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Original Article

Pathology Section

# Chimerism Revisited: Perspective of an Immunopathologist

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## ABSTRACT

**Introduction:** Chimerism analysis is an important diagnostic tool not only for assessing the risk of relapse after allo-Haematopoietic Stem Cell Transplant (HSCT) in patients with malignant diseases, it is also the predominant method for monitoring post-transplant engraftment status in both haematological malignancies and non malignant haematological disorders.

**Aim:** To show the chimerism data observed in post-HSCT cases of the hospital over last four years and to emphasise on timely and close monitoring of these patients in the laboratory.

**Materials and Methods:** The present study was a retrospective study in which 40 HSCT cases comprising of both haematological malignancies and non malignant haematological disorders were monitored in the molecular laboratory of Army Hospital (Research and Referral), at regular intervals by Short Tandem Repeats-Polymerase Chain Reaction (STR-PCR) for quantification of donor chimerism. The pretransplant workup included Human Leukocyte Antigens (HLA) typing of all recipients and donors, serum Panel Reactive Antibody (PRA) testing and Single Antigen Bead (SAB) assays for detecting donor specific antibodies in all haploidentical transplants.

**Results:** Male patients formed majority, with only nine female cases. After complete HLA matching and preconditioning, 35 patients underwent matched related stem cell transplant, two were Matched Unrelated Donor (MUD) and three haploidentical transplants. Complete donor chimerism at D+90 was reported in 19 patients (73%) of haematologic malignancies with two cases of relapse and five showing evidence of Graft Versus Host Disease (GVHD). Amongst non malignant disorders 10 patients (71.4%) showed complete donor chimerism at D+90 with two cases of GVHD.

**Conclusion:** Post-HSCT, progressive chimerism monitoring is an essential molecular test that predicts engraftment status of the patient by verifying the dynamic relationship between recipient and donor cells.


**Keywords:** Allogeneic haematopoietic stem cell transplantation, Graft versus host disease, Haploidentical transplant, Minimal residual disease, Short tandem repeat

## INTRODUCTION

Allogenic HSCT is a potentially curable treatment option for patients suffering from both haematologic malignancies and various non malignant haematologic conditions like aplastic anaemia, haemoglobinopathies, storage disorders and primary immunodeficiency diseases etc., [1,2]. Allogenic HSCT is performed after chemo/radio ablation of the recipient marrow followed by infusion of donor stem cells that facilitates sustained donor cell engraftment. Post-HSCT, what is desirable is a state of engraftment where donor cells completely replace the recipient's haematopoietic lineage. This type of donor immune reconstitution is synonymous to tolerance [3]. Though efforts to develop tolerance inducing strategies and agents are gaining rapid recognition, it still remains a distant possibility. Allogenic HSCT is associated with complications related to use of preconditioning regimen, HLA match, type of graft (T cell deplete or replete), underlying molecular aberrations of the disease etc., therefore, a need for frequent monitoring of transplant recipient for development of sinister events like graft failure, relapse or GVHD. Various molecular techniques are used to predict re-emergence of disease clone or recipient haematopoiesis. It is here that the role of immuno/haematopathologist becomes crucial. For all patients of allogenic HSCT, a surveillance protocol for engraftment evaluation post-HSCT is followed using chimerism studies. Chimerism testing helps predict graft rejection or recurrence of disease. Chimerism testing is routinely done between 30 and 100 days after transplantation. Post-HSCT, the recovery of haematopoiesis in recipient depends on ability of donor stem cells to generate progenitor cells and repopulate the marrow niche. This dynamic donor-recipient mix is named chimera which is measured qualitatively and quantitatively in post-transplant patients [4-6].

Regular monitoring of chimerism status provides an early indication of incipient relapse of underlying malignancy. Increasing mixed chimerism warrants initiation of immunotherapy with Donor Lymphocyte Infusion (DLI) and reduction of Immunosuppression (IS). The clinical importance of chimerism has increased further with introduction of Reduced Intensity Conditioning (RIC), as more patients show mixed chimerism [7,8].

Chimerism analysis also serves as a surrogate marker for Minimal Residual Disease (MRD) detection especially in acute leukaemia's without suitable molecular alterations. It remains the only most important tool in predicting early disease relapse or graft failure in non malignant disorders. The authors report here, the spectrum of chimerism data observed at our centre with emphasis on following analgorithmic approach in the post-HSCT period which may serve to monitor and predict disease status.

