Genetic Differences by Platelet-Specific Antigens Used for Monitoring Allomyelotransplant Engraftment

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Monitoring of allomyelotransplant engraftment is essential for evaluation of the function of transplanted cells and timely diagnosis of disease relapse. The possibility of using differences by HPA genes for monitoring of survival of transplanted hemopoietic stem cells was studied. Differences by HPA genes in HLA-identical sibs can be used for the analysis of chimerism after myelotransplantation.

Key Words: *hemopoietic stem cell transplantation; monitoring of engraftment; chimerism; HLA identical sibs; HPA genes*

Transplantation of hemopoietic stem cells (HSC) is widely used in the treatment of oncological diseases. In practical hematology stem cells are transplanted primarily to patients with hemoblastosis, but this treatment was also used in other blood diseases. Complete engraftment of transplanted stem cells promotes their long functional activity (recovery of hemopoiesis and immune system).

After allogenic transplantation of HSC, the recipient becomes a hemopoietic chimera and carries cells from genetically foreign donor. Informative differences between the donor and recipient are used during the posttransplantation period for evaluation of the type of hemopoietic chimerism, monitoring of engraftment, evaluation of transplanted cells function, and timely diagnosis of disease relapse [23]. Several types of chimerism are distinguished, with different prognostic significance for the clinical status of the recipient [11]. Recipients whose hemopoietic and lymphoid cells during the posttransplantation period belong to the donor are regarded as those in a state of complete chimerism [13]. Circulation of both own and donor cells after HSC transplantation without hematological signs of relapse or graft rejection is determined as a state of stable mixed chimerism, which, as a rule, precedes the onset of complete donor chimerism [14]. Mixed chimerism can be a result of circulation of patient's own normal hemopoietic cells or residual leukemic clone [24]. Dynamic follow-up of informative markers is essential for evaluation of patient's status after HSC transplantation. Quantitative increase in recipient's markers usually indicates the relapse of the underlying disease.

Detection of genetic differences between donor and recipient by polymorphic gene sites or their protein products precedes identification of chimerism after HSC transplantation. If it is impossible to evaluate chimerism directly at a molecular or biochemical level, the differences can be detected at the level of final products, for example, terminal sugars (blood groups) [7,8,10,25]. Erythrocyte phenotyping is a simple, rapid, and highly sensitive procedure (0.04-3.00%) [15]. A disadvantage of this approach is possible distortion of the results due to erythrocyte transfusions during the posttransplantation period. Long-term circulation of erythrocytes in the recipient blood (up to 30 days) makes impossible evaluation of engraftment at a certain period. In addition, there is a lag between recovery of hemopoiesis evaluated by peripheral blood erythrocyte markers and HSC engraftment in the bone

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marrow determined at the molecular level by genes encoding glycosyltransferase synthesis. Hemopoiesis recovery in the bone marrow anticipates the emergence of donor phenotype erythrocytes in the peripheral blood by 1-3 months [21].

Cytogenetic analysis is widely used for detecting differences between donor and recipient cells by sex chromosomes. Standard G-differential staining of chromosomes shows chromosome aberrations or constitutive peculiarities of donor or recipient before and after HSC transplantation [22]. The disadvantage of the method is long procedure, obligatory presence of dividing cells, high percentage of false-positive results, low sensitivity (5-10%), and difficult performance. The advantage of cytogenetic analysis is convenient monitoring of residual pathological clone in patients [20]. The study of donor-recipient differences by sex chromosomes by the quantitative fluorescent *in situ* hybridization is a simple highly sensitive method requiring small volumes of material for the analysis [1,2,6,9].

High sensitivity of PCR-based methods detects any number of cells of different populations. Molecular monitoring of chimerism is particularly important in the absence of cytogenetic markers of the disease.

PCR-studies of genetic variations in non-coding DNA sequences (microsatellites, short tandem repeats, variability in the number of tandem repeats, *etc.*) revealed individual differences in humans, which should be taken into consideration in evaluation of chimerism [3-5,16-18,26,28]. Real-time PCR is used for dynamic quantification of recipient DNA after HSC transplantation. The method is based on the use of fluorescein-labeled sequence-specific probes placed between the direct and reverse primers. During the synthesis of daughter DNA the fluorescent probes is cleaved and the fluorescent signal is emitted and analyzed using a special device. Real time PCR is commonly used for evaluating individual differences in diallele markers [12].

