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

[1]S Zainina , [2]CF Leong, [2]NH Hamidah, [2]O Ainoon & [3]SK Cheong

[1]Department of Pathology, Faculty of Medicine and Health Sciences
Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[2]Department of Pathology, Hospital Universiti Kebangsaan Malaysia
Jalan Yakob Latif, Cheras, Kuala Lumpur, Malaysia
[3]Department of Medicine, Clinical School, International Medical University
Seremban, Negeri Sembilan, Malaysia

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.[1,2] 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-*

*Corresponding author: *zainina@medic.upm.edu.my*

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, Uppsala, 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 $MgCl_2$, 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 $16.0x10^9$/l and the highest $231.0x10^9$/l. CML Patient 6 had the biggest spleen size at

Table 1. Patients characteristics

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

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*

Table 2. Short tandem repeats (STR) analysis and *bcr-abl* transcript

pt name	bcr-abl	D30		D60		D100		D130		D160		D190	
	pretransp	chimerism	bcr-abl	chimerism	bcr-abl	chimerism	bcr-abl	chimerism	bcr-abl	chimerism	bcr-abl	chimerism	bcr-abl
CML 1	pos	NC	pos	MC	pos	MC	pos	CC	neg	CC	neg	CC	neg
CML 2	pos	MC	pos	MC	pos	MC	pos	CC	neg	CC	neg	CC	neg
CML 3	pos	MC	pos	MC	pos	CC	neg	CC	neg	CC	neg	CC	neg
CML 4	pos	NC	pos	NC	pos	NC	pos	NC	pos	CC	neg	CC	neg
CML 5	pos	NC	pos	graft failure		NC	pos	CC	neg	CC	neg	CC	neg
CML 6	pos	MC	pos	NC	pos	NC	pos	NC	pos	NC	pos	NC	pos
CML 7	pos	MC	pos	CC	neg	CC	neg	CC	neg	CC	neg	CC	neg
CML 8	pos	MC	neg	CC	neg	CC	neg	CC	neg	CC	neg	CC	neg
CML 9	pos	MC	pos	MC	pos	MC relapse	pos	MC	pos	MC	pos	NC relapse	

CC – Complete chimerism
NC - No chimerism
MC – Mixed chimerism

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 *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.* [4] who found the age at presentation to be 34.9 years while Hamidah *et al.* [5] 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-abl* transcripts. Our study appears to support this belief as complete chimerism was observed to correlate with clearance of *bcr-abl* transcripts. Seven out of nine patients attained complete chimerism simultaneously with the disappearance of the *bcr-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-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 cytolytic activity against residual tumour cells. So this could be one of the possibilities why his mixed chimerism state appeared to eliminate his *bcr-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-abl* transcripts by the graft.

CONCLUSION

This observational cohort study showed that full chimerism is essential for disappearance of *bcr-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

[1] Holyoake, Dr Tessa L. Recent advances in the molecular and cellular biology of chronic myeloid leukaemia: lessons to be learnt from the laboratory. Br J Haematol 2001;113:11-23.

[2] Geary CG. The story of chronic myeloid leukaemia. Br J Haematol 2000; 110:2-11.

[3] Lee, Stephanie J. Chronic myeloid leukaemia. Br J Haematol 2000;111: 993-1009.

[4] SC Ng, P Kuperan. Correspondence: age at diagnosis of chronic myeloid leukaemia. Malaysian J Pathol 1990;12(2):111

[5] NH Hamidah, SK Cheong, O Ainoon. Correspondence: age at diagnosis of chronic myeloid leukaemia. Malaysian J Pathol. 1999;13(2):123.

[6] Kolb, Schattenberg, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, Ljungman P, Ferrant A, Verdock L, Niederwieser D, Van Rhee F, Mittermuller J, De Witte T, Holl E, Anari H. Graft versus leukaemia effect of donor lymphocyte infusions in marrow grafted patients. Blood 1995; 86: 2041-7.

[7] Lawler SD, Baker MC, Harris B, Morgenstern GR. Cytogenetics study on recipients of allogeneic bone marrow using the sex chromosome as a markers of cellular origin. Br J Haematol 1984;56:431-42.

[8] Walker H, Singer CRJ, Pattereson J, Goldstone AH. The significance of the host haemopoietic cells detected by cytogenetic analysis of bone marrow from recipients of bone marrow transplant. Br J Haematol 1986;62: 385-95.

[9] Bretagne S, Vidaud M, Kuentz M, Cordonnier C. Mixed blood chimerism in T cell depleted marrow transplant recipients: evaluation using DNA polymorphism. Blood 1987;70:1692-709.

[10] Schimitz M, Godde Salz E, Lofhler H. Cytogenetics study on recipients of allogeniec bone marrow transplant after fractionated total body irradiation. Br J Haematol 1985;60: 239-52.

[11] Schattenberg A. DeWitte T, Salden M, Vet J. Mixed haemopoietic cimerism after allogeneic transplant with lymphocyte depleted bone marrow not associated with a higher incidence of relapse. Blood 1989;73:1367-78.

[12] Denis C, Ramana T, Christine M, Keith D, Barbara G, Kenneth C, Anderson A, Lee M, Jerome R. Natural history of mixed chimerism after bone marrow transplantation with CD6 depleted marrow : a stable equilibrium. Blood 1990;75(1): 296-304.

[13] Chaterine J, Antoinette C, Edwin P,Enrica O, Donna N, Robert J, Jerome R. Reconstitution of T cell receptor repertoire diversity following T cell depleted allogeneic bone marrow transplantation related to haematopoietic chimerism. Blood 2000;95(1):352-9.

[14] Warren EH, Greenberg PD, Riddel SR. Cytotoxic T lymphocyte define human histocompatibility antigens with a restricted tissue distribution. Blood 1998; 91:2197-207.