## MATERIALS AND METHODS

The present study was a retrospective study in which 40 patients were analysed who underwent HSCT at Army Hospital (Research and Referral) over four years i.e. January 2015 to June 2019 and analysed over next six months. Patients and donors gave written informed consent and study was approved by Hospital Ethical Committee. The study consisted of 40 patients of haematological malignancies and non malignant haematological conditions who underwent HSCT. They were monitored at regular intervals for disease relapse, graft failure or GVHD, postallogenic HSCT by chimerism. Recipients included those suffering with haematological malignancies and non malignant disorders for which a bone marrow transplant was done.

**Inclusion criteria:** All patients with atleast six months of initial clinical and laboratory follow-up were included in the study.

**Exclusion criteria:** Cases that were not on regular follow-up and non compliant patients were excluded from the study.

### Study Procedure

The HLA typing was done by Sequence Specific Primer (SSP) method in the laboratory for all patients [9]. For haploidentical match transplants the pretransplant immunological monitoring also included performing screening of serum Panel Reactive Antibodies (PRA) in the recipient for Class-I and Class-II HLA antigens by flow cytometry and further using luminex platform for SAB assay for detecting donor specific antibodies. Blood and bone marrow samples were taken at various time periods after transplantation and tested for chimerism using Amp FLSTR Identifiler kit (Applied Biosystems). STR-PCR chimerism for engraftment purpose was performed every 30 days for first six months, then at three monthly intervals for two years post-transplant and six monthly thereafter. This time interval protocol was strictly adhered especially, in cases of haematologic malignancies.

**Conditioning:** Intravenous infusion of cyclophosphamide, Fludarabine, busulphan, Antithymocyte Globulin (ATG) and melphalan were used as chemotherapeutic agents in different combinations and dosages. ATG was given primarily to patients with mismatched donors and to MUDs. Post-transplant IS consisted of cyclosporine in combination with short course of methotrexate, mycophenolate mofetil. In cases of haematological non malignant conditions who undergo RIC, the immunosuppressive agents were continued for one year as against malignant cases where the drugs were tapered over a period of three months. So as part of conditioning regime, the more cytotoxic drugs were replaced by less toxic agents that enabled donor engraftment. In absence of GVHD, post-transplant IS was tapered from three months onwards. Patients who had an episode of GVHD thereafter were treated with prednisolone and resumption of full dose IS, if applicable. Patients with evidence of impending relapse detected by increasing mixed recipient chimerism were initiated on chemotherapeutic drugs along with DLI.

**Chimerism testing:**

a) **Isolation of genomic DNA:** DNA was isolated from peripheral blood or bone marrow sample of recipient and donor before the transplant using a Qiagen kit (Qiagen, CA). The column exchange DNA preparation method ensured high quality DNA devoid of PCR inhibitors. DNA quantification was performed using spectrophotometry. Post-transplant, DNA was extracted from recipient at frequent intervals to determine chimerism status.

b) **Short Tandem Repeat (STR-PCR):** The method is based on discrimination of donor and recipient alleles using a set of 15 STR-PCR exhibiting a high degree of size polymorphism. PCR was performed using AmpFLSTRIdentifiler kit (Applied Biosystems, Foster city, CA) according to the manufacturer instructions. All STR markers were amplified in a multiplex PCR reaction (Thermocycler ABI). One µL of PCR product was added to 09 µL deionised formamide/gene scan-500 (LIZ) as per manufacturers protocol, denatured at 95° for two minutes and placed on ice for atleast one minute before electrokinetic injection on the ABI3100 OR 3130 capillary electrophoresis instrument.

c) **Quantification of donor chimerism:** After electrophoresis, the fragments were analysed using Gene mapper software. Post-transplant chimerism value was calculated using average of atleast three informative STR markers [10,11]. The chimerism values were estimated from observed peak areas of the informative markers [12].

## STATISTICAL ANALYSIS

Categorical variables were summarised as percentages. Data in tables were presented as median values and range (minimum-maximum) or as absolute numbers.

