Evaluation of Hematopoietic Chimerism Following Allogeneic Peripheral Blood Stem Cell Transplantation with Amelogenin Marker

Seyed H. Ghaffari PhD*, Bahram Chahardouli MSc*, Ardashir Gavamzadeh MD*, Kamran Alimoghaddam MD*

Background: Monitoring the engraftment of donor cells after allogeneic stem cell transplantation is important for the early diagnosis of graft failure or relapse of disease. The objective of the present study was to evaluate the application of the amelogenin gene for the assessment of chimerism in samples of patients who had received a sex-mismatched stem cell transplantation.

Methods: A polymerase chain reaction technique was developed using a set of amelogenin gene primers alone and/or in combination with short tandem repeats primers and was performed on blood and/or bone marrow aspiration samples of 30 recipient patients after transplantation. The technique was then set up as a routine procedure, from September 2000 through April 2006, more than 1400 samples taken from 300 stem cell transplantation patients suffering from different types of leukemia and nonmalignant hematologic disorders were evaluated for detection of chimerism after transplantation.

Results: The sensitivity of the test was as low as 1 – 2%. The ratio of X/Y fragments was as the mixed chimerism. In 90% of the patients, amelogenin marker was as informative as short tandem repeats markers, as confirmed by the clinical outcome. In 5% of the patients, when there was no pre-bone marrow transplantation sample from either donor or recipient, the applicability of this assay became crucial to our treating physicians.

Conclusion: The application of the amelogenin marker alone or in combination with the short tandem repeats system can be used for relative quantitative analysis of mixed chimerism and for observing kinetics of engraftment in patients who have sex-mismatched bone marrow transplantation. Amelogenin polymerase chain reaction analysis showed an excellent correlation with the short tandem repeats-polymerase chain reaction results.

Keywords: Bone marrow transplantation • chimerism • stem cell transplantation • amelogenin

Introduction

Allogeneic stem cell transplantation (SCT) is extensively used to treat patients with malignancies as well as nonmalignant hematologic diseases. Monitoring the engraftment of the donor cells after allogeneic SCT may be important for the early diagnosis of graft failure. In some cases, detection of mixed chimerism can be predictive of relapse. Genetic markers that allow distinction between the donor and recipient cells, based on allelic differences between the two cell populations, are useful tools for determining hematopoietic chimerism after SCT or donor leukocyte infusions (DLI). Several techniques are available for detecting mixed chimerism after SCT. Analysis of short tandem repeats (STR) or variable number of tandem repeats (VNTR) markers have been used to...
demonstrate chimerism after SCT. Analysis of STR loci by polymerase chain reaction (PCR) is the method of choice for this purpose. To be able to perform this type of analysis, the STR-PCR patterns of both the donor and the recipient have to be known and must be distinguishable. Sometimes, patient’s sample prior to the transplantation is not available, hence, the pretransplantation genotype cannot be determined. One approach to this problem is the sex identification of both male and female cells in the cell mixtures of patients after the sex-mismatched SCT. In sex-mismatched transplantation settings, information on the ratio between donor and recipient can be obtained efficiently and rapidly by using few techniques such as fluorescent in situ hybridization (FISH) with probes specific for X- and Y-chromosomes or using PCR-based amplification with primers specific for a gene which present on both X- and Y-chromosomes. In humans, the amelogenin gene is present on both the X- and the Y-chromosomes, which have been utilized for sexing in forensic case-work and prenatal identification. However, there are size differences in this gene between these chromosomes; when samples from the female patients were analyzed with amelogenin, only one peak was detected while the male patients showed two peaks with a 1:1 ratio. Therefore, the relative ratio of these peak areas was used to calculate ratio of the donor and the recipient cells mixed in recipient’s peripheral blood samples. The application of this assay can be used not only for PCR-based sex determination but also for analyzing relative quantification of mixed chimerism and for observing kinetics of engraftment.

The aim of the present study was to evaluate the application of the amelogenin gene for assessment of chimerism in peripheral blood leukocyte (PBL) and/or BM samples taken from patients who had received sex-mismatched SCT.

Patients and Methods

Patients

The patients studied had been treated by sex-mismatched allogeneic SCT (n=30) at Hematology, Oncology and BMT Research Center, Shariati Hospital, Tehran, Iran, between January 2001 and August 2003. PB samples were obtained from 30 patients undergoing sex-mismatched allogeneic SCT. The corresponding donor samples were obtained from the donor directly or from the graft.

DNA preparation

DNA from PBLs or PBL fractions of the donors and patients before and after SCT were analyzed. PBLs were collected six months after SCT, at three to six-month intervals and at the time of relapse. After DLI, PBLs were collected every month until complete donor chimerism was achieved. Mononuclear cells and polymorphonuclear cells (granulocytes) were isolated by Ficoll-Isopaque density gradient centrifugation. DNA was isolated by salting out method. DNA was also extracted using silica membranes (QiAmp blood Kit; Qiagen, Hilden, Germany) as recommended by the manufacturer. DNA concentration was measured by UV spectrophotometry at 260 nm.

