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State of Chimerism as a Promising Tool for Follow up of Hematopoietic Stem Cell Transplantation in Egyptian Patients

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Abstract
Follow up of patients after HSCT by chimerism detection can detect the outcome either engraftment, relapse or rejection. Many variables affect the result of chimerism either increasing or decreasing chimerism. Lineage specific chimerism is specific and sensitive than chimerism detection by whole blood. Here in this study the importance of these variables on the outcome of chimerism detection is studied.
Subject and method: *This study was conducted at Ain Shams University Hospital during the periodbetween January 2011and January 2015. 46 patients were included (16 child and 30 adult). They weretransplanted for malignant and non malignant hematological diseases from totally matched siblings.Themedian duration of follow-up was one year. Chimerism status was detected for all patients by polymerase chain reaction based on amplification of variable number tandem repeat (VNTR) markers. Six VNTR loci were detected in every subject pre-transplant in order to detect an informative locus to be used in follow uppost-transplant. Also lineage specific chimerism and dilution experiment were conducted on some patients.*
Results: *One patient showed no informative locus pre-transplantation, 37 patients showed complete donor chimersm, two patients retained recipient pattern post-transplantation and six patients showed MC. Lineage cell specific chimerism and dilution experiment were conducted for patients who showed failure or mixed chimerism.*
Conclusion: *Chimerism detection by VNTR is dependable method to follow up patients after BMT however,some cases need some modifications as repeated analysis, short duration, lineage specific in comparison to whole blood analysis, dilution experiments in order to predict the outcome.*
Keywords: *Chimerism, Hematopoietic stem cells, Hematological diseases, Immunodeficiency.*

Introduction

Allogeneic- hematopoietic stem cell transplantation (HSCT) has become the main treatment of a wide range of malignant and nonmalignant hematological disorders [1-4]. The main goal of post-transplantation monitoring in HSCT is to predict negative events, such as disease relapse, graft rejection and graft-versus-host disease, in order to intervene with appropriate therapy. The recurrence of the disease is still the most important barrier to the success of this treatment [5]

Successful allogeneic HSCT is associated with engraftment of donor cells in the recipient's bone marrow, a condition known as complete chimerism (CC). Engraftment with co-existence of both donor and recipient-derived haemopoeitic cells, the so called mixed chimerism (MC) is considered non complete engraftment which may

be a risk factor for the development of subsequent relapse or graft rejection.Somestudies indicate that MC is associated with complications, while others disagree with this finding [6,7]. [8] Two common types of MC are known decreasing and increasing MC. MC can be decreasing or increasing depending on the ratio between donor and recipient cells .Many variables affect the evolution of chimerism as increasing MC levels in hematopoietic stem cell transplantation (HSCT) performed after hematological malignancies may indicate disease relapse, graft failure, or rejection[1-3,9-11]. On the other hand, decreasing MC, often seen after tapering of immunosuppr-ession after transplant or after donor lymphocytes infusion (DLI), may be an early predictor of graft-versus host disease (GvHD) and of its more desirable counterpart graft versus- tumor effect. Further-more, MC may be remain stable over time and be compatible with prolonged remission, particularly in nonmalignant diseases, where MC may indicate a tolerant state associated with a low incidence of GvHD[12,13]. Apart from full or complete donor chimerism (CC), a 2001 international workshop recognized split chimerism (one cell lineage complete donor and another complete host). In the latter case, it is important to determine whether the percentage of recipient DNA is stable, increasing or spontaneously decreasing over time[14].

Eventually, determining chimerism may also be useful to monitor response to a DLI or help to decide on administering prophylactic DLI in specific situations (e.g., to potentiate graft-versus-tumor effect or to prevent incipient graft rejection in some cases of increasing MC [15].