## RESULTS

The patient demographic profile is detailed in [Table/Fig-1]. The median age was 18.5 years (range 02-47). Sixteen (40%) were sex mismatch HSCT was performed at this centre. Thirty-one patients were male (77.5%%) and nine were female (22.5%).Twenty six cases were of haematologic malignancies (65%) and 14 of non malignant conditions (35%) who underwent stem cell transplant.

| Variables | n (%) |
|---|---|
| **Age** at HSCT, median (range), years | 18.5 (02-47) |
| **Gender** | |
| Male | 31 (77.5) |
| Female | 09 (22.5) |
| **Sex mismatch** HSCT | 16 (40) |
| **Stem cell source** | |
| Peripheral blood | 36 (90) |
| Bone marrow | 04 (10) |
| **Donor type** | |
| MRD | 35 (87.5) |
| MUD | 02 (5) |
| Haploidentical | 03 (7.5) |
| **Diagnosis** | |
| Haematologic malignancies | 26 (65) |
| Non malignant disorders | 14 (35) |
| **Conditioning** | |
| Myeloablative | 14 (35) |
| Non myeloablative | 26 (65) |
| **GVHD** | 07 (17.5) |

**[Table/Fig-1]:** Demographic characteristics of our patients.

**HLA matching:** Complete HLA match at HLA-A, B DR locus occurred in 35 (87.5%) patients for whom matched related donor transplant was performed. Two cases were of HLA MUDs (5%) and three cases (7.5%) of haploidentical transplant for whom the donor was from within the family. The two successful MUD cases, was a three-year-old male child suffering from severe combined immunodeficiency disorder and other a case of Acute Myeloid Leukaemia (AML). Two haploidentical transplant cases included were diagnosed patients of WiskottAldrich syndrome and third a case of AML. The AML haplotransplant patient relapsed after three months whereas the two patients with underlying primary immunodeficiency disorder suffered episodes of GVHD.

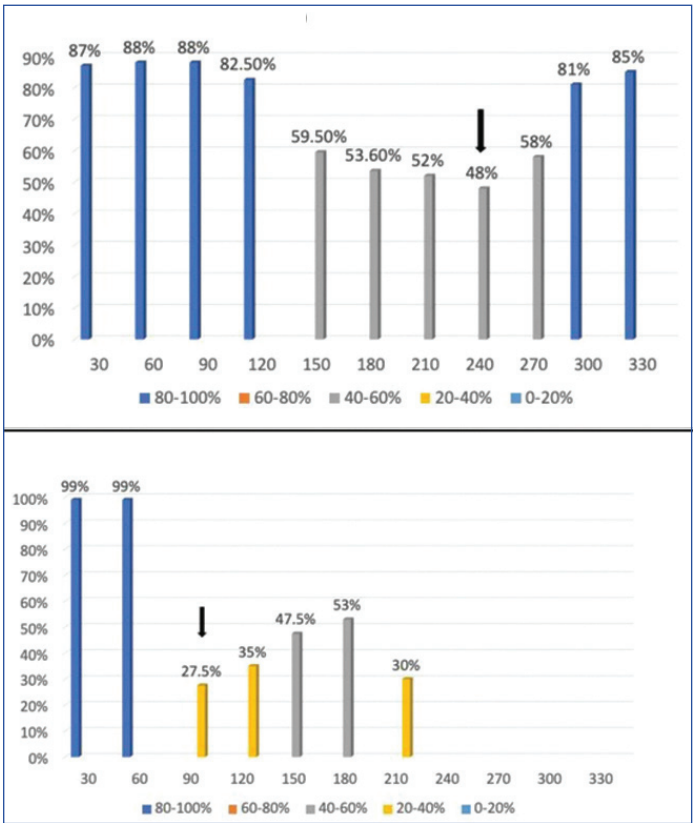
**Chimerism status/Prevalance of chimerism:** The follow-up chimerism status for all cases was atleast initial six months. The median Day+30 whole blood chimerism level was 99.6% (range 87-100%) and 96.75% (87-100%) for haematologic malignancies and non malignant conditions, respectively, whereas the D+90 values were 74.8% (range 88-99.2%) and 99% (range 81-99%) [Table/Fig-2]. Complete donor chimerism at D+90 was reported in 19 patients (73%) of haematologic malignancies with two cases of relapse and five showing evidence of GVHD. Amongst non malignant disease category, 10 patients (71.4%) showed complete donor chimerism at D+90 with two cases affected with GVHD. Follow-up and correlation with MRD levels was done only in two haematological malignancy cases with disease relapse.

| Type | Median donor chimerism at D+30 | Median donor chimerism at D+90 |
|---|---|---|
| Haematological malignancy | 99.6% | 74.8% |
| Non malignant haematological disorder | 96.75% | 99% |

**[Table/Fig-2]:** Median donor chimerism observed at D+30 and D+90.

Highlighted, here is the longitudinal follow-up of chimerism pattern as observed and its clinical implication in two pertinent cases

from the study cohort. As is seen in [Table/Fig-3] the graph shows increasing mixed recipient chimerism values in a patient of AML who despite chemotherapeutic therapy, subsequently relapsed. Correlation with MRD was suggested ongoing relapse though his bone marrow aspirate was in morphological remission. Patient was treated with azacytidine, chemotherapeutic agent, to which he did not respond.




