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Whole-comparative Genomic Hybridization and "Cell Code" Estimation: An Application for Assessment of Cellular Chimerism

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Abstract
Whole-comparative genomic hybridization (W-CGH) allows one to identify copy number differences in highly repeated DNAs between two genomes. It allowed the identification of nuclear markers that can be used to distinguish cell populations from different individuals in a chimeric situation. We discuss the reliability of W-CGH accomplished with fluorescent *in situ* hybridization (FISH) and digital image analysis (DIA) to analyze the degree of chimerism in patients after allogeneic hematopoietic stem cell transplantation (HSCT).

Key words: W-CGH, FISH, degree of chimerism.

Abreviations
HSCT: Hematopoietic Stem Cell Transplantation
W-CGH: Whole-Comparative Genomic Hybridization
FISH: Fluorescent *in situ* Hybridization
DIA: Digital Image Analysis

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a common therapeutic procedure for most cases of leukemia. After HSCT a chimera is produced: cells from the transplant donor (D) and engrafted cells from the host (H) coexist within the same individual. The degree to which the D lymphohematopoietic system restores normal hematopoiesis in H through the generation of a stable chimera is a critical step to establish the success of the engraftment or the relapse of the disease [1, 2]. For these reasons the study and follow up of the degree chimerism in patients after HSCT has a great relevance in order to detect a successful engraftment, graft failure or rejection.

Several methods have been described to determine the degree of chimerism. These approaches are based on the identification of genetic markers that discriminate between H and D cell populations. Classical markers include red blood cell phenotyping [3, 4], inmunoglobulin isotype analysis [5] and conventional cytogenetic methods [6, 7, 8]. Molecular biology provided efficient tools to develop alternative and more sensitive methods [9, 10, 11]. Nowadays, probably the most widespread is polymerase chain reaction (PCR) with variable number of tandem repeats/short tandem repeats (VNTR/STR-PCR) [11] that offers a quite sensitive method for the study of chimeras [12, 13]. However, all those techniques only provide semi-quantitative evaluations of the degree of chimerism. i.e. they do not count and compare the number of D and H cells. This problem is partially solved only in sex-mismatched transplants by using FISH with sex chromosome-specific probes [14, 15]. Similarly, quantitative PCR is a very feasible technique [11, 13], but the localization of markers to distinguish D and H complicates the procedure, thus decreasing its efficiency [16]. Hence, nowadays determining the degree of chimerism after HSCT is still a complicated issue [17, 18, 19].

Whole-Comparative Genomic Hybridization (W-CGH) is a rapid method to identify polymorphisms related to highly repetitive DNA sequences [20]. Highly repetitive DNA sequences encompass the blocks of constitutive heterochromatin, which are concentrated close to centromeres. The size of those blocks of heterochromatin is determined by the number of copies building them, which is mendelian inherited [21]. If the number of repeats of a sequence building certain heterochromatic region is consistently different between two individuals then these chromosomal polymorphism in the constitutive heterochromatin is a good candidate to discriminate or characterize the cells and genomes of these individuals. The application of our procedure is based on locating those particular kinds of markers to distinguish two individuals (putative D and H) by means of W-CGH. Afterwards, using FISH and digital image analysis (DIA), each individual can be classified according to a "cell code" related to the detected marker: the size of the heterochromatin blocks in a selected chromosome (or in various). The main contribution of this technique is to reveal all the differences existing (at the technique resolution) between the two individuals compared in the size of the heterochromatin blocks of their chromosomes. Hence, all the putative markers are revealed after a single experiment. In the same manner that FISH is employed as a common tool to study sex-mismatched cases. Subsequent FISH-DIA of the marker detected by W-CGH, will allow us to discriminate the origin (H or D) of each cell present in the chimeric H based on its relative size. It provides the ability to quantify the degree of chimerism even in sex-matched cases.

In the current investigation we created artificial situations in order to recreate chimerism environments, and to show the potential of a rapid protocol based on

W-CGH followed by FISH-DIA to identify the procedure of different cell populations.