In the past decade, more than 40 studies have addressed the possible role of chimerism analysis in the detection of minimal residual leukemia after SCT. Whether relapse can be detected early enough for useful intervention in the form of immunotherapy is highly dependent on the sensitivity of the technique. STR- or VNTR-based PCR has a moderate sensitivity of 1–5%, compared with the conventional morphologic definition of relapse as ≥5% leukemic blasts in the bone marrow. As few cases, CC in peripheral blood was established at the time of clinical relapse in the bone marrow. The sensitivity of the technique may be increased by the use of lineage-specific analysis of cell subsets enriched for cells that may contain minimal residual disease, such as cells with the immunophenotype of the initial leukemia cells [14].lineage specific chimerism allows for precise monitoring of patients after allogenic stem cell transplantation. The unquestion-able advantages of this method are high sensitivity and specificity. The method itself is cost and time consuming requires qualified staff and appropriate equipment .lineage specific chimerism may be worth applying in selected patients with high risk of relapse or graft failure.The optimal timing of these diagnostic intervene-tions is a critical issue and has to be further optimized as,only very frequent monitoring of chimerism status by highly sensitive methods might identify impending relapse and allow early immunological intervention [16].

The main goal of this study is to detect lab variables affecting chimerism state by using lineage specific chimerism and compare it with whole blood chimerism and also using a semi-quantification method in case of mixed chimerism in order to provide a complementary information to predict the outcome of HSCT and allow rapid interference in case of relapse or rejection .Also detect any limitations, modifications and recom-mendation which may increase sensitivity of this techniquefor the benefit of laboratory that don't have access to either DNA sequencer or genetic analyser.

Patients and Methods

The current study was conducted at Ain Shams University Hospital during the period between January 2011 and January 2015. 46 patients wereincluded (16 child and 30 adult). They were transplanted for malignant and non malignant hematological diseases from totally matched siblings. The median duration of follow-up was one year (table 1).

Table1 shows the descriptive data for the patients.

Diagnosis & number of patients total (46 pairs)	VNTR pre-transplantation	Age	Regimens	Chimerism status	Final out come after 1,2,6,9,18 and 24 month
Hematological malegnancies : n=40 (CML,ALL,AML and CLL)	One patient(no informative locus) and the rest , 1-3 informative loci could be founded	19-35 years (60% males,40%females)	Myeloablative	One patient :non informative locus & 37 patients : CC& 2 patients : retained recipient pattern	The same results
n=6 Lymphoma,SCID,aplastic anemia and bthalassemia	1-3 informative loci could be founded	1-8 years	Non myeloablative	MC	4 patient : MC changed to CC, one patient : persistent MC and the last patient relapsed

In order to follow up those patients a previously described semi-quantitative polymerase chain reaction approach based on the amplification ofvariable number tandem repeat (VNTR) markerswas used to detect of chimerism status. Six VNTRloci were detected in every subject (Apo-B, DIS80, YNZ-22,HRAS, 33.1,33.6) pre-transplant in orderto detect an informative locus to be used in followup post-transplant. VNTR loci are defined asbeing useful if analysis of recipient and donorsamples prior to BMT showed a unique band forthe recipient and another unique band for thedonor, or if they showed a unique band for the recipient only. Patients who exhibited completedonor hematopoiesis with all markers tested at all times were defined as donor or complete chimerism (CC).All patients with CC subjected to dilution experiments. Patients who exhibited mixed populations of donor and host cells on more thanone occasion with at least two different markersafter day +45 or when the recipient wastransfusion- independent were considered MC.Patients follow up at 1,3,6,9,12,15,18 and 24months was done . If any patient showed mixed chimerism at any period post-transplant, thepatient was followed up every 2 weeks to assist his clinical situation in order to interfere early.

Any mixed chimerism result for patient was subjected to dilution experiment and lineage specific chimerism in order to detect level of mixed chimerism and to evaluate chimerism status at cell lineage level.

Methods

A) Sampling

Two ml of venous blood were withdrawn aseptically into sterile EDTA vacutainer tube for V NTR polymorphism testing by PCR.

B) Analytical methods

DNA extraction was performed using Wizardwhole blood genomic DNA extraction kit Supplied by Promega (*).DNA was extracted from total, T-cell, and myeloid subsets using QIA amp DNA Blood Mini Kit (Cat. No. 51106; Qiagen) according to the manufacturer's instructions in case of mixed chimerism.