Restriction fragment length polymorphism analysis is the method of choice for identification of chimerism after HSC transplantation. The method is based on donor-recipient differences in restriction sites for specific bacterial fragments (restrictases). This method is sensitive for evaluation of the type of chimerism during the early posttransplantation period, because DNA is isolated from all nuclear cells [20].

As many markers (labels) as possible should be used for identification of cell nature and DNA source, because informative genetic differences can be found in only 80% donor-recipient pairs of close relatives. In order to evaluate the donor-recipient differences, 15 highly incident gene allele variants have to be identified in 98% cases.

Gene polymorphism based on single nucleotide substitution is the main source of human genome variety [19]. Polymorphism of HPA genes (human platelet antigens) is mainly due to replacement of a single nucleotide in the structure of allele-specific sites. Phenotypical expression of HPA system antigens is determined by several genes located in the long arms of chromosomes 5, 17, and 22 (HLA antigens are located on chromosome 6). In accordance with the genetic laws, gene location at different chromosomes suggests their independent inheriting. Hence, HLA-identical sibs can differ from each other by HPA genes. We hypothesize that HPA genetic differences can be used for monitoring of allomyelotransplant engraftment.

MATERIALS AND METHODS

PCR was carried out using peripheral blood leukocyte and bone marrow nuclear cell DNA after selective erythrocyte lysis. DNA was isolated using special filters by the method offered by Protrans company. DNA concentration and purity were evaluated on a spectrophotometer: one optical density unit (OD) corresponded to 50 μg/ml, purity corresponded to the ratio of parameters at λ=260 nm and λ=280 nm (OD260/OD280). DNA with OD260/OD280 equal to 1.65-1.80 was used in the reaction.

PCR with allele-specific primers (Protrans) was carried out according to manufacturer's instruction. Matrix reaction mixture consisted from 46 μl buffer P with oligonucleotides, 94 μl buffer Y with salts, and 1 μl Taq polymerase (Bio-Rad). After ingredients were thoroughly mixed, 10 μl mixture was put into a strip well (negative control). Then 34 μl DNA sample (50-150 ng/μl) was added to the tube with the main mixture and thoroughly mixed. The reaction mixture (10 μl/well) was put into Domino HPA System strip wells with lyophilized allele-specific primers on the bottom. The strips were centrifuged at 500*g* for 5 min for more complete contact of primers with the reaction mixture. The amplification program consisted of 30 cycles. Primary denaturing of DNA was carried out at 94°C for 2 min, subsequent ones for 10 sec. Primer annealing and synthesis of the daughter DNA was carried out at 65°C for 1 min (10 cycles), while the conditions of subsequent 20 cycles were as follows: 61°C, 50 sec (annealing); daughter DNA synthesis at 72°C (30 sec).

The results were interpreted in UV (λ=312 nm) after DNA staining in 1% ethidium bromide and

electrophoresis in 2% horizontal agarose gel (20 min, 200 V). Amplification products were seen as red-orange bands. The results of amplification of internal control were taken into account. The reaction was considered correct if two bands were obtained (internal control and test DNA sample am-

TABLE 1. HPA Label for Evaluation of Engraftment of Closely Related Allomyelotransplant

