DOI: 10.1038/bmt.2016.5

Bone Marrow Transplantation (2016) **51,** 874–875

www.nature.com/bmt

LETTER TO THE EDITOR

Chimerism interpretation with a highly sensitive quantitative PCR method: 6 months median latency before chimerism drop below 0.1%

Bone Marrow Transplantation (2016) **51,** 874–875; doi:10.1038/bmt.2016.5; published online 15 February 2016

Despite better results with hematopoietic stem cell transplantation (HSCT) over the years, high rates of relapse, which in acute leukemia can reach 30–70% of cases, remain a major challenge.[1–2] The treatment of relapse has better outcomes if performed early. Studies show that the detection of mixed chimera after transplantation can predict for relapse in most cases and it may allow for earlier appropriate treatment, with higher chances of success.[3-5]

A variety of techniques has been developed to determine post-transplant chimerism. The use of PCR-based amplification of STRs has become the gold standard for quantitative chimerism analysis but, although the high informativity and good accuracy of the method, its sensitivity is relatively low and the detection level of a minor genotype is between 0.4 and 5%.[6–9] Some studies have proposed RQ–PCR-based methods that allow for the detection and quantification of hematopoietic chimerism, with sensitivities $\leqslant 0.1\%$ of the minor genotypes.[10]

In this study, we tested a method of quantitative PCR of insertions–deletions (indels) diallelic polymorphisms.[11–13] RQ–PCRs were performed with TaqMan technology at an ABI Prism 7500 apparatus (Applied Biosystems, Foster City, CA, USA). The TaqMan reaction is based on the 5′ nuclease activity of Taq DNA polymerase to cleave an oligonucleotide probe during PCR, thereby generating a detectable signal that is analyzed in a dedicated thermocycler.[14] We analyzed samples from 41 adult patients submitted to HSCT at the Bone Marrow Unit of the University of Campinas between 2005 and 2010. Patients' DNA samples were appropriately stored and their concentrations were measured with Thermo Scientific Nanodrop 1000 (Wilmington, DE, USA). We first analyzed all pre-HSCT samples of patients and their respective donors for the presence of 13 indels polymorphisms. The primers and probes for the polymorphisms studied came in a set produced by the Spanish company Sistemas Genómicos (Valencia, Spain). The set also included the β globin gene for DNA quality control. We used a total of 25 ng of DNA and all samples were satisfactorily amplified. When a polymorphism was present in patient DNA and absent in donor DNA, it was considered informative and selected for further analysis. We found informative polymorphisms in 83% (34) of patients, half of them had more than one. Patients had at least three post-HSCT samples and were tested for one or two polymorphisms up to 1 year post HSCT, or longer, if relapse occurred later and samples were available. Two patients were excluded because of inadequate samples. The median number of samples was 7.5 per patient and we used 100 ng of DNA for the tests. A complete table with the characteristics of patients and the distribution of the polymorphisms studied can be found in the Supplementary Appendix.

Descriptive analyses were done and presented through graphics and tables. The time of relapse was defined as the date of first hematologic relapse. To investigate clinical correlations, Pearson correlation coefficient was applied and differences in quantitative variables were analyzed by Mann–Whitney or *t*-test, when satisfactory, whereas categorical data were compared by χ^2-test. Kaplan–Meier method and log-rank test were also applied for survival analysis. *P*-values below 0.05 were considered significant. SPSS (statistical package for social sciences) version 21.0 (Armonk, NY, USA) and the GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) were used.

Almost half (46.9%) of the patients relapsed, most of them (73.3%) in the first 6 months. The median overall survival for relapsed patients was 9 (2–98) months and, at the end of the observation period, only one patient (6.6%) was alive. The main cause of death was disease progression. Among non-relapsed patients, five (29.4%) died, four in 9 months post HSCT, mostly from infectious complications. Five-year overall survival in non-relapsed patients was significantly higher than in the relapsed group ($P = 0.001$, log-rank test).

The quantitative PCR method was able to quantify the chimerism in almost all 249 post-HSCT samples and it was highly sensitive, with a minimum level of detection reaching 0.01%. For the analysis of post-HSCT samples, we used 100 ng of DNA. Only one patient had an inadequate amplification of the DNA in almost all his samples because of bad DNA quality. As shown in Figure 1, the chimerism kinetics was very different between groups of relapsed and non-relapsed patients. The relapsed group showed a steep initial drop and then a very early and fast rise in chimerism before hematologic relapse. The non-relapsed group showed, initially, a less deep and more progressive fall in the percentage of recipient cells, followed by a continuous and slow fall over time. Although we could not find a cutoff level of chimerism that could predict relapse, the difference in the kinetics was evident. Also, curiously, there was a median period of about 6 months before chimerism clearance reached levels below 0.1% in non-relapsed patients.

