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VNTRs Analysis for Detection of Chimerism Status after Hematopoietic Stem Cell Transplantation in Egyptian Patients.

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ABSTRACT

Chimerism analysis is an important method for monitoring allogenic post hematopoietic stem cell transplantation (HSCT). Variable number tandem (VNTR) analysis is considered an informative technique to follow up chimerism state after HSCT. The aim of our study is to illustrate the role of VNTR analysis as a method for evaluation of state of chimerism following HSCT in our laboratory. This study included 35 pairs undergoing HSCT. Informative loci pre-transplantation using six VNTR loci were detected. After transplantation the informative loci were used to detect chimerism status. After DNA extraction from blood samples, amplification of VNTR loci was performed using a conventional PCR protocol. Amplified product of DNA samples was run on 2% agarose, stained with ethidium bromide together with DNA ladder to identify the site of the band. Only 29 pairs were followed up after HSCT for chimerism analysis every three months. Five recipients died after HSCT and one pair failed to detect informative locus. For the 29 patients who were followed: 24 recipient showed complete chimerism (CC), 3 recipients showed mixed chimerism (MC) and 2 recipients retained recipient type (failure)after HSCT. VNTR analysis using a panel of six loci is suitable to detect state of chimerism after HSCT.

Keywords: HSCT, Chimerism, VNTRs, Informative locus.

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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.[6] Two types of MC are known decreasing and increasing MC. Mixed chimerism can be decreasing or increasing depending on the ratio between donor and recipient cells.[7]

Chimerism monitoring can predict HSCT outcome success or failure.[8-9] Complete chimerism is very important for sustained engraftment and for preventing relapse of the underlying disease.[1-3,8-10] An increase in the proportion of host cells in the post- HSCT period strongly suggests a risk of disease recurrence.[11,12]

Chimerism detection depends on the utilization of the difference between the donor and recipient polymorphic genetic markers.[6] Various techniques including erythrocyte phenotyping , cytogenetic analysis , fluorescent in situ hybridization (FISH) ,restriction fragment length polymorphism (RFLP), short tandem repeat / variable number tandem repeat (STR/VNTR) analysis and real-time quantitative PCR for SNPs are used for chimerism analysis post transplantation.[14-18] However, several limitations have been associated with erythrocyte phenotyping , cytogenetic analysis and fluorescent in situ hybridization (FISH) as low sensitivity, limitation to sex-mismatched transplantations, high DNA requirement ,time-consuming, and limited degree of polymorphism .[16-18]

STRs and VNTRs are the tandem repetitive blocks of DNA. When the repetitive sequence is 15-50 nucleotides long it is termed VNTR , and when it is 2-6 it is termed STR . The main two tasks following HSCT are: first is to identify informative markers which can be used in following up patients post transplantation. The second task is to estimate quantity of donor-specific cells in the recipient.[7,19]

PCR- based amplification of highly polymorphic STR/VNTR system is considered to be one of the most informative, accurate, quantitative or semi-quantitative, cost effective and sensitive technique.[20,21] Due to their high polymorphic content they can be used in evaluating hematopoietic chimerism state and in determining the origin of leukemic cells in patients with recurrent disease after allogenic HSCT. As to identify an informative locus, pre-transplant samples are screened for informative VNTR or STR loci. An informative locus is one for which at least one recipient allele has a different number of repeats than the donor allele(s).[20-21]

The aim of this study is to clarify the role of VNTR analysis by conventional PCR as a method for evaluation of the state of chimerism after allogeneic HSCT.

SUBJECTS AND METHODS

Subjects

The current study was conducted at Clinical Pathology Department of Ain Shams University Hospital during the period between June 2013 and June 2015. A total of 35 pairs (donors and their recipients) were included in the study. Two groups were included namely: pediatric (6 patients) and adult group(29 patients) . The mean age of the pediatric group was 7.5 years with range of 1.5-13 years. The age of adult group ranged from 20-37 years with mean of 28 years.

