RESEARCH PAPER

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Utility of saliva and hair follicles in donor selection for hematopoietic stem cell transplantation and chimerism monitoring

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Selection of an HLA identical donor is a critical pre-requisite for successful hematopoietic stem cell transplantation (HSCT). Most transplant centers utilize blood as the most common source of DNA for HLA testing. However, obtaining blood through phlebotomy is often challenging in patients with conditions like severe leucopenia or hemophilia, pediatric and elderly patients. We have used a simple in-house protocol and shown that HLA genotypes obtained on DNA extracted from saliva or hair are concordant with blood and hence can be used for selection of donors for HSCT or organ transplantation. Similarly, for post-HSCT chimerism monitoring, non-availability of pre-transplant DNA samples poses a major limitation of reference STR fingerprints. This study shows that DNA obtained post-HSCT from hair follicles can be used to generate pre-transplant patient specific fingerprints while the STR profiles obtained in saliva samples cannot as these display a mixed state of chimerism.

Introduction

Allogeneic hematopoeitic stem cell transplantation (HSCT) is the preferred treatment for several hematological diseases, metabolic disorders and a variety of immunodeficiencies. The process of HLA matching for selection of a suitable donor for HSCT is a key step that requires low to high resolution HLA genotyping using multiple molecular technologies and appreciable laboratory expertise. Peripheral venous blood is the most preferred source of DNA for such genetic testing due to high yields (~10–15 ng/μl of whole blood) and customary practice. However, alternates to phlebotomy might be quite useful for the pediatric population, as well as for patients with severe aplastic anemia (SAA) or hemophilia.

One essential component of evaluating the success of an HSCT outcome is the post-transplant chimerism based monitoring of engraftment and an early detection of rejection and/or molecular relapse. This involves STR/VNTR based analyses similar to the DNA fingerprinting and is a highly sensitive technique that can quantify percent donor cells up to 0.1%. The major advantage of the technique is the ability to detect an early molecular relapse, and thus help in instituting timely donor lymphocyte infusions (DLI). Chimerism testing relies upon comparing fingerprints of post-HSCT recipient with that of the donor and that of pre-transplant patient sample every time a test is performed. However, in practice, the pre-transplant patient-derived DNA sample is often not available or may be insufficient for recurrent testings at different post-HSCT time points. In such situations, alternate sources of DNA like buccal swabs or mouthwashes have been tried, but these are not always suitable because these tissues get gradually replaced by donor-derived cells following transplantation.[1]

These limitations encouraged us to examine alternative sources of DNA that could be adopted for donor recipient HLA matching and/or chimerism testing. Isolation of DNA from multiple sources including buccal epithelial cells,[2-4] mouthwashes,[5,6] formalin-fixed paraffin embedded tissues,[7] sperm cells,[8] saturated spit wads,[9] saliva,[8,10] buccal swabs[11] and treated cards[2] have often been tried for epidemiological studies and forensic applications. None of these have, however, been examined extensively for transplant related applications like HLA analysis for donor selection or chimerism evaluation. Although saliva has been used as a common source of DNA, limited information is available on the suitability of DNA obtained from saliva for HLA testing,[12] or from hair for HLA class II typing,[13] there are no published reports, to the best of our knowledge, on using such alternate sources of DNA for donor selection for HSCT in clinical practice.

Recently, studies by Hong et al.,[14] Rovo A et al.[15] and Seifert B et al.[16] have shown that the hair follicles are devoid of plasticity unlike adult stem cells and that these may serve as a reliable source

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of recipient profile in the absence of pre-transplant reference DNA during post-HSCT evaluation. More recently, however, Jacewicz and coworkers,[17] using a relatively sensitive RQ-PCR assay system, suggested that hair follicles are not devoid of transdifferentiation and that these may possess cells of both recipient as well as the donor origin. It therefore appears plausible to examine the advantage of using hair as an alternate and useful source of DNA for chimerism monitoring. In this study, we have sought to investigate the suitability of two alternate sources of DNA, saliva and hair follicles for donor-recipient HLA matching and for chimerism monitoring in patients undergoing allogeneic HSCT. The study showed that both these sources provide sufficient quantity and quality of DNA suitable for HLA genotyping for routine donor selection and may actually be convenient resources to supplement unrelated donor search activities. Further, for STR based chimerism monitoring, DNA obtained from hair follicles (but not saliva) could be utilized as a pre-transplant patient reference since hair follicles retained their original genomic integrity following successful donor HSC engraftment. On the contrary, saliva or mouthwashes contain a mixture of cells including lymphocytes as well as epithelial cells and hence are more likely to develop mixed state of chimerism and is thus not suitable to obtain information on the pre-transplantation genotype of the recipient.[18] These observations are relatively debatable and remain an issue of technique specificity and sensitivity, probable detection limits and the levels of chimerism (or micro-chimerism) of the donor constituent cells.