We tried to correlate the chimerism kinetics with the risk of relapse and the diagnosis, but we didn't find statistical significance. It is evident, though, that those patients who relapse have a very early rise in the population of recipient cells. As early as 6–8 weeks post transplantation, we started to see a difference in chimerism levels, but probably due to the limited sample size, there was no statistical significance, only a trend at 12–14 weeks ($P = 0{,}06$). The median time to hematologic relapse was 18 weeks. This helps explain why there was no response to donor lymphocyte infusions among all five patients who were submitted to it after detection of mixed chimerism by variable number tandem repeat (VNTR)/STR and all of them died of disease progression. Early intervention upon evidence of relapse is crucial for prognosis in this population.

It was not possible to correlate the chimerism values with any other clinical parameter. The samples were heterogeneous at the time of collection and the number of patients was probably insufficient to be able to demonstrate a statistical difference between relapsed and non-relapsed patients based on the chimerism value. It is necessary to evaluate a larger number of patients in a prospective study.

The chimerism technique by real-time PCR of insertion–deletion diallelic polymorphisms was easy to perform and very accurate for

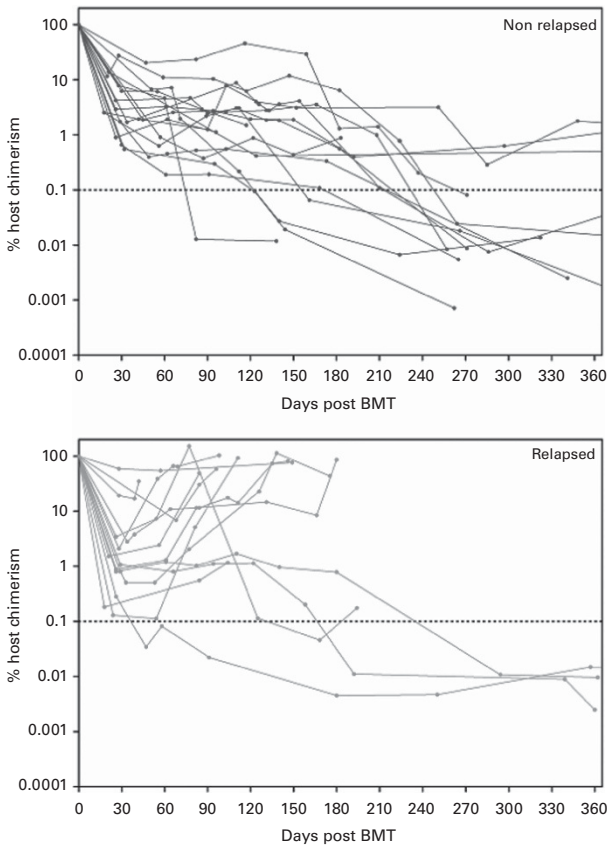

Figure 1. Differences in chimerism kinetics reveal a long time to clearance of host cells to $< 0.1\%$ in non-relapsed patients, up to 1259 days post transplantation (median 189 days), and a very early rise in host cells in relapsed patients.

the detection of chimerism after HSCT. It was able to detect very low levels of recipient cells in the studied post-HSCT population. Although it was not able to determine a cutoff value that could be predictive of relapse, it showed that the chimerism kinetics is very different between relapsed and non-relapsed patients. There are still very few reports on the use of indels for longitudinal chimerism evaluation post HSCT and some important questions remain unanswered and need further investigation. Is it possible that the remaining recipient cells in the first 6 months post transplantation observed in the non-relapsed patients were responsible for triggering a more efficient GvL effect? Are these residual recipient cells normal or leukemic cells? Is initial mixed chimerism beneficial? The predominant type of recipient cells that remain after conditioning could be responsible for the results of the transplant. It is been reported, for example, that filters used in bone marrow harvest may contain on average 21% long-term culture-initiating cells and 15% fibroblast colony-forming units of the total progenitor cell content and that adding these cells to the harvest significantly reduced the incidence of grade II–IV GvHD and the transplant-related mortality.[15] It highlights the importance of other bone marrow cells, such as mesenchymal cells in the mechanisms of engraftment. The evaluation of chimerism at such low levels could help us understand the complex mechanism of genetically distinct cells interacting in different post-transplantation settings. This could also lead to a better optimization of the current nonmyeloablative conditioning regimens for better GvL effect with lower adverse effects.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We would like to thank São Paulo Research Foundation for funding this research and the Laboratory of Molecular Analysis of Polymorphism of the University of Campinas for providing the DNA samples.