Materials

Two individuals, a female (F) and a male (M) involved in chimerism evaluation procedures (by means of FISH of sex chromosomes), were employed in our investigation (with their consent and according to the principles of the Declaration of Helsinki).

Methods and Statisics

Chromosome and Interphase Nuclei Spreads

Peripheral blood lymphocytes were extracted and cultured for 72h in RPMI 1640 (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) medium supplemented with 1.5% phytohaemagglutinin, 10% fetal calf serum and antibiotics. Cells were arrested at metaphase with colcemid (10 mg/ml) for 90 minutes. Chromosome slides were prepared by exposing the cell suspension to 0.075 M KCl for 20 minutes and rapidly fixing in fresh fixative (methanol-acetic 3:1); the cells were spread onto clean slides and allowed to dry.

Two sets of slides were used for FISH. For "Cell Code" estimation: slides obtained from F and M individuals were prepared. For chimerism evaluation, slides were prepared from F and M cell cultures which were mixed in three different concentrations: 1µlF-1µlM, 2µlF-1µlM and 3µlF-1µlM. Slides with unknown degree of chimerism were also employed. Slides prepared from male individuals were employed for W-CGH.

W-CGH Mixed Probe

For the W-CGH experiment, F and M DNAs were extracted from peripheral blood samples according to standard procedures. The concentration of each DNA sample was carefully measured using a DNA-spectrophotometer (Amersham-Pharmacia Biotech, Piscataway, NJ, USA). Two DNA samples (F and M) of equivalent concentrations were labeled with biotin-14-dATP (F) and digoxigenin-11-dUTP (M) employing a commercial nick translation kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). After DNA labeling, probe size fragments were tested to be in the range of 600-2000 bp in a 1% agarose gel. F and M probes were mixed at equal concentrations and the resulting mixed probe (F probe + M probe) was precipitated with ethanol. After air drying, the probe was dissolved in hybridization buffer [50% (vol/vol) formamide/ 10%(wt/vol) dextran sulfate/ 2X standard saline citrate (SSC), pH 7] to a final concentration of 20 ngr/µl, denatured at 70°C for 10 minutes, and placed on ice for 5 minutes.

FISH Probes

Biotin-labeled chromosome 9-specific satellite III probe (Appligene-Oncor, Illkirch, France) (0.5 µl/probe/slide) and digoxigenin-labeled chromosome X-specific satellite probe (DXZ1)(Appligene-Oncor, Illkirch, France) (0.5 µl/probe/slide) were dissolved in hybridization buffer (9 µl/slide) [50% (vol/vol) formamide/ 10% (wt/vol) dextran sulfate/ 2X Standard saline citrate (SSC), pH 7], denatured at 70°C for 10 minutes, and incubated on ice for 5 minutes.

Probes Hybridization

Metaphase slides were incubated in 2XSSC at 37°C for 60 minutes and dehydrated in 70%, 85% and 100% ethanol. After air drying, slides were denatured in 70% formamide/ 2XSSC pH 7 at 70°C for 2 minutes, and dehydrated again. The probe (F + M mixed probe for W-CGH experiment; and chromosome 9 probe plus chromosome X probe for FISH experiment) was applied to the slides and hybridized at 37°C in a moist chamber overnight. After hybridization, the slides were washed in 50% formamide at 42°C for 15 minutes and in 2XSSC at 37°C for 8 minutes. A non-specific antibody blocking solution [BSA 10% (wt/vol)/ Tween 20 20% (vol/vol)/ 2XSSC, pH 7] was applied for 5 minutes at 37°C. Slides were then incubated for 25 minutes at 37°C in the antibody solution, with a single layer of FITC-avidin and Rhodamine anti-digoxigenin antibodies (Appligene-Oncor, Illkirch, France) for simultaneous localization of the two probes in green (G) and red (R), respectively (F probe + M probe in W-CGH experiments and chromosomes 9 and X satellites in FISH). And finally mounted with anti-fade solution (Vectashield). The FISH experiment samples were counterstained with DAPI (4´,6-Diamidino-2-Phenylindole) (100ngr/µl).