Results

DNA preparations. A comparison of the OD 260/280 and 260/230 absorbance ratios and the yield of DNA preparations obtained from three different sources, namely, blood, saliva and hair follicles is shown in **Table 1**. The average 260/280 and 260/230 ratios of all the samples tested were corrected for background absorbance at 320 nm and were found to be in the range of 1.69 to 2.13 and 1.8 to 2.98 respectively, indicating that preparations were free from protein and polysaccharide contaminations respectively. The overall yield of DNA obtained was comparable in all the three sources i.e., saliva (39.4–96.3 μg/ml in healthy and 1.17–114 μg/ml in patients), peripheral blood (9.46–57.45 μg/ml in healthy and 2.45–69.23 μg/ml in patients) and hair (0.1–0.9 μg/6 hairs in healthy and 0.1–0.8 μg/6 hairs in patients).

A comparison of DNA preparations electrophoresed in 0.8% agarose gels confirmed an overall integrity, good quality and quantity in all isolations whether prepared from peripheral blood, hair or saliva.

HLA typing. HLA class I and class II typing was performed using three molecular techniques viz., PCR-SSP, reverse line strip hybridization (RLS) and reverse SSO by Luminex. A representative PCR-SSP gel image for HLA-A typing using blood, saliva and hair simultaneously in one of the individuals is shown in **Figure 1**. Similar typing results were obtained by RLS hybridization, and reverse SSO techniques. All the three DNA sources tested, i.e., peripheral blood, saliva and hair follicles gave absolutely consistent HLA typing results in each individual and irrespective of the original source of DNA utilized (data not shown).

Chimerism monitoring. Well resolved STR based chimerism profiles were obtained with all the patient DNA samples tested and these were not affected whether isolated from hair (**Fig. 2**) or saliva (**Fig. 3**) or peripheral blood (**Figs. 2 and 3**). Gene Mapper analysis showed sharp STR allele specific peaks and there were no stutters or nonspecific amplifications in all the tests performed.

Hair-derived DNA retain pre-HSCT recipient fingerprints. An example of the hair-derived STR profile corresponding to FES marker and its comparison with blood-derived profile in a patient with Thalassemia major at d +153 post-HSCT is shown in **Figure 2**. The STR profile seen in recipient's blood post-transplant was completely the same as the donor (allele peaks 220 and 224 bp) (**Fig. 2C and D**). This is in contrast to the profile obtained in the hair sample (216 and 220 bp peaks) (**Fig. 2B**) which showed similarity to the original pre-recipient blood reference profile (**Fig. 2A**) instead of the donor (**Fig. 2C**). Similar observations were made in all other patients suggesting that hair, unlike saliva, retained the original patient profile and could be utilized as a pre-transplant reference for chimerism studies employing STR based Genescan analyses on automated DNA sequencer.

Saliva-derived DNA exhibit mixed chimerism fingerprints. **Figure 3** illustrates an example of chimerism profile obtained in a CML patient at d +1861 post-HSCT with 100% donor chimerism in peripheral blood (**Fig. 3C**). A saliva sample was collected from this patient and processed in parallel with the blood and was found to possess peaks corresponding to both patient (222 + 232 bp) and donor (226 + 232 bp) specific profiles (**Fig. 3D**). As expected, the saliva being composed of lymphocytes as well as epithelial and other cell types, displayed a mixed profile of pre- and post-transplant STR fingerprints. Hence, the saliva-derived DNA may be utilized for HLA typing purposes but cannot substitute for a pre-recipient DNA reference sample for chimerism analysis.