The 35 pairs were studied in order to detect informative loci pre-transplantation using 6VNTR loci to be able to detect chimerism status post-transplantation using the detected informative loci . Apo-B, YNZ-22, HRAS, 33.1, 33.6 and D1S80 were the six VNTR loci examined.

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Patients were followed up post HSCT every 3 month for detection of state of chimerism . Nine pairs were followed up for 3month, 9 pairs were followed up for 6 month , 7 pairs were followed up for 12 month and 4 pairs followed up for 15 month. 6 pairs were excluded from the study as 5 recipients died and one pair had non informative locus. Table (1) shows patient's characteristics.

Table 1: Patient's Characteristics

Diagnosis	AML (11) ALL(5) Aplastic anemia(11) SCID(1) B-thalassemia(2) Fanconi anemia(2) Lymphoma(2) MSD (1)
Follow up (months)	9 pairs were followed up for 3month. 9 pairs were followed up for 6 month. 7pairs were followed up for 12 month. 4 pairs followed up for 15 month. 6 pairs were excluded from the study as 5 recipients died and one pair had non informative locus.
Regimens	Myeloablaive (29) Non myeloablative(6)
Stem cell source	Peripheral blood (34) Cord blood (1)
T cell depletion	None
Sex	60% males 40% females
Donors	33 HLA- matched related donor(brothers) 1 patient from mother 1 patient from grand mother
GVHD prophylaxis	All

Methods

Sampling

Two ml of venous blood was withdrawn aseptically into sterile EDTA vacutainer tube for VNTR polymorphism testing by conventional PCR.

Analytical methods

DNA extraction was performed using Wizard whole blood genomic DNA extraction kit Supplied by Promega (*). PCR amplification of six different VNTRs loci (Apo-B, YNZ-22, 33.6, 33.1, D1S80 and H-Ras) was performed. All oligonucleotide primers were synthesized commercially (Promega) Primer sequences and amplification cycles (Tables 2, 3) were obtained from previously published data [3,4,17] with some modification. All reactions were performed 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 2.5 units Taq polymerase (Promega). Amplified products were separated on 2% agarose gel containing ethidium bromide for 1 hour at 100 volts, visualized using ultra violet transilluminator and photographed. Alleles were characterized by their molecular weight determined relative to a 100bp DNA ladder (Promega) run as a marker.

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Table 2: Primer Sequence of the Six VNTR Loci.

Gene	Sequence	Reference
APO-B	5' CCTTCTCACTTGGCAAATAC 3' 5' ATGGAAACGGAGAAATTATG 3'	(Ref .11)
33.1	5' CGTGTCACCCAC_AAGCTTCT 3' 5' TGCTTTCTCCACGGATGGGA 3'	(Ref.22)
YNZ-22	5' GGTCGAAGAGTGAAGTGCACAG 3' 5' GCCCCATGTATCTTGTGCAGTG 3'	(Ref.22)
33.6	5' TGTGAGTAGAGGAGACCTCAC 3' 5' AAAGACCACAGAGTGAGGAGC 3'	(Ref.17)
D1S80	5'GAAACTGGCCTCCAAACACTCCCCGCCG 3' 3' GTCTTGTTGGAGATGCACGTGCCCCTTGC 3'	(Ref.5)
HRAS	5' TTGGGGGAGAGCTAGCAGGG 3' 5' CCTCCTGCACAGGGTCACCT 3'	(Ref.22)

Table 3: PCR conditions of Six VNTR Loci

VNTR Locus	Denaturation	Annealing	Extension	Cycles
Apo-B	94ºC, 1min	58ºC, 6min		26
33.1 , 33.6 and H-ras	95ºC, 30 seconds	65ºC, 1min	72ºC, 30 seconds	25
YNZ-22	95ºC, 1min	59C, 1min	72ºC,2 min.	30
D1S80	94ºC, 1min	66ºC, 1min	70ºC, 5min.	28

The sizes of alleles ranged from 435 bp to 1006 bp for Apo-B, from 666 bp to 2375 bp for 33.1, from 364 bp to 993 bp for 33.6, from750 bp to 1500 bp for 33.4, from 238bp to 938bp for YNZ-22, from 488 bp to 768 bp for D1S80 and from 1000 bp to 2500 bp for H-ras (Figs. 1).