Amelogenin assay

Amelogenin gene amplification allows detect both male and female cells by a single-step PCR reaction simultaneously. The female cells were detected by a single amplification product of 106 bp, and the male cells gave rise to two products of 106 bp and 112 bp, respectively. For each DNA sample, a PCR was performed using primer A, 5'-CCCTGGGCTCTGTAAAGAATAGTG-3', and primer B, 5'-ATGAGAGCTTAAACTGGGAGCTG-3'. The PCR assay was performed in a final volume of 30 µL containing 50 – 100 ng DNA, 100 pmol of each amplification primer, 1 U Taq polymerase, and 3 µL 10× PCR buffer comprising 50 mM KCl, 0.1 M Tris HCl (pH 8.8), 25 mM MgCl2, and 2 mM of each dNTP.

PCR was carried out for 30 cycles (each cycle consisted of 45 sec at 95°C, 45 sec at 60°C, and 45 sec at 72°C), with an initial denaturation at 95°C for five min. For electrophoresis and visualization of the amplification products a 2% agarose gel prestained with ethidium bromide was loaded with 8 µL of PCR product and 2 µL loading buffer. The gel was run at 100 V for approximately one hour. DNA amplification was confirmed by the presence of a band of the appropriate size in each PCR reaction with the exception of the negative controls. Six percent polyacrylamid gels were used and DNA was visualized with DNA silver staining system.

Calculation of mixed chimerism

The picture of the gel was analyzed by Quantity
One and Multi-Analyzer (BioRad) and the intensity of bands were calculated. The degree of mixed chimerism was calculated based on the assumption that the DNA present in the sample should reflect the corresponding cell content and that the alleles of donor and recipient should amplify accordingly. We determined the ratio of donor and recipient by calculation of the proportion of the peak areas corresponding to donor signals as compared with the sum of peak areas of the donor and the recipient signals for amelogenin marker (Figure 1).

**Results**

Figure 2 shows the PCR product obtained with amelogenin primers. The female cells were detected by a single amplification product of 106 bp (AMGX); the male cells gave rise to products of 112 bp (AMGY) and 106 bp (AMGX).

To test the sensitivity of the assay, we mixed male WBC with female WBC at ratios of 0.5% to 80% and 50 ng of DNA (about 10^4 cells genomic equivalent) was amplified in each PCR reaction. The sensitivity was determined as the lowest concentration of DNA that produced a visible band on a silver-stained polyacrylamid gel (Figure 2).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Schematic illustration of calculations performed for the quantitative assessment of mixed chimerism. Calculation of mixed chimerism in heterozygous/homozygous peak constellations with one shared allele.

Analysis of the amplified products indicated that the sensitivity of the assay was as low as 1 – 2% for this marker.

We investigated 30 allogeneic SCT suffering from different types of leukemia (n=18) or nonmalignant hematologic disorders (n=12) by close molecular monitoring during one to twelve months after transplantation. Twenty-eight patients were in complete remission and displayed a full donor hematopoiesis, as demonstrated by amelogenin marker analysis; two patients had mixed chimerism and rejected the transplant. Figure 3a demonstrates an example of engraftment in a patient with full chimerism as determined by amelogenin marker analysis. Figure 3b demonstrates an example of graft rejection in WBC of a patient with ALL using amelogenin-PCR analysis.

In the course of the present study, several attempts were made for using a combination of different STR and amelogenin markers. It was hoped to set up a multiplex-PCR assay containing both the STR and the amelogenin markers that could be used for the detection of chimerism in our SCT patients. We have observed that in multiplex assays, the STR marker can be employed in combination with amelogenin marker for detection of
Amelogenin as a marker of hematopoietic chimerism following blood SCT

chimerism. In Figure 4, amelogenin marker used with two STR markers (D16S539 and D4S2366) by multiplex PCR. Using amelogenin marker in sex-mismatched SCT, 100% (30/30) of all samples were found to be informative. Using combination of three STR markers, 83% (25/30) of all samples were found to be informative for at least one STR marker. As illustrated in the electropherogram of the amplification products from a female donor and a male recipient before SCT and from the recipient after SCT (Figure 4), the STR and amelogenin markers both revealed minor bands indicating a mixed chimerism after SCT.

Between September 2000 and April 2006, more than 1400 samples from 300 BMT patients suffering from different types of leukemia and nonmalignant hematologic disorders were evaluated for detection of chimerism after the transplantation. About 51% of patients received sex-mismatched SCT. In 90% of cases, amelogenin marker was as informative as STR markers, as confirmed by clinical outcome.