Image Capture and Analysis

Slides were analyzed using a DIA platform based on a Leica DMLB fluorescence microscope equipped with a charge-coupled device camera (Cool Snap, RS Photometrics, Trenton, NJ, USA) with three independent green, red and blue filters. Images were captured as .tiff files employing Cool Snap software.

For W-CGH DIA, quantitative longitudinal measurement of G and R probe fluorescence intensities (G and R grey levels) was performed along chromosomes 9-9' using Visilog 5.1 software (Noesis, Vélizy, France), variations in G and R were measured in the range of 0 to 255 grey levels. Excel software (Microsoft Corporation, Redmond, WA, USA) was employed for statistical analysis and for the estimation of the mean G to R ratio to quantify the different contribution to the final hybridization of each of the DNAs in the mixed probe.

FISH images DIA was performed employing Leica Qwin Software (Leica Microsystems, Wetzlar, Germany) in order to measure the satellite size of both homologous chromosomes 9-9' (Sum of total G grey levels of the detected area) and the interphase nuclei surface size (Sum of total blue -B- grey levels of the detected area). Variations in G and B were measured in the range of 0 to 255 grey levels. The relative size of the chromosomes 9-9' satellites to the whole nucleus (G to B ratio) was computed independently for F and M. In order to compare their means, t-student was performed; and Chi-2 was used to contrast the ob-

served and expected results employing Excel software (Microsoft Corporation, Redmond, WA, USA).

Results

W-CGH and Chromosomes 9-9' Heterochromatin Blocks

After W-CGH, the fluorescence signal of a particular chromosome region is a mixture of both DNA probes hybridized (G labeled and R labeled). While chromosome arms usually show yellow-like (G + R) fluorescence, some pericentromeric heterochromatin blocks show significant G or R dominance (Fig. 1). The dominant fluorescence of one of the two channels (G or R) indicates a higher contribution to the final hybridization of that probe (over the other) and, hence, a higher number of copies of the sequence involved in that genome, and vice versa.

Samples from two different sex individuals (F and M) were employed to perform W-CGH. The F-probe was revealed in G and the M-probe in R. The mixed (F + M) whole genome-probe was hybridized to male-derived metaphase chromosomes. DIA was performed over ten selected metaphases after background subtraction plus G and R channel compensation. A similar DNA hybridization of both DNA probes (F-G and M-R) was detected along chromosome arms, that is, each arm was stained homogeneously yellow (G + R). Although some of the chromosome regions showed a similar response to that of the chromosome arms, other chromosomes (notably chromosomes 1, 9, 15, 16 and Y) exhibited a predominance of the G or R channel (Fig. 1) in their heterochromatin blocks.

We focused our attention on the pericentromeric regions of chromosomes 9-9' that showed the highest G predominance (Fig. 2) (other chromosomes data not shown). Both homologous chromosomes 9-9' were studied in 10 digital images. And the mean G to R ratio in that chromosomal regions was 1.87, revealing a high predominance of G over R (> 1.75, used as cut-off threshold in CGH experiments) (Fig. 2B). The interpretation of that differential fluorescence is that hybridization of the F probe in this chromosomal region is substantially higher than that of the M probe, and led us to conclude that the genome of F presents a larger amount of highly repeated sequences on its pericentromeric heterochromatin of the chromosomes 9-9'.

Characterization of Individuals: "Cell-Code" Estimation

In order to define a characteristic to distinguish the two analyzed individuals (F and M), we employed the most remarkable difference previously detected between them by W-CGH, i.e. the different sizes of their chromosomes 9-9' heterochromatin blocks. Using a single DNA probe, a numeric interval that defines the cells belonging to each individual (M and F) by its own "cell code" (CC) was derived from the relative size of the blocks of pericentromeric heterochromatin. The CC for the pericentromeric region of the chromosomes 9-9' (9CC) was defined as the amount of chromosome 9 probe hybridized and revealed in G, against the background obtained with DAPI (B by blue channel). The sum of total G grey levels of both chromosomes 9-9' heterochromatin blocks areas, and the sum of total B grey levels of the whole nucleus area was measured for each image of the 50 images captured, and the G to B ratio calculated for each cell. This ratio characterizes the two individuals on the basis of the relative size of their chromosomes 9-9' heterochromatin blocks with respect to their total nuclear DNA.