Simultaneous analyses of post-transplant blood, hair and saliva fingerprints. In this study, 18 patients out of 24 had 100% donor chimerism while six patients had mixed chimerism based on blood-derived fingerprints at various time points ranging from d + 55 to d + 1855 post-transplant as shown in **Table 1**. All these patients were tested for the status of chimerism in their hair, saliva and blood samples simultaneously and the results obtained are summarized in **Figure 4**.

As shown in **Figure 4A**, complete donor chimerism was observed in blood samples in 18 patients at different time points post-transplant. In analogy, the hair-derived profiles did not show any donor contribution in these patients and remained at 0 baseline. The saliva-derived fingerprints displayed mixed chimerism ranging from 10% to 88.7% maximum. Careful analysis of saliva STR profiles revealed a trend where the mixed chimerism profile showed a gradual skewing toward higher donor type at the later post-HSCT time points as compared with early post-HSCT period, though in different patients.

Table 1. Details of the study subjects, status of DNA yield, HLA allele typing and % donor chimerism in post-HSCT patients using DNA obtained from peripheral blood, saliva and hair follicles

S. No	AGE/SEX	STATUS	d + Post-transplant	% Donor chimerism in			Blood			Saliva			Hair		
				Blood	Hair	Saliva	A 260/280	A 260/230	Concentration (μg/ml)	A 260/280	A 260/230	Concentration (μg/ml)	A 260/280	A 260/230	Concentration (μg/ 6 hair)
1	26/M	Healthy	-	-	-	-	1.75	2.82	27.39	1.7	1.91	47	1.79	2.46	0.6
2	30/M	Healthy	-	-	-	-	1.78	2.41	12.38	1.9	2.15	39.4	1.78	2.98	0.2
3	23/F	Healthy	-	-	-	-	2.01	1.97	42.35	1.89	2.05	89.2	1.76	2.05	0.5
4	40/F	Healthy	-	-	-	-	1.79	2.72	9.46	1.84	1.98	96.3	1.7	2.39	0.1
5	26/M	Healthy	-	-	-	-	1.74	2.89	57.45	2.06	2.06	88.2	1.74	2.4	0.9
6	38/M	Healthy	-	-	-	-	1.77	2.1	38.65	1.9	1.9	46.2	2.13	2.7	0.6
			Mean (Range)				1.81 (1.74–2.01)	2.49 (1.97–2.89)	31.28 (9.46–57.45)	1.88 (1.7–2.06)	2.01 (1.9–2.15)	67.72 (39.4–96.3)	1.82 (1.7–2.13)	2.5 (2.05–2.98)	0.48 (0.1–0.9)
7	18/M	CML	98	100	0	12.3	1.72	2.45	13.5	1.78	2.12	108.1	1.73	2.67	0.2
8	39/M	CML	440	100	0	26.8	1.81	2.54	36.73	1.87	1.81	55.74	1.7	2.97	0.6
9	32/M	CML	255	100	0	18.6	1.7	2.78	57.83	1.87	2.11	86.85	1.95	2.64	0.4
10	7/M	ALL	55	100	0	7.8	1.77	2.69	27.31	1.9	1.92	74.3	1.86	1.96	0.1
11	24/M	ALL	700	100	0	68.8	1.75	2.7	30.63	1.96	2.3	98.6	1.76	2.58	0.3
12	22/F	AML	77	100	0	11.2	1.76	2.98	69.23	1.89	1.87	90.2	1.87	2.69	0.2
13	49/M	AML	180	100	0	15.5	1.72	2.75	26.80	1.76	2.02	85.3	1.96	2.05	0.4
14	32/F	SAA	482	100	0	28.3	1.79	1.98	4.68	1.72	1.98	114	1.79	2.86	0.6
15	18/M	SAA	512	100	0	32.1	1.78	2.73	2.45	1.77	2.33	89	1.83	1.95	0.1
16	12/M	SAA	70	100	0	10.5	1.78	1.97	6.9	2.01	2.87	68.87	1.79	2.36	0.15
17	34/M	CML	1855	100	0	88.7	1.84	1.95	21.4	1.76	1.93	1.17	2.03	2.52	0.38
18	44/M	SAA	4140	68.2	0	72.86	1.91	2.0	6.4	2.03	2.22	4.36	2.06	1.98	0.45
19	21/M	ALL	207	97	0	54.8	1.84	2.2	10.9	1.92	1.98	1.59	1.73	1.88	0.48
20	22/M	SAA	1749	22.75	0	14.26	1.75	2.1	25.1	2.02	2.32	15.6	1.98	2.12	0.8
21	35/M	SAA	68	100	0	9.2	1.88	2.22	33.2	2.1	2.12	5.6	1.8	2.1	0.3
22	12/F	Thal maj	152	46.9	0	12.9	1.9	1.98	45.1	1.87	2.1	10.4	1.7	2.2	0.2
23	13/M	Thal maj	400	100	0	25.1	1.78	1.87	36.7	1.9	1.97	12.5	1.78	2.32	0.33
24	13/F	CML	630	100	0	66	1.76	1.88	45.8	1.77	1.9	20.5	1.8	2.24	0.26
25	17/M	SAA	507	100	0	30.4	1.89	2.24	29.8	1.86	2.0	33	1.9	1.98	0.29
26	13/M	Thal maj	153	100	0	10	1.9	2.11	22.6	1.89	2.2	15.6	1.97	2.1	0.7
27	35/M	SAA	109	78.86	0	42.9	1.85	1.97	26.7	1.92	2.1	22.5	1.88	2.1	0.23
28	10/M	SAA	414	85.1	0	45.2	1.79	1.98	35.7	1.97	1.8	23.4	1.7	2.2	0.25
29	39/F	AML	1143	100	0	88.7	1.99	2.14	39.5	1.96	2.2	32.5	1.88	1.88	0.65
30	39/F	CML	1181	100	0	88.1	1.69	2.0	32.4	1.99	2.1	25.6	1.87	2.0	0.55
		Mean (Range)		91.62 (22.75–100)	0	36.71 (7.8–88.7)	1.81 (1.69–1.99)	2.26 (1.87–2.98)	28.64 (2.45–69.23)	1.9 (1.72–2.1)	2.09 (1.8–2.87)	45.64 (1.17–114)	1.85 (1.7–2.06)	2.26 (1.88–2.97)	0.37 (0.1–0.8)