..* Promega corporation. 2800 woods Hollow Road. Madison, WI 537 11-5399 USA. **www.promega.com**

RESULTS

All pairs (35 pairs)were subjected to analysis of 6 loci of VNTR(D1S80,APO-B,YNZ-22, HRAS, 33.1 and 33.6) pre-transplantation in order to get informative loci. . DIS 80 was informative in 24 patients , Apo B was informative in 20 patients , YNZ-22 was informative in 8 patients , 33.6 was informative in 8 patients , 33.1 was informative in 2 patients , and HRAS was informative in one patient . Failure to detect an informative locus was recorded in one pair . Follow up of the patients, using informative loci , was done every three month to detect the state of chimerism post HSCT . However, only 29 pairs were followed up as 5 recipients died after allogenic HSCT either from infection, GVHD, graft failure and /or relapse and failure to detect informative locus in one pair.

Of the 29 patients studied, 24 recipient showed CC, 3 recipients showed MC and 2 recipients retained recipient type (failure) after HSCT.

The first case of MC showed MC after 3M then converted to CC during follow up at 6,9 and 12 months. The second patient showed MC during follow up at 3M, 6M , 9M then converted to CC after 1 year. The last patient showed MC during follow up at 3M then retained recipient type at 6M and 9M then converted to MC(after receiving donor lymphocyte infusion) during follow up at 12 and 15 month. Table (4) summarizes the state of chimerism of the studied patients. Figures (1, 2) show photography of agarose gel of some of studied patients.

Table 4: Chimerism Status of studied Patients

Chimerism Status	2 patients : failure -(patient type) 24 patients : CC . 2 patients : MC then converted to CC . 1 patient : MC then recipient type and finally MC .

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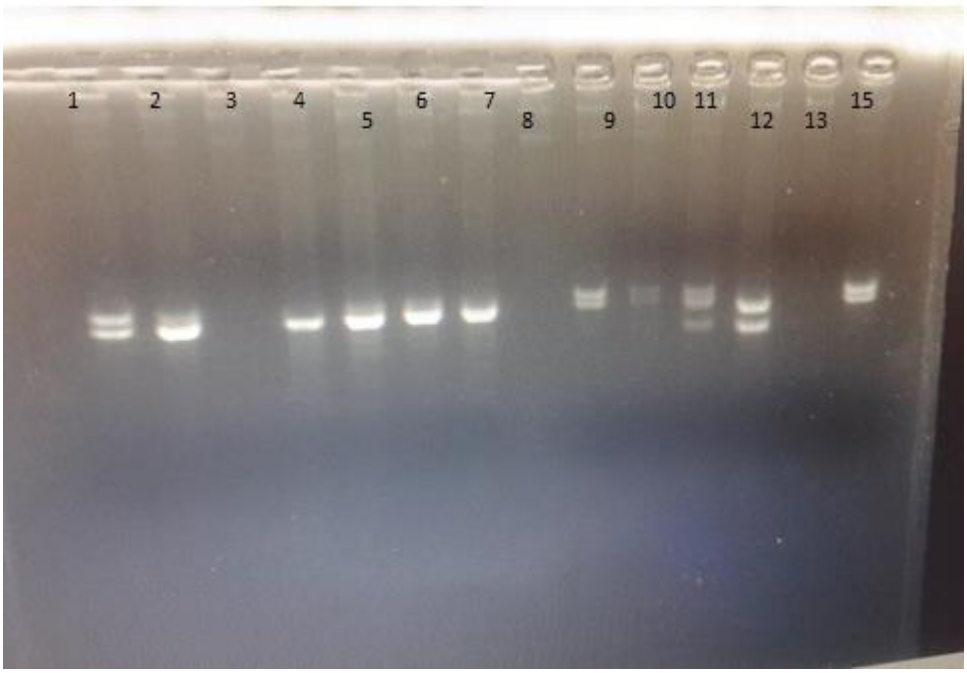