No.	HLA-identical sibs	HPA genotype	HPA gene label in recipient after HSC transplantation
1	Ab-va (pt, f) Ab-v (brother)	1a/**a**, -2a/**a**, -3a/**b**, -5a/**a** 1a/**b**, -2a/**b**, -3a/**a**, -5a/**a**	Emergence of $1b^+$-, $2b^+$- and disappearance of $3b^+$-cells
2	T-v (pt, m) T-v (brother)	1a/a, -2a/a, -3a/**a**, -5a/**b** 1a/a, -2a/a, -3a/**b**, -5a/**a**	Emergence of $3b^+$- and disappearance of $5b^+$-cells
3	Kur-na (pt, f) Kur-na (sister)	1a/**b**, -2a/a, -**3a**/**a**, -5a/a 1a/**a**, -2a/a, -**3b**/**b**, -5a/a	Emergence of $3b^+$- and disappearance of $1b^+$-cells
4	Sht-va (pt, f) Sht-v (brother)	**1a**/**a**, -2a/a, -3a/**b**, -5a/a **1b**/**b**, -2a/a, -3a/**a**, -5a/a	Emergence of $1b^+$- and disappearance of $1a^+$, $3b^+$-cells
5	Sh-kov (pt, m) Sh-kova (sister)	1a/b, -2a/**b**, -3a/**a**, -5a/a 1a/b, -2a/**a**, -3a/**b**, -5a/a	Emergence of $3b^+$- and disappearance of $2b^+$-cells
6	Sh-rov (pt, m) Sh-rova (sister)	1a/a, -2a/a, -3a/b, -5b/b 1a/a, -2a/a, -3a/**a**, -5**a**/b	Emergence of $5a^+$- and disappearance of $3b^+$-cells
7	Drug-n (pt, f) Drug-n (brother)	**1a**/**a**, -2a/a, -3a/**a**, -5a/**b** **1b**/**b**, -2a/a, -3a/**b**, -5a/**a**	Emergence of $1b^+$-, $3b^+$- and disappearance of $1a^+$-, $5b^+$-cells
8	Mus-na (pt, f) Boch-v (brother)	1a/a, -2a/a, -3b/**b**, -5a/**b** 1a/a, -2a/a, -3**a**/b, -5a/**a**	Emergence of $3a^+$- and disappearance of $5b^+$-cells
9	Sh-na (pt, f) Zh-kin (brother)	1a/a, -2a/a, -3a/**b**, -5a/**b** 1a/a, -2a/a, -3a/**a**, -5a/**a**	Disappearance of $3b^+$- and $5b^+$-cells
10	Ab-na (pt, f) Gul-v (brother)	1a/a, -2a/a, -3a/b, -5a/**b** 1a/a, -2a/a, -3a/b, -5a/**a**	Disappearance of $5b^+$-cells
11	Kel-n (pt, m) Kud-va (sister)	1a/a, -2a/**b**, -3a/b, -5a/a 1a/a, -2a/**a**, -3a/b, -5a/a	Disappearance of $2b^+$-cells
12	Versh-na (pt, f) Versh-na (sister)	1a/a, -2a/a, -3b/b, -5a/**b** 1a/a, -2a/a, -3b/b, -5a/**a**	Disappearance of $5b^+$-cells
13	Kha-v (pt, m) B-va (sister)	1a/a, -2a/a, -3b/b, -5a/**b** 1a/a, -2a/a, -3b/b, -5a/**a**	Disappearance of $5b^+$-cells
14	M-kyan (pt, m) M-kyan (sister)	1a/a, -2a/a, -3a/**b**, -5a/b 1a/a, -2a/a, -3a/**a**, -5a/b	Disappearance of $3b^+$-cells
15	Ber-noi (pt, m) Ber-noi (brother)	1a/a, -2a/a, -3b/b, -5a/**b** 1a/a, -2a/a, -3b/b, -5a/**a**	Disappearance of $5b^+$-cells
16	Myak-v (pt, m) Myak-v (brother)	1a/**b**, -2a/a, -3b/b, 5a/a 1a/**a**, -2a/a, -3b/b, 5a/a	Disappearance of $1b^+$-cells
17	Af-va (pt, f) Af-v (brother)	1a/b, -2a/**b, -**3a/a, -5a/b 1a/b, -2a/**a, -**3a/a, -5a/b	Disappearance of $2b^+$-cells
18	Ut-nov (pt, m) Luz-na (sister)	1a/a, -2a/**b**, -3a/a, -5a/a 1a/a, -2a/**a**, -3a/a, -5a/a	Disappearance of $2b^+$-cells
19	Zim-rev (pt, m) Zim-rev (donor)	1a/**b**, -2a/b, -3a/a, -5a/a 1a/**a**, -2a/b, -3a/a, -5a/a	Disappearance of $1b^+$-cells
20	Z-tsev (pt, m) M-na (sister)	1**b**/b, -2a/b, -3a/b, -5a/a 1**a**/b, -2a/b, -3a/b, -5a/a	Emergence of $1a^+$-cells
21	Ov-va (pt, f) Ov-v (brother)	1a/a, -2a/a, -3**b**/b, -5a/b 1a/a, -2a/a, -3**a**/b, -5a/b	Emergence of $3a^+$-cells
22	Zin-va (pt, f) Zin-va (sister)	1a/a, -2a/a, -3**b**/b, -5a/**a** 1a/a, -2a/a, -3**a**/b, -5a/**b**	Emergence of $3a^+$-, $5b^+$-cells
23	Sht-rev (pt, m) Sht-rev (brother)	1a/a, -2a/b, -3a/a, -5a/**a** 1a/a, -2a/b, -3a/a, -5a/**b**	Emergence of $5b^+$-cells

Note. Differences between donor and recipient HSC are shown with bold letters. pt: patient; f: female; m: male.

plification products). The results were considered specific, if the lengths of amplified DNA corresponded to the marker lengths.