OD ratios corrected with absorbance at 320 nm. CML, chronic myeloid leukemia; AML, acute lymphoid leukemia; SAA, severe aplastic anemia; Thal maj, thalassemia major.

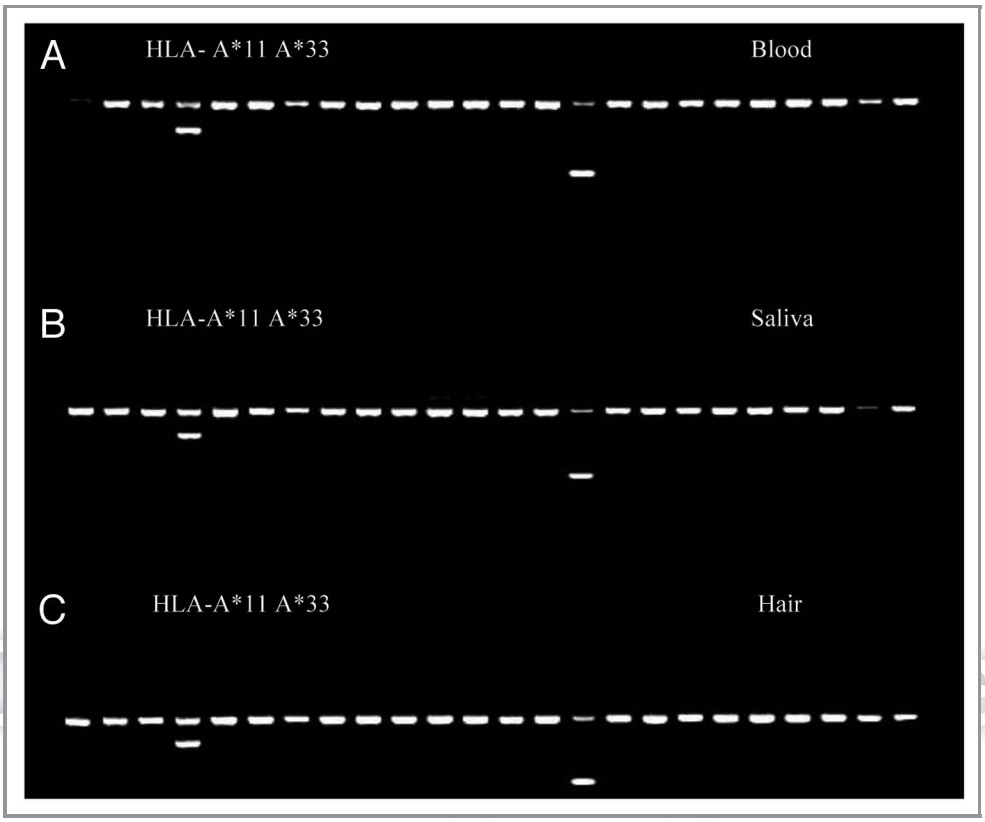

Figure 1. An agarose gel (2%) showing representative SSP based typing of HLA-A alleles using DNA samples isolated from (A) peripheral blood, (B) saliva and (C) hair in the same individual.

In patients with mixed chimerism prevailing in blood (**Fig. 4B**), a mixed profile was also seen in their saliva samples. However, the relative percentage of the donor chimerism was relatively lesser in the DNA obtained from saliva than that of the blood in all the patients except one on d + 4140 where it was marginally greater in saliva-derived DNA than that of blood (**Fig. 4B and Table 1**).

Discussion

The main impetus for this study came from difficulties encountered frequently with peripheral blood sample collection in a variety of situations particularly when dealing with patients with low total leukocyte counts. This often led to inadvertent delays in histocompatibility testing and complications in post-HSCT monitoring of engraftment. Major practical difficulties encountered include collecting enough DNA from patients with Severe Aplastic Anemia who have leucopenia or the pediatric group of patients for HLA matching purposes. Often, it is necessary to store additional aliquots of DNA to be used as a pre-transplant reference during post-HSCT chimerism monitoring and is not possible in such patients. Hence, we opted to investigate the suitability of alternative DNA sources for the above tests in these patients rather than waiting for them to recover their total leukocyte counts (TLCs) in the peripheral blood. The idea was to avoid the unwarranted delays that the latter could cause in reporting HLA typing results and subsequently the HSCT.

The study enrolled 30 volunteers, 24 of whom were patients who underwent allogeneic sibling HSCT while six were healthy individuals. Samples were collected for DNA extraction from three different sources, i.e., blood, saliva and hair in these subjects. The DNA preparations from all the three sources (blood, hair and saliva) were found to be relatively pure, of good quality and intact as confirmed both spectrophotometrically as well as by agarose gel electrophoresis. Consistent HLA typing results were obtained using SSP, rSSO and SBT (data not shown) from all three sources despite the fact that saliva harbors a substantial proportion of microbes and may be contaminated with RNA. These observations are in agreement with the findings of other studies.[12,19] Further, the protocol described is relatively cost effective as it excludes costs incurred on phlebotomy or procurement of commercial kits available for DNA isolation.

This approach of using saliva and hair follicles as alternates to phlebotomy has several advantages: (1) ideal for pediatric or elderly patients, (2) quick, easy and non-invasive, (3) excludes requirements of a trained phlebotomist, precludes trauma caused by needle pricks, (4) best suited for patients with leucopenia (SAA) or hemophiliacs, (5) offers an easy option to establish saliva/hair banks to supplement unrelated marrow donor registries particularly in low-resource settings, (6) is suitable for material transfer and storage and (7) adaptable to other genetic testing set-ups.

Saliva as a fine and acceptable source of human genomic DNA[19,20] is now well established. Our results indicate that it can