PCR amplification of six different VNTRs loci (Apo-B, YNZ-22, 33.6, 33.1, D1S80 and H-Ras) were performed. All oligonucleotide primers were synthesized commercially (Promega), primer sequences and amplification cycles were obtained from previously published data [17,18] with some modification . All reactions wereperformed in a volume of 50ul containing 25 ul, master mix ready to use (Promega), 24 pmol each primer (Promega), 250ng template DNA, 15 ul deionized water and and 2.5 units Taq polymerase (Promega).

Cell Lineage specific chimerism:

In case of mixed chimerism samples, for the analysis of post-transplant chimerism at cell lineage level immune-magnetic selection using magnetic beads directly conjugated to anti-CD3and anti-CD33 monoclonal antibody (Dynal, Oslo, Norway) was used. 1–2 ml of peripheral blood, obtained at various time-points following HSCT,were directly incubated with CD3 and CD33Dynabeads and incubated at room

temperature ona blood tube rotator for 30 min. Selected cellswere then isolated using a magnetic particle concentrator (MPC, Dynal) and washed twice with 0.1% (w/v) BSA/PBS. DNA extraction forCD3 and CD 33 cells was performed. VNTR analysis was performed for every cell lineage forthe informative locus.

Quantification of donor chimerism

Quantification of the degree of mixed chimerismis determined by mixing pre-transplant recipient and donor DNA in dilution experiments. Mixes of recipient and donor DNA are then subjected to PCR amplification for one or more information loci.

Preparation of donor/recipient dilution series:

Prior to use, each DNA solution is incubated at37°C for 30 min to ensure homogeneity. DNA is quantified by UV spectro-photometry (Gene Quant Pro DNA Calculator, Pharmacia Biotech) and samples are then diluted to 10 ng/ul. A 50:50mix of donor: recipient DNA is prepared andincubated at 37°C for 30 min.This solution is then used as the starting point forthe creation of two separate serial dilution series:

(1) where the 50:50 mix is diluted out in donorDNA, creating 25%, 10 %,5%,2.5% recipientDNA dilutions; and (2) where the 50:50 mix isdiluted out in recipient DNA, creating 75%, 90% recipient DNA dilutions.

The combination of these dilutions series creates adilution series that covers 2.5%–90% recipient DNA dilutions. 250ng template DNA of eachdilution point is then subjected to PCRamplification, according to the method describedabove. Mixing experiments are carried out induplicate and standard curves are generated fromthe mean values. Following electrophoresis, donorand recipient alleles are identified according totheir respective size. The degree of mixed chimerism seen in any post-transplant sample is compared to that particular patient's standardcurve.

All Products were separated on 2% agarose gel containing ethidium bromide for 1 hour at 100volts, visualized using ultra violet transilluminator and photographed. Alleles were characterized by their molecular weight determined relative to a100bp DNA ladder (Promega) run as a marker

RESULTS

This study was conducted on 46 patients.Failure to detect an informative locus was recorded in one pair. 37 patients showed complete donor chimersm status throughout their post- HSCT period (1,3,6,12,18,24 month) (figure1). Two patients retained recipient pattern posttransplant-ation (rejection or primary non-engraftment) and six patients showed mixed chimerism. Lineage cell specific chimerism were conducted for patients who showed failure or mixed chimerism and dilution experiment were conducted for patients all patients.