Serological HLA typing was carried out in the microlymphocytotoxic test [27] with panels of specific sera (Hisans Firm and National Blood Transfusion Station, Ministry of Health of Belarus Republic).

RESULTS

The study was carried out in 78 HLA identical sibs (42 normal subjects and 36 patients with leukemia). There were 39 HLA identical pairs of brothers and sisters: patient/healthy subject (36 pairs) and 3 pairs of healthy sibs. Distribution analysis of HPA genes showed their complete identity in 8 sibs pairs (20.51%). Nineteen pairs of sibs (48.72%) differed by one allele gene, seven (17.95%) pairs by 2 genes, and 4 pairs (10.26%) by 3 allele genes of different HPA locuses. A total of 46 HPA differences were detected. Eleven (23.91%) pairs of sibs differed by locus 1 allele genes, 7 (15.2%) by locus 2 genes, 16 (34.8%) pairs by locus 3 genes, and 12 (26.09%) pairs differed by locus 5 genes. Hence, HPA polymorphism in HLA identical siblings was due to differences mainly in locuses HPA-3, HPA-5, and HPA-1 genes.

Transplantation of HSC was carried out in 30 patients from HLA identical brother or sister, nonreactive in a mixed lymphocyte culture. The following nosological entities were observed: 12 patients with chronic myeloleukemia (chronic phase), 6 with acute oligoblastic leukemia, 3 with acute lymphoblastic leukemia, 3 with acute prolymphocytic leukemia, 4 with acute myeloblastic and acute myelomonoblastic leukemias, 1 with acute nondifferentiated leukemia, and 1 with alveolar rhabdomyosarcoma. Engraftment of transplanted donor HSC was analyzed on days 30, 60, and 90 after transplantation, every 3 months during the first year, and then every 6 months. The longest period of observation was 3 years. Donor and recipient HPA typing was carried out by 2 allele variants of genes of 6 locuses (HPA-1-HPA-5, HPA-6w) before HSC transplantation and by the gene alleles in which differences were detected after transplantation. A total of 106 bone marrow DNA samples and 36 peripheral blood cell DNA samples were analyzed.

Differences by HPA genes in comparison with donors were detected in 23 of 30 patients after HSC transplantation. Seven patients were HLA- and HPA-identical to sibs.

HPA alleles not coinciding in HSC recipient and donor were considered informative for monitoring. Disappearance of recipient markers and emergence of donor marker indicated repopulation of transplanted stem cells. Emergence of recipient markers after their absence usually indicated a relapse of the disease. The informative value of the markers could be two-directional or unidirectional (Table 1).