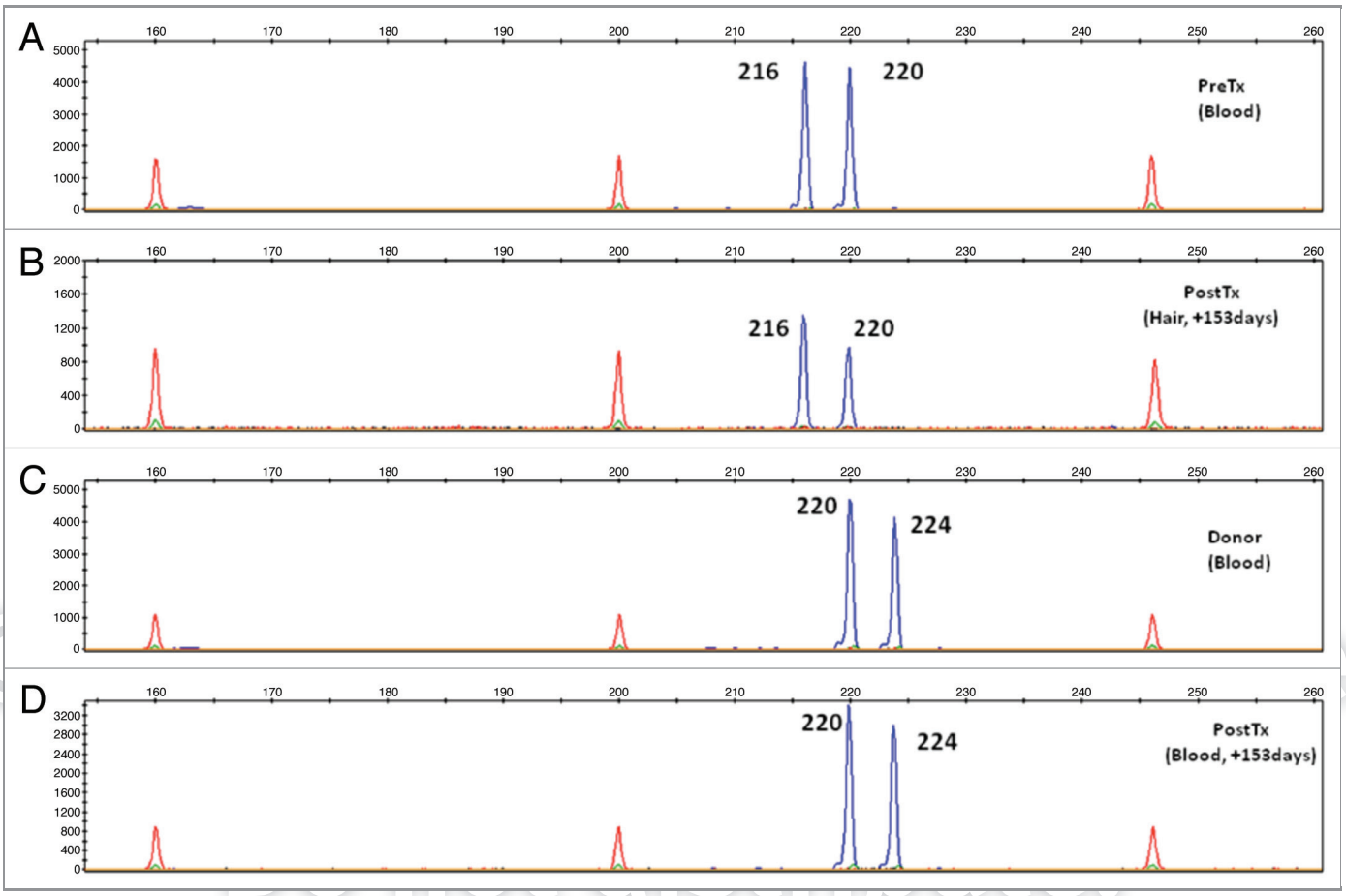

Figure 2. Hair-derived DNA retain pre-HSCT recipient fingerprints. A comparison of representative STR marker FES resolved on ABI 3130xl automated DNA analyzer using Genemapper software. Panels (A–D) show DNA fingerprint profiles monitored for a Thalassemia major patient on d + 153 post-HSCT. Panel (A) shows recipient's pre-transplant blood fingerprint (B) fingerprints on DNA obtained from post-transplant recipient's hair, (C) shows donor's blood-derived fingerprint and (D) shows the profile obtained from recipient's blood post-HSCT. Note the fingerprint of patient's hair post-transplant (B) remains the same as pre-transplant recipient (A) rather than of donor (C).