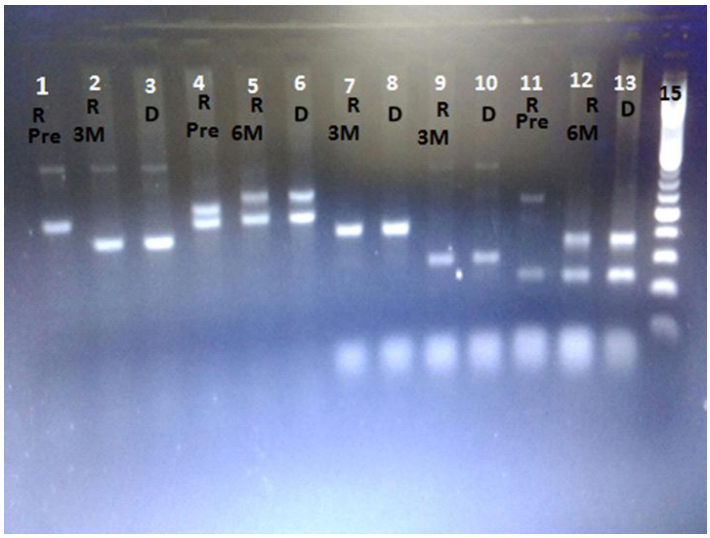

Figure (1): photograph of 2% agarose showing 5 pairs post transplantation showing full chimerism Lane 1,2,3 (1st pair),lane 4,5,6 (2nd) pair testing for D1S80 locus:

Case of CC lane 1,2,3 (R,R3M,D),Case of CC lane 4,5,6 (R,R6M,D)

Lane 7,8 (3rd pair),lane 9,10 (4th) pair and lane 11,12,13 (5th) pair testing for YNZ locus: Case of CC lane 7,8 (R3M,D) ,case of CC lane 9,10 (R3M,D) ,Case of CC lane 11,12,13 (R,R6M,D)

Ladder lane 15: DNA molecular weight 100bp ladder (rang from 100bp-3000bp).

Abbreviations: R-Pre = recipient pre- transplant-tation; R 3,6 M= recipient 3,6 month post transplantation;D=donor)

Concerning patients with complete chimerism (37 pairs) 34 pairs showed 100% donor chimerism by dilution experiments. The remaining 3pairs ,two pairs showed 95%-97.5% doner chimerism , the third pair showed 75%-95% donor chimerism, the 3 pairs converted to 100% donor chimerism spontaneously after one month without any intervention (figure2).

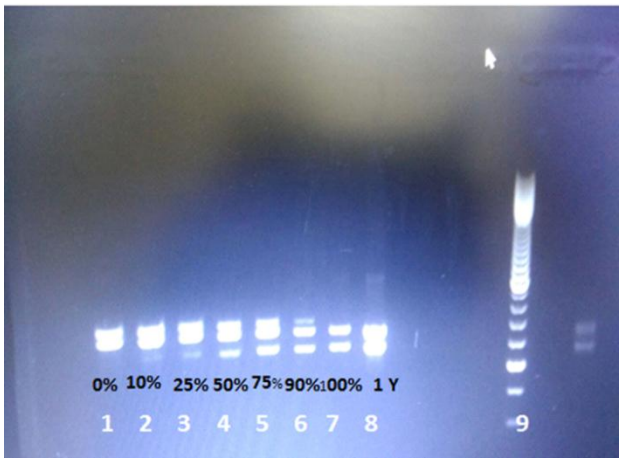

Figure (2): photograph of 2% agarose showing follow up for patient one year post transplantation by dilution experiment of apo-B:
Lane 1,2,3,4,5,6,7: (0%,10%,25%,50%,75%,-90%,100% donor).
Lane 8: case of CC 100% donor (R-1Y).
Lane 9: DNA molecular weight 100bp ladder (rang from 100bp-3000bp).
Abbreviations: R 1Y= recipient 1 year post transplantation).

Concerning the two patients who showed failure or retained recipient pattern, separated myeloid and lymphoid cells showed the same result as whole blood. Also dilution experiment was done in-order to detect any micro-chimerism (<5% donor blood), however, the two patients showed 0% donor blood which ensures the same whole blood result.