Figure 1: Photograph of 2% agarose of DIS80 showing :Lane 1 & 2 patient and donor showing informative locus. Lane 4&5 : patient and donor showing CC .Lane 6&7:patient and donor showing CC. Lane 9&12: patient pre-transplant and donor &lane 11 : patient showing MC post transplant .

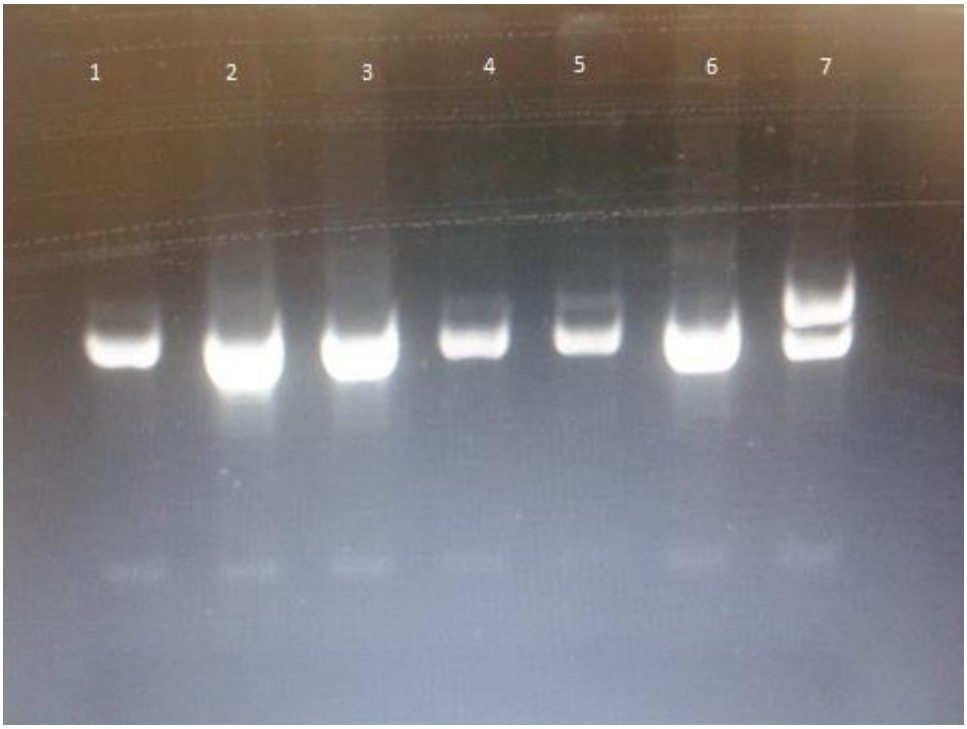

Figure 2: Photograph of 2% agarose of Apo B showing : lane 1 : patient pre –transplantation and lane 2 donor , lane3: patient post transplantation showing CC. Lane 4&5: another donor and patient post transplantation showing CC. Lane 6 & lane 7 : another pre-transplantation pair showing informative locus

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DISCUSSION

Detection 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]

Many previous studies used PCR-based analysis of VNTR loci for studying of chimerism state after HSCT. This method 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.[7-21]

In the current study, 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 get an informative locus between pairs to evaluate post- transplant chimerism state. An informative locus is one for which at least one recipient allele has a different number of repeats than the donor allele(s). These is in accordance with Kamel et al.,[20] Mossallam et al.,[21] and Kletzel et al.[7] All previous investigators used VNTR method as a method for detection of chimerism state Kletzel et al .,[7] 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.