**[Table/Fig-3]:** Graphic representation of longitudinal monitoring of chimerism by STR-PCR in two patients of Thalassaemia and AML, respectively. Arrows indicate points of therapeutic intervention due to decreasing donor mixed chimerism. This is followed positive response to treatment depicted by increasing donor chimerism in patient of thalassaemia (1a) and shows decreasing donor chimerism with relapse in a patient of AML (1b).

The second case was of haemoglobinopathy (Thalassaemia) [Table/Fig-3a] who throughout longitudinal follow-up on chimerism maintained a state of stable mixed status with periods of complete donor chimerism. There was no evidence of increasing recipient lineage cells and patient remained symptom as well as transfusion free.

## DISCUSSION

Allogenic stem cell transplantation is currently a well-established procedure for treatment of various malignant and non malignant haematologic diseases. It not only helps in restoring normal haemopoiesis but is also the preferred therapeutic modality for malignant disorders due to graft versus leukaemia effect where donor T cells show immune reactivity against the host leukaemic cell epitopes [13]. Chimerism analysis by STR-PCR method helps to determine the genetic origin of haematopoietic cells in patient circulation, post-transplant [14,15]. It is one of the most significant tools for monitoring donor engraftment. In this study, authors evaluated the engraftment status of 40 cases with diverse haematological disorders (malignant and non malignant). The chimeric status helped predict relapse in a case of AML showing no overt clinical evidence. Gambacorta V et al., in their study have shown that reappearance of host specific haematopoietic chimerism has been associated with relapse [16]. If performed at frequent intervals in post-transplant period it helps to predict disease relapse, level of engraftment and information on GVHD/GVT effects [17]. This may prevent catastrophic event by timely therapeutic intervention either by reduction of IS, use of immunotherapy (DLI) or use of chemotherapeutic agents like azacitidine [18]. In malignant disorders, the chief concern is to maintain a balance between graft failure or disease relapse and GVHD by close laboratory monitoring. In non malignant disorders, timely monitoring helps to prevent graft failure by optimisation of IS therapy. In the 14 cases of non malignant diseases, authors observed that a stable mixed chimerism status was adequate to control the underlying disease. This was contrary to haematological malignancies where complete donor derived haematopoiesis is desirable for cure [19]. Persistence of host haematopoietic lineage cells in malignancies is definitively suggestive of relapse or impending relapse. However, in non malignant diseases where GVL has no significance the main purpose is to lower risk of GVHD. Seven patients with 100% donor chimerism were found to have GVHD. Hence, a stable mixed chimerism (where both donor and host cells co-exist) is an accepted norm, provided the dynamics of chimerism status is monitored serially [20]. If an increase in ratio of recipient haematopoiesis (as depicted by increasing mixed chimerism) is observed there is need for therapeutic intervention. Also, it is important to note that a stable mixed chimerism in non malignant disorders is sufficient to revert the clinical manifestations of the primary disease, as was observed in present study. Arwen S et al., in their study have demonstrated that stable long-term MC did not negatively affect patient well-being and long-term outcome [21]. Few of present study Thalassaemia and primary immunodeficiency disorder patients post-transplant remained in good health suggestive of immunologically synergistic environment between donor and recipient cells.