Concerning mixed chimerism patients: four patients showed transient decreasing mixed chimerism (increase toward the donor and decrease recipient part). They showed MC at first and then converted to complete chimerism afterwards. One patient showed stable mixed chimerism as first showed 75%MC, third month showed 50%- 25%MC then relapsed at seventh month 0%MC.After medical interference the case showed again mixed chimerism 50%-70% at ninth month, then finally stable MC 70%- 90% at 12,15,18 month. The last patient showed increasing mixed chimerism (towards the recipient) as at first month showed 50%MC, at second month patient relapsed. The patient then undergone a second transplantation, after that showed MC 50%-75%. Cell lineage specific chimerism and dilution experiment showed increasing chimerism as whole blood result (Figure 3).

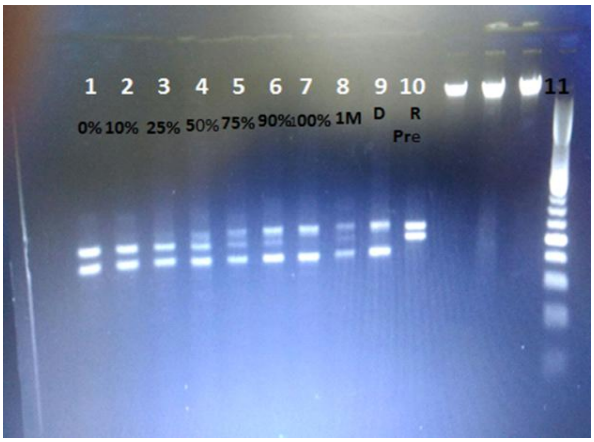

Figure (3) : photograph of 2% agarose showing follow up for patient one month post transplant-tation by dilution experiment of DIS80 and another pair pre-transplantation.
Lane 1,2,3,4,5,6,7: (0%,10%,25%,50%,75%,-90%,100% donor)
Lane 8:case of MC 75% (R 1M)
Lane 9,10: informative locus (D, R- Pre)
Lane 11: DNA molecular weight 100bp ladder (rang from 100bp-3000bp).
Abbreviations: R-Pre =recipient pre- transplant-ation; R 1M= recipient 1 month post transplanta-tion; D=donor , MC =mixed chimerism).

Discussion

Allogenic HCST has become the main treatment in many malignant and nonmalignant hematolo-gical diseases in adults and children[19]. Chimerism detection is the key stone in following up patients after HSCT[20]. Chimerism monitoring can identify and predict HCST outcome. Various techniques as cytogenetic analysis, fluorescent in situ-hybridization, restricted fragment length polymorphism, STR/VNTR analysis and realtime

quantitative PCR are used for chimerism analysis post-transplantation[21]. However, previous techniques had many limitation which open the gate to find the most suitable method which can be applied to all cases. Nowadays, more sensitive methods for determining chimerism based on real-time PCR. However, STR/VNTR still keeping its importance and its privacy as dependable method in comparison to real-time PCR. Many investigator as Kamel et al.,[21] Mossallam et al.,[22] andKletzel et al[23], used VNTR method as a method for detection of chimerism state as it has many advantages, namely: speed, sensitivity and ease of analysis of polymorphic sequences .Also, it can be applied in all cases, doesn't depend on sex-mismatch and needs only small amounts of blood [22,23]. Kletzel et al,[23] compared between real-time PCR and VNTR analysis in order to find an accurate and efficient methodology to asses chimerism. They demonstrated a complete correlation between the two methods. VNTR analysis was equally efficient as compared with real-time PCR. They added, that although real-time PCR is a simple and rapid method, it is highly sensitive and vulnerable for false positive. Consequently, they recommended a confirmation by VNTR analysis as dependable technique.

Asdetection of chimerism state has become routine procedure for evaluation of engraftment of post HSCT. Successful allogenic HSCT is associated with CC. Many studies recorded that CC relates to a more frequent and more severe GVHD, less relapse and longer disease-free survival. On other hand, mixed chimerism associates with less GVHD, higher frequency of relapse and shorter disease free survival. The outcome of different types of chimerism state developing after HSCT in many cases are in parallel with the prognosis of the disease.[7] Also, in patients with hematologic malignancies it has been a matter of intense debate whether these persistent or reappearing host cells represent malignant or non-malignant recipient hematopoietic cells, or a combination of both [24,25,26].