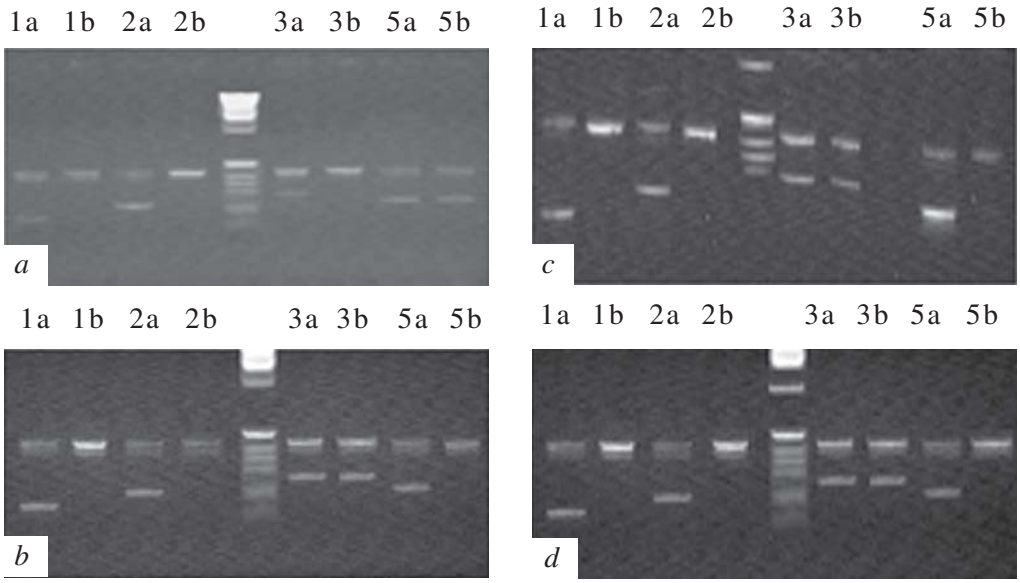

Fig. 1. Monitoring of engraftment of hemopoietic stem cells by HPA genes in patient T-v. *a*) genotype of patient T-v (m) before HSC transplantation: HPA-1a/a,-2a/a,-3a/**a**,-5a/**b**; *b*) genotype of HLA identical T-v's brother: HPA-1a/a,-2a/a,-3a/**b**,-5a/**a**; *c*) genotype of patient T-v on day 30: HPA-1a/a,-2a/a,-3a/**b**,-5a/**a** (donor chimerism); genotype of patient T-v on day 60: HPA-1a/a,-2a/a,-3a/**b**,-5a/**a** (donor chimerism).