Table 1. Results from three experiments of artificial chimerism.

CHIM	O-F	O-M	Unk	E-F	E-M	CHI-2
F1-M1- exp a	25	25	0	25	25	0.000
F2-M1- exp a	32	16	2	32	16	0.000
F3-M1- exp a	36	11	3	35.25	11.75	0.007
F1-M1- exp b	20	27	3	23.5	23.5	0.766
F2-M1- exp b	35	15	0	33.33	16.66	0.122
F3-M1- exp b	34	16	0	37.5	12.5	0.002
F1-M1- exp c	22	26	2	24	24	0.188
F2-M1- exp c	29	19	2	32	16	0.586
F3-M1- exp c	30	16	4	34.5	11.5	1.855

On each experiment (a, b and c) three different degrees of chimerism were recreated: F1M1- female 1 vs. male 1, F2M1- female 2 vs. male 1, F3M1- female 3 vs. male 1.
The number of cells observed within the 9CC-F (O-F) and 9CC-M (O-M) intervals and the number of cells expected, are displayed (E-F and E-M, for male and female respectively). The origin of each of 50 cells (O-M and O-F) was determined comparing G to B ratios obtained with previously stated 9CC values for M and F. Statistical analysis to compare observed (O) and expected (E) results was performed. Chi-2 (ft = 1, $\alpha = 0.05$) revealed no significant differences between O and E values in all the experiments (Chi-2 < 3.6).
(Unk- Number of cells within the overlapping interval; exp- Experiment).

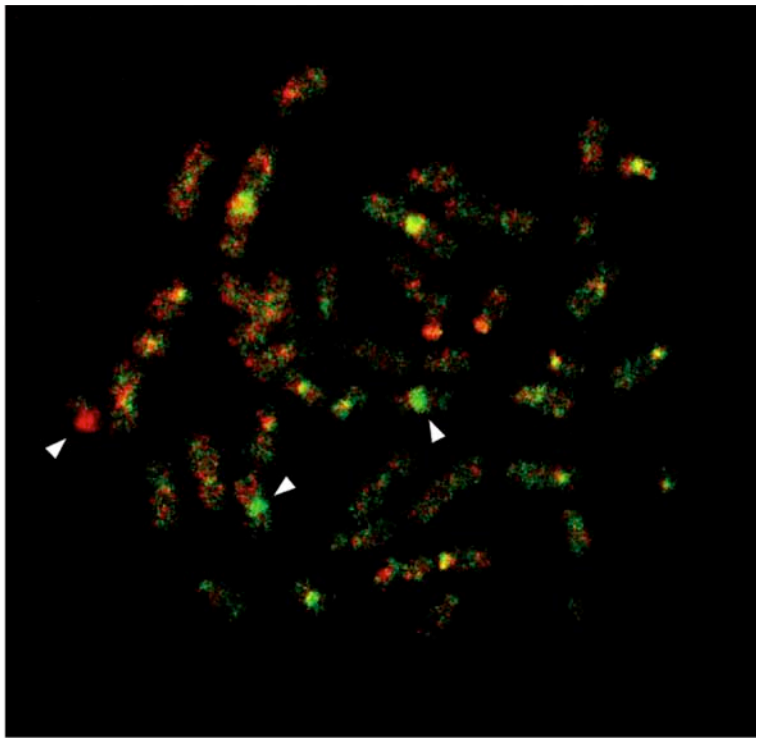

Fig. 1. A- W-CGH over a male metaphase spread. F DNA probe was labeled in G and M DNA probe was labeled in red (R). Chromosome arms showed yellow-like fluorescence (G + R) while some pericentromeric heterochromatin blocks showed significant G or R dominances. Note R fluorescence dominance in chromosome Y and G fluorescence dominance in chromosomes 9-9' pericentromeric heterochromatin (arrowheads).