McCann and Lawler [27] reported that detection of molecular evidence of relapse, as detected by mixed chimerism, may provide a window of opportunity to intervene with approach such as donor lymphocyte infusion prior to evidence of clinical relapse.

Although, Antinetal,[28] recommended that at least 3 loci should be used and the more the number of loci used the better the chances to get an informative locus. Also, Zhou et al.,[29] reported that the use of more than one locus marker is also needed for consecutive donor chimerism evaluation, since loss of specific chromosomal regions during clonogenic evolution may occur in several hematological malignancies. Also to avoid misclassification of a heterozygous individual as homozygous as observed with YNZ-22, as larger alleles fail to amplify efficiently under standered conditions when present in heterozygosity with another much smaller alleles which may lead to wrong diagnosis . So in the present study, the previous 6 loci of VNTR (D1S80, ABO.B, YNZ-22, HRAS, 33.1 and 33.6) were applied to all patients pre-transplantation in order to increase the discriminative power to 100% as previously stated, also to get an informative locus between pairs to evaluate post-transplant chimerism state.

All patients in this study received stem cells from fully matched sibling. Peripheral blood samples was evaluated in all cases. Also dilution experiment as a semiquantification method was applied to all cases ,however , lineage specific chimerism was applied to rejected cases (1ry- non engraftment) and MC cases only. Complete donor chimerism was found in 37/46 patients of this study. They showed CC status throughout their post-BMT follow up period (1,3,6,12,18,24 month), these results confirmed by dilution experiments as , 34/37 were CC (100% donor), 3 /37 were MC at high percentages (75%-97.5% donor) then coverted spontaneously to CC(100% donor) without any intervention. Only two patients rejected (1ry- non engraftment), they retained recipient pattern during post-transplantation follow up. They failed to convert either to CC or to MC during post- transplantation follow up. Lineage specific chimerism and dilution

experiments confirm the same result, they failed to show any engraftment and even at 1.25% dilution showed 0% donor blood.

The remaining 6 patients showed MC,four patients (suffering from lymphoma, B-thalassemia, SCID and aplastic anemia),showed MC and converted spontaneously or after DLI into CC (decreasing MC). Andreani et al., [30] observed that MC detected early after HSCT often moves towards complete chimerism. Also, one patient suffering from SCID showed persistent stable MC during post-transplantation follow up inspite of clinical recovery, this patient shows increasing MC at 1, 3 month,6 (75%, 50%, 25% donor), then relapsed at 9 month (0% donor), then after been subjected to a cycle of donor lymphocyte infusion the patient converted again to stable mixed chimerism (75%-90%) at 12, 18, and 24 month and never show CC. HSCT has the potential to cure primary immune deficiency syndromes (PIDS). Burroughs and Woolfrey[31] stated that the immune defect in PIDS may be corrected by partial reconstitution of normal immune cells, in other words full donor chimerism of the affected cell subset may not be required. This concept may add further rationale to limiting the intensity of the conditioning regimen in these cases. The last patient suffering from aplastic anemia showed increasing progressive MC (50%,25%,10% donor) where the percent of recipient cells increased and the percent of donor cells decreased during pos-transplantation follow up and finally he relapsed, and was subjected to a second transplantation. Park et al [32] concluded in their study that there is a strong correlation between MC and the recurrence of the underlying disease in haematological and non hematological malignancies. They added that progressive increasing MC had the highest risk of relapse. This is in agreement with this case of aplastic anaemia in this study. Molecular evidence of persisting recipient cells may be a reflection of either survival of leukemic cells or survival of normal heamatopoietic cells, or combination of both. Persistent MC in the early post transplant period is caused predominantly by normal recipient cells. Thus MC state reduce the clinical graft versus leukemia (GVL) effect of allo-reactive donor derived effect tor cells especially in case of leukemia and myelodysplastic syndrome (MDS), and thus facilitate the proliferation of residual malignant cells.