Zhou et al.,[23] 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. Bryant and Martin[24] mentioned that the discriminative power of VNTR loci depends on the number of alleles detected and their distribution in the population .Also , Antin etal .,[14] 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. Consequently, extension of the panel of VNTRs used is highly recommended.

Also a previous study[25] in Egyptian population , stated that combination of 6VNTR loci increases the discriminative power to 100%; combination of 5,4,3 VNTR loci had discriminative powers ranging between 66.7–100%, 50–100%, 50–100% respectively. On the other hand, combination of 2 VNTR loci had a discriminative powers ranging between 50 – 83.3%. Depending on these data 6 VNTR loci were used in this study to increase the discriminative power of VNTR loci analysis.

Informative locus was detected in 34 pair out of 35pairs pre-transplantation, while there was failure of detection of an informative locus in one pair out of 35 pairs . Complete chimerism was detected in 24 patients out of 35 patients. Mixed chimerism was detected in 3 patients. Failure of chimerism was detected in 2 patients. This is in accordance with Ginsburg et al.,[28] who reported that by using VNTR analysis ,it is possible to evaluate chimerism state post HSCT using informative loci . McCann and Lawler [29] 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.

MC chimerism was detected in 3 patients. Two patients showed MC early , then converted to complete chimerism on follow up .The first patient showed MC after 3M then showed CC after 6 ,9, and 12 month of follow up. The second patient showed MC after 3M, 6M and 9M then converted to CC chimerism after 1 year of follow up. On the other hand, one patient showed MC after 3M then after 6 and 9 month relapsed to the recipient pattern then after a cycle of donor lymphocyte infusion the patient converted again to MC after 12 and 15 month . This is in accordance with Booker [30] & Fan and Chu [31] who stressed on that the ability of detection of state of chimerism to predict graft rejection, graft-vs-host disease or recurrence. It also may help to monitor the efficacy of therapy given to support engraftment.

Similarly, Serrano et al.,[32] found that myeloid MC preceded cytogenetic relapse by 2-12 months which enables early therapeutic interventions. In addition, identification either MC is decreasing or increasing is mandatory .Many previous studies had detected the type of mixed chimerism by doing a dilution experiments by mixing recipient pre-transplant DNA and donor genomic DNA in different percentages and subsequently amplified. Then stain PCR products with ethidium bromide after separation by agarose gel electrophoresis and the degree of MC was expressed as the percentage of host DNA. These is in accordance with many previous investigators as Kamel et al .,[20] , Bader et al .[5] and Kletzel ei al .,[7] who recommended these method to monitor chimerism outcome.

Also, in this study failure to detect an informative locus in one pair was encountered. This confirms the importance of extending the panel of VNTR as recommended by Antin etal .[14]

Finally, 2 patients were recorded as CC by laboratory analysis but relapsed clinically .This may be attributed to using whole blood in this study only as a sample to evaluate chimerism . Many investigators as Serrano et al ., [32] , Bader et al ., [5] , Antin etal ., [14] Breuer et al., [33] , Goh etal ., [22] ,Yang et al ., [34] Bacher et al .,[35] and Kletzel et al.,[7] had stressed the importance of lineage specific chimerism detection. As patient may be CC on whole blood but MC on meyloid or lymphoid lineage according to the type of the disease.

CONCLUSION

VNTR analysis by conventional PCR is a sensitive easy to perform rapid method for detection of chimerism state after HSCT provided that a large panel of loci is used. Sequential analysis for chimerism state is an important tool to assess engraftment, relapse , and therapeutic interventions . Detection of type of mixed chimerism by dilution experiments and the use of lineage specific chimerism may enforce the results of VNTR analysis for follow up of HSCT .

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