DC Vicente[1], ABA Laranjeira[2], ECM Miranda[1], JA Yunes[3] and CA de Souza[1]
[1]HEMOCENTRO, University of Campinas, Campinas, Brazil;
[2]Centro Integrado de Pesquisas Oncohematológicas na Infância (CIPOI), University of Campinas, Campinas, Brazil and
[3]Department of Medical Genetics, Faculty of Medicine, University of Campinas, Campinas, Brazil
E-mail: danycvi@hotmail.com

REFERENCES

1 Rivera GK, Pinkel D, Simone JV, Hancock ML, Crist WM. Treatment of acute lymphoblastic leukemia: 30 years' experience at St Jude Children's Research Hospital. *New Engl J Med* 1993; **329**: 1289–1295.
2 Kiehl MG, Kraut L, Schwerdtfeger R, Hertenstein B, Remberger M, Nicolaus K *et al.* Outcome of allogeneic hematopoietic stem-cell transplantation in adult patients with acute lymphoblastic leukemia: no difference in related compared with unrelated transplant in first complete remission. *J Clin Oncol* 2004; **22**: 2816–2825.
3 Formánková R, Sedlácek P, Krsková L, Říhová H, Srámková L, Star J *et al.* Chimerism-directed adoptive immunotherapy in the prevention and treatment of post-transplant relapse of leukemia in childhood. *Haematologica* 2003; **88**: 117–118.
4 Dey BR, McAfee S, Colby C, Sackstein R, Saidman S, Tarbell N *et al.* Impact of prophylactic donor leukocyte infusions on mixed chimerism, graft-versus-host disease, and antitumor response in patients with advanced hematologic malignancies treated with nonmyeloablative conditioning and allogeneic bone marrow transplantation. *Biol Blood Marrow Transplant* 2003; **9**: 320–329.
5 Bader P, Kreyenberg H, Hoelle W, Dueckers G, Handgretinger R, Lang P *et al.* Increasing mixed chimerism is an important prognostic factor for unfavorable outcome in children with acute lymphoblastic leukemia after allogeneic stem-cell transplantation: possible role for pre-emptive immunotherapy? *J Clin Oncol* 2004; **22**: 1696–1706.
6 Bader P, Beck J, Frey A, Schlegel PG, Hebarth H, Handgretinger R *et al.* Serial and quantitative analysis of mixed hematopoietic chimerism by PCR in patients with acute leukemias allows the prediction of relapse after allogeneic BMT. *Bone Marrow Transplant* 1998; **21**: 487–495.
7 Casarino L, Carbone C, Capucci MA, Izzi T, Ferrara GB. Analysis of chimerism after bone marrow transplantation using specific oligonucleotide probes. *Bone Marrow Transplant* 1992; **10**: 165–170.
8 Oliver DH, Thompson RE, Griffin CA, Eshleman JR. Use of single nucleotide polymorphisms (SNP) and real-time polymerase chain reaction for bone marrow engraftment analysis. *J Mol Diagn* 2000; **2**: 202–208.
9 Alizadeh M, Bernard M, Danic B, Dauriac C, Birebent B, Lapart C *et al.* Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. *Blood* 2002; **99**: 4618–4625.
10 Maas F, Schaap N, Kolen S, Zoetbrood A, Buño I, Dolstra H *et al.* Quantification of donor and recipient hemopoietic cells by realtime PCR of single nucleotide polymorphisms. *Leukemia* 2003; **17**: 621–629.
11 Weber JL, David D, Heil J, Fan Y, Zhao C, Marth G. Human diallelic insertion/deletion polymorphisms. *Am J Hum Genet* 2002; **71**: 854–862.
12 Wilhelm J, Reuter H, Tews B, Pingoud A, Hahn M. Detection and quantification of insertion/deletion variations by allele-specific real-time PCR: application for genotyping and chimerism analysis. *Biol Chem* 2002; **383**: 1423–1433.
13 Jiménez-Velasco A, Barrios M, Román-Gómez J, Navarro G, Buño I, Castillejo JA *et al.* Reliable quantification of hematopoietic chimerism after allogeneic transplantation for acute leukemia using amplification by real-time PCR of null alleles and insertion/deletion polymorphisms. *Leukemia* 2005; **19**: 336–343.
14 Heid CA, Stevens J, Livak KL, Williams PM. Real time quantitative PCR. *Genome Res* 1996; **6**: 986–994.
15 Vicente D, Podestà M, Pitto A, Pozzi S, Lucchetti S, Lamparelli T *et al.* Progenitor cells trapped in marrow filters can reduce GvHD and transplant mortality. *Bone Marrow Transplant* 2006; **38**: 111–117.

Supplementary Information accompanies this paper on Bone Marrow Transplantation website (http://www.nature.com/bmt)