Many researcher as Bader et al.[33], Huismanetal.,[14] studied the importance of lineage specific chimerism and the need for serial investigations at short time interval to detect the respective time widow of interest. Some patient relapsed despite showing CC in peripheral blood 1 week before relapse. Also, some patients relapse in BM without prior detection of recipient cell in peripheral blood. In case of increasing MC confirmed chimerism analysis of subpopulation must be initiated. They Analyze different leucoc-yte subpopulation (granulocytes, monocytes, T-cell,NK, Bcell).

Levrat et al [7] found that CML patients with MChad a significantly increased risk of relapse. They noticed a progressive increase in autologous Tcells which preceded the reappearance of autologous monocytes and granulocytes. The percentage of autologous cells in whole peripheral blood increased over time were involved before malignant cells finally appeared in the peripheral blood. Also, Roux et al [34] found that in case of CML, B cellsand NK cells of the patient remained of donor origin, even at the time of relapse. They assumedthat at the time of relapse the percentage of MCwill increase because, in addition to the recipientT cells, leukemic cells might reappear and that thetype of cell lineage involved may depend on the type of leukemia. However, Bader et al.,[33] demonstrate that the affected cell lineage were independent of the type of leukemia in contrast to the hypothesis of Roux etal.,[34], Also, Bader et al.[33]demonstrate that in case of CML immunotherapy with donor lymp-hocyte infusion may lead to conversion of MC in to CC. And they confirm that the reappearance of recipient cells preceded hematological relapse in patients with acute leukemia may strengthen the hypothesis that these re-emerging host cells weaken the GVL effect of donor effector cell, possibly as the result

of the induction of tolerance towards host antigens.

Preuner et al [35] and Bader et al.,[33] Patients with ALL who were not completely chimeric on day 28 after transplantation had increased risk of relapse, they concluded that within a median observation time of 4.5 years of ALL patients, the appearance of recipientcells after a period of pure donor chimerism in the CD34+ and CD8+ leukocyte subsets revealed adynamic indicative of significantly elevated riskof relapse or imminent disease recurrence.

Depending on previous studies, which revealed that Lineage restricted chimerism is a very useful tool as to follow up post-transplant results [16]. So, in this study, patients rejected (1ry- non engraftment and MC cases were also analyzed for lineage restricted chimerism for myeloid and Tcell lineages with a median follow up period of one year. But, there was no difference in the state of chimerism between whole blood and either myeloid or T cell. This differences with previous studies attributed to 3 causes first: differences in the diagnosis of previous cases as they suffered from lymphoma, B-thalassemia, SCID and aplastic anemia, in contrast to previous studies which were applied on leukemia and MDS .Secondly : in the present study , lineage specific chimerism was analyze by T-cells and granulocytes only. In contrast to Bader et al .,[33] hypothesis, who analyze different leucocyte subpopulation (granulocytes, monocytes,T-cell, NK, Bcell). Third cause was that eight patients only were subjected to lineage specific chimerism, which is small number sample . Finally to avoid non informative cases we extend our panel by 2 loci extra namely 33.4 (VNTR locus)and ZP3 (gene loci). Nowadays, our panel consist of 8 loci.

Conclusion and recommendations for the benefit of laboratory that don't have access to either DNA sequencer or genetic analyser:

1) Start screening patientspre-transplantation by 6-8 VNTR loci to get informative locus.
2) Follow up patients by at least 2 different loci or more if possible.
3) As relapse develops very rapidly,early detection of increasing patient signals requires very frequent measurements.
4) In case of MC follow up every 1-2 weeks by lineage specific chimerism and dilution experiment, as only sensitive methods as lineage specific chimerism might identify impending relapse and allow early intervention
5) In case of acute leukemia follow up every 1-2 weeks by lineage specific chimerism and dilution experiment as patients who do not develop CC or decreasing MC within 6 months of SCT have a high risk of pending relapse and mortality.
6) Lineage specific chimerism must be screened by different subpopulation (granulocytes, monocytes, T-cell, NK, B cell) independent of diagnosis
7) Extend your panel by VNTR loci to avoid non informative cases

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