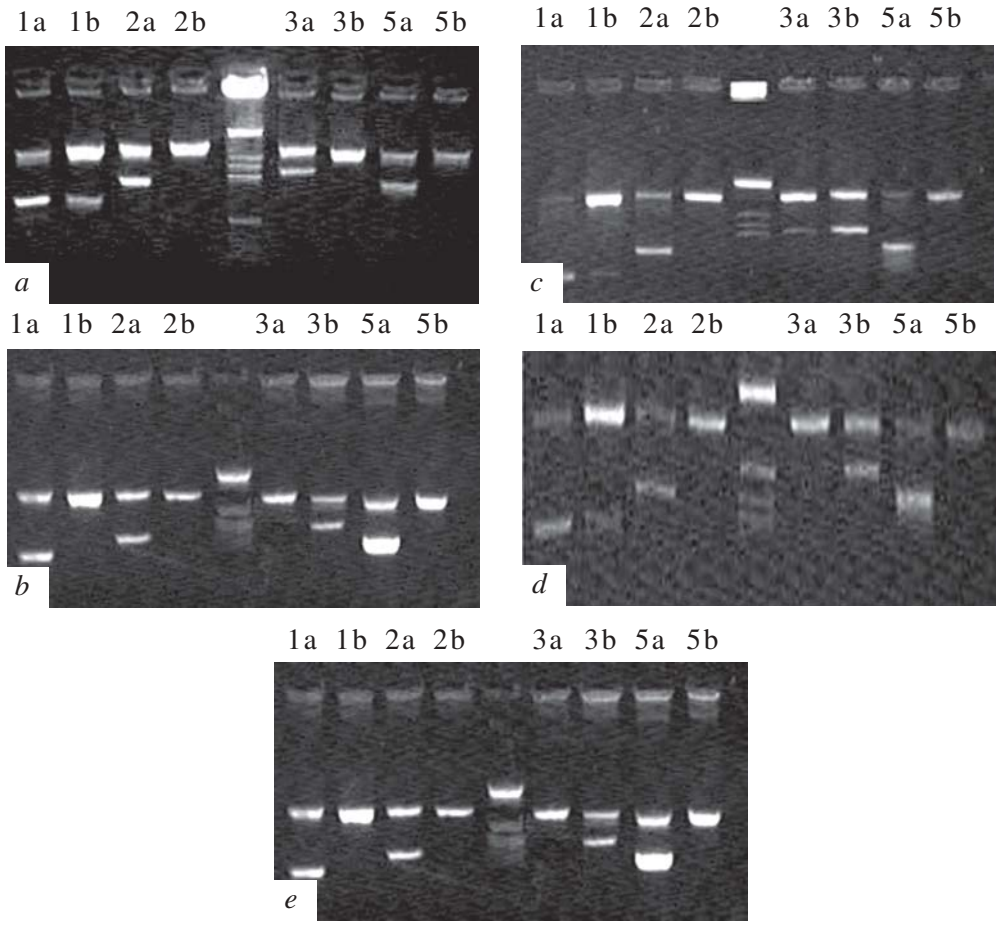

Fig. 2. Monitoring of hemopoietic stem cell engraftment by HPA label in patient Kur-na (f). *a*) genotype of patient Kur-na before HSC transplantation: HPA-1a/**b**,-2a/a,-3**a**/**a**,-5a/a; *b*) genotype of HLA-identical sister: HPA-1a/**a**,-2a/a,-3**b**/**b**,-5a/a; *c*) genotype of patient Kur-na on day 30: HPA-1**a**/**b**,-2a/a,-3**a**/**b**,-5a/a (mixed chimerism); *d*) genotype of patient Kur-na on day 60: HPA-1**a**/**b**,-2a/a,-3**b**/**b**,-5a/a (mixed chimerism); *e*) genotype of patient Kur-na on day 90: HPA-1**a**/**a**,-2a/a,-3**b**/**b**,-5a/a (donor chimerism).

Two-directional markers were detected in 8 sibs pairs (Nos. 1-8). The siblings differed by allele genes of several locuses or genes of the entire HPA locus and were homo- or heterozygotic by differing alleles.

Unidirectional marker was detected in 11 patients (Nos. 9-19), differing from HSC donor by one allele HPA gene (the patient was heterozygotic by allele HPA gene, while the donor had the homozygotic variant of this gene). The type of chimerism could be determined by disappearance of cells with recipient HPA marker.

In 4 cases (Nos. 20-23) we detected differences by HPA alleles between siblings, when the patient was homozygotic by an allele and only the heterozygotic donor marker could emerge during repopulation of transplanted stem cells. In this case it was difficult to define the type of chimerism by only molecular methods. The findings of these studies were to be compared with the data of cytological and cytogenetic analysis of the bone marrow.

Hence, the detected marker could be used for monitoring HSC engraftment by the molecular method alone in 19 of 23 patients genetically differing from donors by HPA.

Patient T-v (m) had a genotype HPA-1a/a,-2a/a,-3a/a,-5a/b, his HLA-identical brother had a genotype HPA-1a/a,-2a/a,-3a/b,-5a/a (pair No. 2). Allomyelotransplant engraftment was monitored by the appearance of cells with donor HPA-3b-allele and disappearance of recipient HPA-5b gene. On days 30 and 60 the type of donor hemopoiesis was determined by the analysis of bone marrow nuclear cell DNA (Fig. 1).

Patient Kur-na (f) differed from her sister (bone marrow donor) by HPA-1 locus allele gene and the entire HPA-3 locus genes. Patient's genotype was HPA-1a/b,-2a/a,-3a/a,-5a/a, donor's genotype was HPA-1a/a,-2a/a,-3b/b,-5a/a (pair No. 3). Engraftment of transplanted stem cells could be evaluated by disappearance of HPA-1b and HPA-3a alleles and appearance of HPA-3b gene. On day 30 the

presence of recipient's markers HPA-1b, HPA-3a, and donor marker HPA-3b was determined in DNA samples from patient's bone marrow nuclear cells; on day 60 only recipient marker HPA-1b was detected, indicating decreasing mixed chimerism. On day 90 HPA genotype of bone marrow nuclear cells corresponded to the donor one (HPA-1a/a,-3b/b), that is, donor chimerism was identified (Fig. 2). The period of observation was 3 years; the patient was in a clinical hematological remission.

Before HSC transplantation the genotype of patient Sht-va (f) with acute nondifferentiated leukemia was defined as HPA-1a/a,-2a/a,-3a/b,-5a/a, while her brother's genotype was HPA-1b/b,-2a/a, -3a/a,-5a/a (pair No. 4). Mixed hemopoiesis type was identified at all stages of observation, which was paralleled by unstable clinical hematological remission and development of a relapse (the patient died). Identification of mixed chimerism is prognostically unimportant if there are no markers of the tumor cell clone. Only dynamic analysis of patient's bone marrow nuclear cell or peripheral blood cell DNA detects the increase or decrease in recipient's markers. The appearance of additional her own HPA-3b marker in this patient on day 60 indicated increasing mixed chimerism.

Hence, recipient-donor differences by HPA genes show the type of chimerism at certain periods after HSC transplantation, predict possible development of molecular relapse and clinical hematological exacerbation of the disease.

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