failed to clear. Patient CML 7 achieved complete chimerism status and disappearance of the bcr-abl transcripts faster than the other patients described before and this status persisted throughout the study period. Patient CML 8 was the unique patient; at day 30 post-transplant, he showed mixed chimerism as well as disappearance of the bcr-abl transcripts. Subsequently, he achieved complete chimerism which persisted. Patient CML 9 showed mixed chimerism and persistently positive bcr-abl
transcripts. At day 100 post-transplant, he showed signs of transformation to acute leukaemia with his peripheral blood showing presence of blast cells. He immediately received donor lymphocyte infusion but it failed to kill the leukaemic cells. Subsequent analysis showed no chimerism and presence of the \textit{bcr- abl} transcripts.

**DISCUSSION**

In the CML patients studied, 55.5\% were Chinese whereas 44.4\% were Malays. There was a marked gender difference in this group of patients with 88.8\% being male and 11.1\% being female. The median age at presentation was 32 (range 15-49 year old) compared to SC Ng et al. \cite{SCNg} who found the age at presentation to be 34.9 years while Hamidah et al. \cite{Hamidah} reported the mean age at presentation to be 34.8 years. It appears that Malaysian CML population is
younger compared to the West. The reason is not clear but could be due to the lower average age of the Malaysian population compared to the population in the West.\(^4\)

In this study, the chronic leukaemia of CML Patient 9 transformed into acute leukaemia after day 90 post-transplantation. Though initially, he attained mixed chimerism, promising for engraftment, the mixed chimerism failed to sustain. At the time he showed haematological transformation into acute leukaemia, he had received donor lymphocyte infusion (DLI). Donor lymphocyte can restore complete remission but is only effective in about 70% of cases with a failure rate of 30%.\(^6\)

Some studies indicate that mixed chimeras may be at higher risk of relapse\(^7.8,9\) while other studies do not corroborate this finding.\(^9,10,11\) No conclusion can be drawn from our small series. To date, many investigators believed that CML patients need complete donor chimerism to eliminate the bcr-\(\text{abl}\) transcripts. Our study appears to support this belief as complete chimerism was observed to correlate with clearance of bcr-\(\text{abl}\) transcripts. Seven out of nine patients attained complete chimerism simultaneously with the disappearance of the bcr-\(\text{abl}\) transcripts. In one of the studies carried out\(^12\), the authors found immune reconstitution after transplantation showing a complete chimerism state had significantly higher subsets of CD2, CD3, CD4, CD5 and CD6 cells than that of the mixed chimeric state.

In our series, one patient (CML 8) managed to eliminate his bcr-\(\text{abl}\) transcripts while he was only at mixed chimerism state. The reasons for such a finding could be as follows:

1. NK cells after transplant are higher in a mixed chimeric state\(^12\). NK cells are capable of cytolysis activity against residual tumour cells. So this could be one of the possibilities why his mixed chimerism state appeared to eliminate his bcr-\(\text{abl}\) transcripts.
2. The persistence of recipient cells in a mixed chimeric state results in the continued presence of allo-antigens (e.g. minor histo-compatibility antigens) unfamiliar to the donor origin of T cell precursors\(^13\). Evidence suggests that such minor histo-compatibility plays an important role in the GVHD and graft versus leukaemia (GVL) effect.\(^13\) An increasing number of minor histo-compatibility antigens have been identified and it has been shown that the expression of some minor histo-compatibility antigens is restricted to haematopoietic cells and leukaemia cells.\(^14\)
3. It could be that the residual disease in this patient is more sensitive and fragile to immune control by the graft compared to the other patients.
4. The last reason could be that he underwent the transplantation at a stage where his residual disease is very low and this facilitated clearance of bcr-\(\text{abl}\) transcripts by the graft.

**CONCLUSION**

This observational cohort study showed that full chimerism is essential for disappearance of bcr-\(\text{abl}\) transcripts. However, one patient did not require full chimerism state to clear the disease. Further studies need to be carried out to elucidate the mechanisms of tumour clearance without full chimerism.
REFERENCES