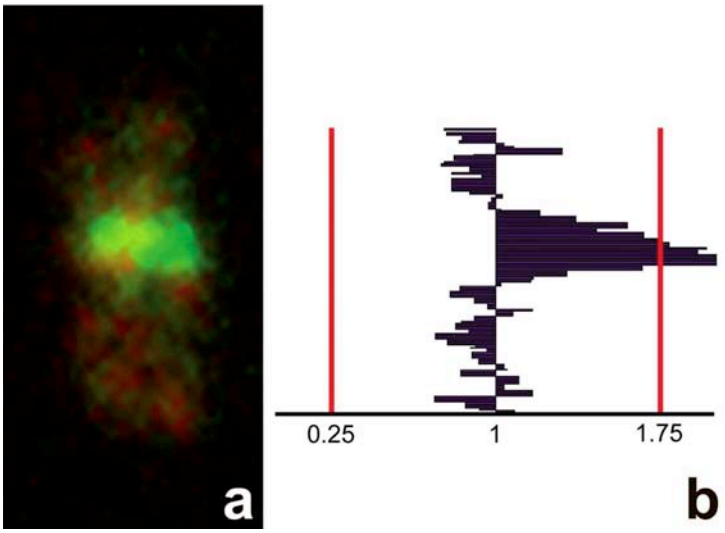

Fig. 2. A- Selected chromosome 9 B- Longitudinal representation of the G to R ratio of the selected chromosome 9, revealing G over R dominance (>1.75, red line) in the pericentromeric heterochromatin.

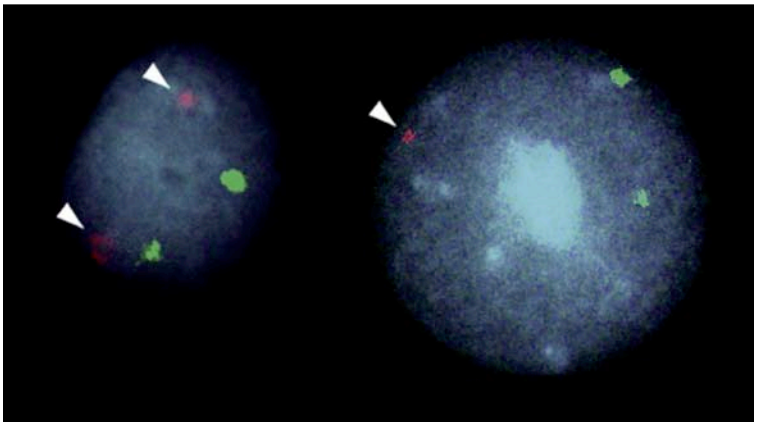

Fig. 3. FISH over F (left) and M (right) interphase nuclei. Chromosome 9 pericentromeric heterochromatin probe revealed in G shows the differential size of these blocks between F and M. Chromosome X heterochromatin specific probe is revealed in R (arrowheads) and is used as a control of the origin of the cell (M or F).

We calculated 9CC of each individual as the interval of values of its G to B ratio for the 50 cells measured.

Results show that G to B ratio for F (9CC-F) varies within the interval 0.0018-0.0097 (mean = 0.00486, variance = 0.00284) while that of M (9CC-M) varies within the interval 0.00004-0.0019 (mean = 0.00126, variance = 0.00055). Although both intervals overlap to a certain degree (0.0018-0.0019), significant differ-

ences were found between the mean of G to B ratios of both individuals (t-Student = 6.8 > 2.03, $\alpha = 0.05$).