**Discussion**

Transplantation with hematopoietic stem cell HLA-identical sibling donors is successfully performed in our BMT center to treat patients with hematopoietic malignancies. Follow-up of hematopoietic donor and evaluation of chimerism after allograft BMT is crucial. Thus, development of an accurate, sensitive, and fast method for confirming the lymphoid and myeloid donor engraftment is a major interest.

PCR techniques using a set of amelogenin gene primer were performed on whole WBC and/or BMA samples, and in some cases on isolated T-cell and granulocyte subsets of recipient patients. Since the amelogenin gene is present on both X- and Y-chromosomes, samples from female subjects were expected to show a single PCR product and samples from male subjects were expected to show two PCR products of equal intensity (ratio 1:1).

In the current study, we were interested in the applicability and predictive value of amelogenin marker in the clinical setting for quantitative assessment of mixed chimerism after hematopoietic SCT. In order to simulate different clinical situations and to test the linearity, precision and sensitivity of the amelogenin-PCR for quantitative assessment of mixed chimerism in sex-mismatched SCT patients, cell mixtures of male and female individuals, covering the whole range between 0.5% and 80% were prepared. In this experiment, a linear correlation was found between the proportion of cells mixed and the calculated ratio of the donor and recipient according to the peak area of the corresponding bands. A minor cell population representing 1 – 2% was reproducibly detected in several independent experiments. This level of sensitivity and reproducibility was not limited to male patients with female donor or vice versa; any male/female cell mixture could be analyzed. The test was especially informative and useful when we were monitoring female or male patients in process of rejecting or engraftment of their grafts (Figure 5).

The feasibility of the assay and the accuracy of
quantitative value obtained with amelogenin marker were compared with the STR-PCR analysis. In a separate experiment, multiplex PCR amplification systems were developed using well-characterized, polymorphic STR loci. Nine STR loci (D4S2366, D16S539, TH01, D13S317, TPOX, CSF1PO, VWA, FES/FPS, F13A01, D7S820, and CSF1PO) were combined to generate three triplex STR systems for rapid, accurate, and reliable analysis (data submitted for publication). To each system, the amelogenin primer set was also added.

The multiplex STR-PCR systems with amelogenin marker were performed on 30 sex-mismatched recipient pairs. Comparison of values obtained with amelogenin PCR analysis in patients transplanted from sex-mismatched donor showed an excellent correlation with the STR-PCR results. Actually, the use of multiplex STR alleles together with amelogenin marker increase the reliability of the results and assay becomes more informative.

Today, this test is routinely used, alone or in combination with multiplex STR systems, at our

### Figure 3.

Example of engraftment and rejection in two allogeneic SCT patients by multiplex PCR. The SCT multiplex PCR using amelogenin and STR markers was performed to analyze patient's PB samples pre- and post-SCT. a) Example of engraftment in a patient with CML. b) Example of graft rejection in a patient with ALL. D=donor; R=recipient; M=male; F=female.
Amelogenin as a marker of hematopoietic chimerism following blood SCT

BMT research center to monitor engraftment and to determine chimerism in SCT patients. More than 51% of our BMT-patients were sex-mismatched patients. In some cases, detection of mixed chimerism was predictive of relapse. In 5% of the cases, when there was no pre-BMT sample from either donor or recipient, the applicability of this assay became crucial to our treating physicians. In

Figure 4. Electropherogram of the amplification products of samples from a female donor and a male recipient before transplantation and a sample from recipient after BMT. A) The multiplex PCR using amelogenin and STR markers was performed to analyze patient’s PB samples pre- and post-SCT. B) Electropherogram of the amplified products of samples which shows peak intensity in arbitrary units. Amplification of the marker AMGX resulted in one peak in female corresponding to the X chromosomes. Amplification of the markers AMGX and Y resulted in two peaks in male corresponding to the X and Y chromosomes. Amplification of all autosomal STR markers used in this set resulted in a normal heterozygous PCR pattern. The DNA samples were heterozygous for D4S2366 and D16S539 in donor (D) and recipient (R) samples. The STR marker D4S2366 and amelogenin marker both revealed an informative PCR results in post-SCT sample.

Figure 5. Example of disease relapse in a ALL patient after SCT by amelogenin marker.
the majority of patients, amelogenin marker in combination with STR markers increases the reliability of the results and the assay becomes more informative, as confirmed by clinical outcome.

In conclusion, we tried to present a novel approach using a PCR amplification of the amelogenin locus for the rapid detection of chimerism in patients who had sex-mismatched SCT. The result shows that the use of amelogenin marker allows the rapid, reproducible, and accurate quantification of mixed chimerism. Comparison of values obtained with amelogenin PCR analysis in patients transplanted from sex-mismatched donor showed an excellent correlation with the STR-PCR results. Taken together, the application of amelogenin marker alone or in combination with STR system can be used for relative quantitative analysis of mixed chimerism and for observing kinetics of engraftment in patients who have sex-mismatched SCT.

References