easily be adapted for HLA typing, particularly for SAA patients having low TLCs as well as pediatric patients. It is thus a useful and equally effective source of DNA, offering the added advantages of being economical, simple in collection and non-invasive, lowering the risk of infections to subjects and laboratory personnel. One of the recent applications of saliva-derived clinical genetics is testing for presence of HLA-B*57:01 corresponding to abacavir hypersensitivity in HIV positive individuals.[21] This approach is not only simple but also precludes the risk of infection among the laboratory personnel. Using saliva as a source of DNA has important relevance in genetic testing procedures since it requires minimum infrastructure for storage, transport and can be easily collected in fields and clinics. Establishing saliva (or hair follicle) banks would be most economical in low-resource settings and could prove of great advantage in supplementing HLA testing for unrelated marrow registries for selection of HLA matched donors.

Saliva contains about 8 million cells/ml, including approximately 1 million cells/ml of leukocytes. Epithelial cells comprise a major portion of cells in saliva. While saliva leukocytes post-HSCT most likely originate from donor HSCs, most epithelial cells are of recipient origin.[18] This explains largely why mixed chimerism was observed in saliva-derived DNA samples despite it being 100% donor type in blood. Further, the study showed levels of donor chimerism were relatively lower in saliva than blood and appeared to increase progressively during later time periods. Although these observations were made in different patients, it is plausible that the overall salivary cellular constituency with donor genotype emerges more gradually and slowly in kinetics than in peripheral blood. The only patient who showed a marginally greater mixed chimerism in saliva than in blood was later found to be undergoing relapse after having maintained complete donor chimerism for $>$ 11 years. This suggests that on one hand, cells with donor genotypes appear late in the buccal cavity lining, on the other hand, in the case of disease relapse following complete engraftment, these tend to lose the donor genotypes also at a slower rate than that in the blood. However, future studies would be needed to prove this hypothesis. Recently, it has been suggested that after allo HSCT, donor cells undergoing apoptosis release donor DNA packaged apoptotic