Detecting Artificial Chimerism: Quantification by "Cell Code"

In order to study the ability of the proposed method, three different chimeric environments were artificially created by mixing F and M cells in known proportions: 1:1, 2:1, 3:1 (labeled as F1:M1, F2:M1, F3:M1). Once the "cell code" interval (9CC, in this case) that characterizes each individual (M and F) had been established, we used this characteristic value to pinpoint the origin of each of the cells found in the different preparations. After standard FISH, we estimated individual 9CCs in 50 cells from each artificial chimeric environment. Values obtained were compared to the 9CCs intervals for M and F, thus each cell was assigned to a F or M origin (O-F or O-M respectively; see Table 1). Additional hybridization of the chromosome X satellite was used as a control to determine the origin of each cell, and provided a control that the origin assigned to each cell based on its 9CC was correct in all cases (Fig. 3). We also employed the 9CC to determine the origin of cells in different situations with an unknown degree of chimerism (data not shown) and the results were once more identical to those obtained with chromosome X as a control.

Those cells of the artificial chimerism environments falling within the overlapping interval of F and M ranges (G to B = 0.0018 - 0.0019) were impossible to assign an origin based on their 9CC, so those cells were not tallied in this study (Unk; see Table 1). The experiments were repeated three times for each artificial chimeric situation. The observed results were compared to those expected, depending on the particular degree of chimerism artificially established to confirm the ability of the approach. No significant differences were observed in any of the three cases (Table 1).

Discussion

The results presented in this investigation support the idea that W-CGH, coupled with standard FISH and DIA, can be a valuable tool to ascertain the origin of the cells in a chimeric situation. Cell characterization is a simple approach that is more advisable than related molecular approaches to study the degree of chimerism after HSCT in some instances [22]. For example, FISH using single or double color for sex chromosomes identification is a common and easy way to quantitatively estimate cell population fluctuations after HSCT of sex-mismatched D and H, and its levels of reliability are almost as high as those offered by PCR for identification of lower levels of chimerism [23, 24, 25]. Our approach using W-CGH opens the future possibility to employ FISH to study the degree of chimerism in sex-matched cases. Additionally it is not necessary to search for markers to distinguish D and H by assaying targets as in quantitative-PCR approach, since W-CGH reveals, in a single *in situ* fluorescence protocol, all the differences existing in their pericentromeric heterochromatin. This fact allows selecting those chromosomes that exhibit the largest differences in constitutive heterochromatin between D and H prior to allogenic HSCT, and subsequently use them as markers of cells origin in the quantification of the degree of chimerism. This would mean that according to our system the first blood samples after cell engraftment could already be analyzed using a predetermined "cell code".

The main point of W-CGH is that multiple differences are revealed in a single experiment. However not all of them are useful for cell discrimination. In fact, we are estimating the range of polymorphisms affecting constitutive heterochromatin which could be more useful to establish a "cell-code" for each individual, and we propose that those chromosomes harboring classical satellite DNA families [especially chromosomes 1 (classic satellites 2 and 3), 9 (classic satellite 3) and 16 (classic satellite 2)] are good candidates for such an approach. Surprisingly, alphoid satellite DNA, present in all the human chromosomes, usually does not offer enough differences for individuals discrimination under our system. In the 30 DNA samples studied by W-CGH we observed the presence of a differential contribution of different satellite DNA families that could be visualized by fluorescence microscopy. Obviously not all of the differences detected can be used for cell discrimination after CC establishment. In fact, when overlapping ranges of CCs of putative D and H are too large, it is not possible to identify the origin of the cells in the chimera. We note that differences less than 600 kb could be rarely detectable by W-CGH [26, 27]. After selecting the candidate/s markers, chimerism evaluation will be easily achieved by FISH and DIA measurement of its results. Hence, this protocol could be of special interest to those labs performing FISH to study the degree of chimerism in sex-mismatched cases. Offering these labs the possibility to study sex-matched cases in the future.

Application of DIA permitted a rapid, feasible and objective analysis of FISH results after W-CGH, and reconstruction of the artificial degree of chimerism. Future improvements like assaying multiple markers to characterize each individual cell population avoiding overlapping ranges, and increasing DIA sensitivity, could eventually make possible automation of procedures for rapid and accurate assessments of chimerism after HSCT.

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