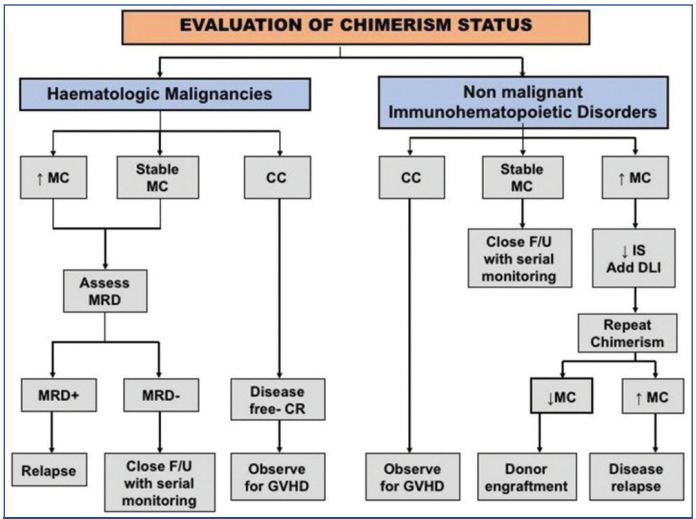
Chimerism needs to be measured longitudinally at frequent intervals to accurately predict disease status. One isolated value has no significance as authors are required to see the increase or decrease graph of chimerism levels [22]. Kristt D et al., in their review have focused on longitudinal and sequential chimerism evaluation for predicting the kinetics of each patient's chimeric state [23]. Single absolute values of percentage chimerism are not useful except for a few rare cases. In present cohort too, the longitudinal monitoring in different diseases (AML and thalassaemia) has helped to create a quantitative record of patient's chimeric status and timely therapeutic intervention helped to prevent relapse.

As of today, various techniques are available for chimerism testing but the most robust is amplification of highly polymorphic STR-PCR informative markers. Chimerism testing has a sensitivity of only 1% when performed on bone marrow or whole blood and hence, should be supplemented with more sensitive assays for MRD detection in haematologic malignancies. The two most sensitive methods being used are multiparameter flow cytometry to detect leukaemia associated aberrant immunophenotypes and real time quantitative Polymerase Chain Reaction (qPCR) for fusion gene transcripts or clonal immunoglobulin or T cell receptor (Ig/TCR) gene rearrangements [24]. These MRD assays have a sensitivity of atleast 1 to 2 log greater than chimerism by STR-PCR method [25,26].

Also, MRD techniques detect residual disease or re-emerging leukaemia clones based on known aberrant surface or genetic markers. Chimerism on the other hand picks up recipient haematopoiesis. Despite, the known fact that patients with complete donor chimerism have shown MRD positivity, it remains one of the imperative tools to monitor donor engraftment especially in non malignant immune-haematologic conditions and in malignant diseases with no known underlying genetic aberrations [27]. Hence, chimerism analysis is complementary to MRD measurement for relapse prediction after transplant in malignant conditions and serves as a surrogate in cases with extramedullary disease or leukaemia/Myelodysplastic syndrome with no known molecular markers [28-30].

In the present study, authors have proposed a diagnostic algorithm for monitoring of post-transplant engraftment by chimerism assay

in a transplant and immunogenetics laboratory [Table/Fig-4]. The algorithm is interpretation of chimerism levels reported in HSCT patients and a step-wise logical correlation with clinical parameters and MRD assays for predicting disease outcome. The sensitivity of the above technique can be increased substantially to 0.1% by using lineage specific leucocyte subsets for testing. These cell subsets may be enriched for cells that may contain MRD. This especially applies to high risk cases when other suitable disease markers are not available for monitoring MRD [31]. By employing variant-allele-specific quantitative PCR-based approaches to detect small DNA insertions or deletion sensitivity can be further increased to a level of 0.01-0.001% [32].




**[Table/Fig-4]:** Diagnostic Algorithm for evaluation of chimerism values in haematologic malignancies and non malignant haematological disorders.
MC: Mixed chimerism; CC: Complete chimerism; MRD: Minimal residual disease; CR: Complete remission; IS: Immunosuppression; GVHD: Graft versus host disease; F/U: Follow-up

## Limitation(s)

Present study included a heterogenous group of haematological disorders, however the limitation was small sample size and short follow-up time of six months of the cases.

# CONCLUSION(S)

Chimerism analysis is an indispensable technique for routine post-HSCT monitoring of donor engraftment. It is especially valuable in setting of RIC as well as patients with no underlying MRD molecular marker. At times, regular time bound chimerism analysis along with clinical assessment may serve the purpose of predicting disease relapse earlier than MRD. Each laboratory should formulate standard operating procedures and guidelines for judicious use of the assay and increasing the test robustness and result reliability. Nonetheless, communication between clinicians, haematologists and immunopathologist accompanied by a methodical step-wise laboratory diagnostic approach can facilitate early relapse diagnosis and timely therapeutic intervention.

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