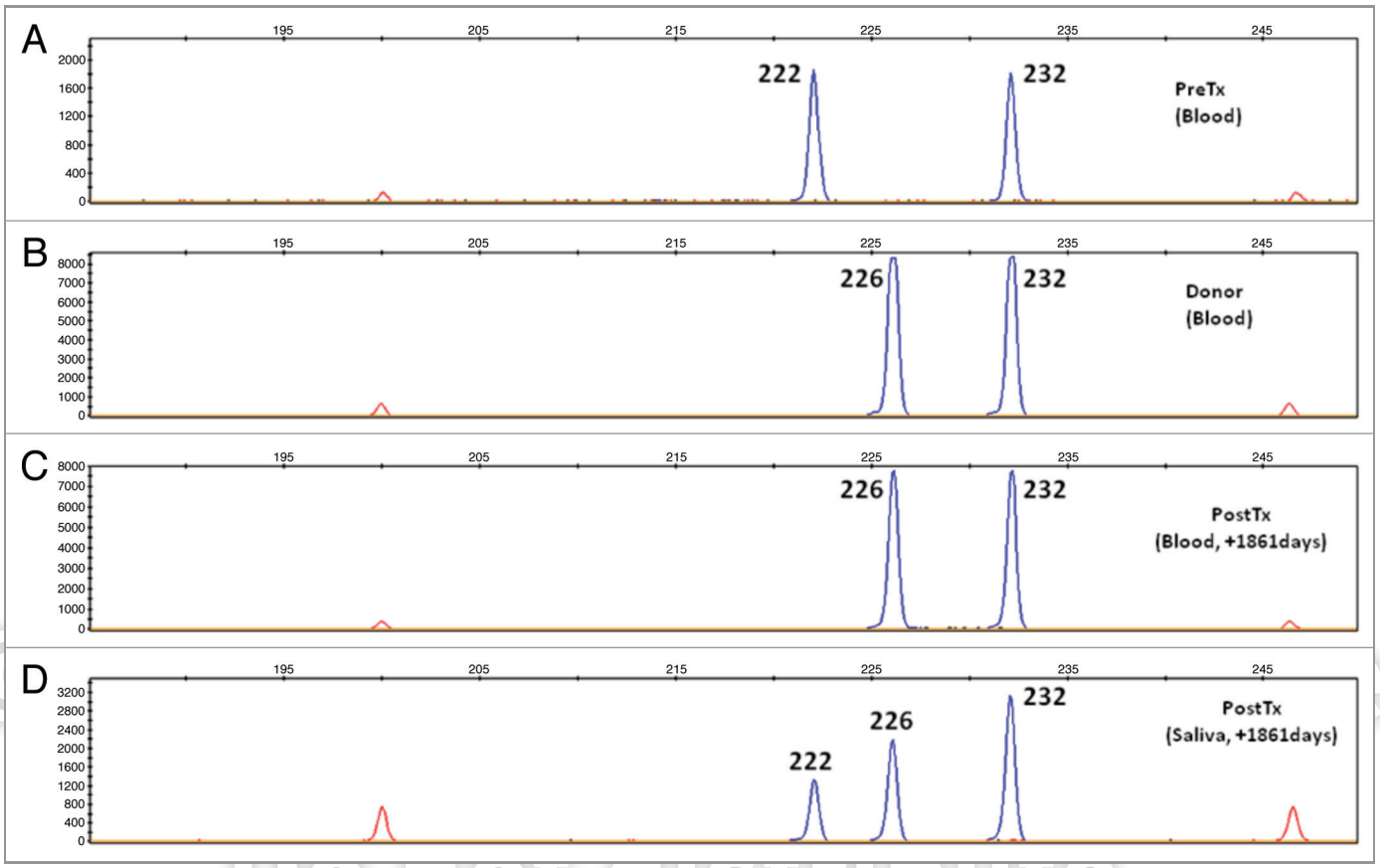

Figure 3. Saliva-derived DNA STR fingerprints display mixed chimerism. A comparison of representative STR marker D21S11 resolved on ABI 3130xl automated DNA analyzer using Genemapper software. Panels (A–D) show DNA fingerprint profiles monitored for a CML patient on d + 1861 post-HSCT. Panel (A) shows recipient's pre-transplant blood fingerprint (B) shows donor's blood fingerprint, (C and D) show the profiles obtained from recipient's blood and saliva respectively on post-HSCT d + 1861 respectively. Note the presence of both donor- and patient-derived mixed chimerism peaks in (D) corresponding to saliva sample.

bodies that are engulfed by the host epithelium and the donor-derived genome is integrated inside nuclei of host epithelium via horizontal DNA transfer hence leading to epithelial chimerism and related genomic instabilities.[22] The exact mechanisms underlying emergence of host epithelial cells with donor genotype are unclear and remain divergent.

Nonetheless, the present study indicates that while saliva-derived DNA offers a useful alternative for donor-recipient HLA matching, this source cannot be used as a reference of the original recipient profile for post-transplant monitoring of chimerism. Similar observations have been made by others.[14-16] The theories explaining this phenomenon range from homing of bone marrow cells to the injured salivary glands[20] to trans-differentiation of hematopoietic stem cells[23] and the presence of associated hematopoietic and non-hematopoietic mesenchymal, endothelial progenitors within the transplanted marrow. Salivary proteome alterations have been reported following allogeneic stem cell transplantation independent of the type of pre-conditioning regimens.[24] These data suggest that changes due to plasticity of hematopoietic donor stem cells or their horizontal DNA transfer like mechanisms can be observed frequently in saliva. Our results support these observations and conclude that saliva cannot substitute for pre-transplant recipient genetic profiling for chimerism testing.

As opposed to saliva, the DNA obtained from hair was found to retain the original recipient genetic integrity even after post-HSCT owing to the suspected lack of plasticity or other unknown mechanisms. Similar observations have been made earlier by Hong and coworkers[14] while others failed to confirm this.[17] The former studies utilized STR analyses and showed that hair lacks plasticity. The latter study, however, used highly sensitive Y-chromosome specific STRs and RQ-PCR with the sex-determining region Y (SRY) based TaqMan florescent assays and showed that the donor-specific DNA could be found in recipient's hair follicles though at very minimal levels. Understanding the underlying molecular mechanisms and verifying whether hair transdifferentiation occurs or not is currently beyond the scope of this study. Nevertheless, our results provide evidence that DNA extracted from hair follicles could be used successfully not only for donor selection but also for chimerism monitoring and as a reliable substitute for pre-transplant DNA sample when routinely utilized STR based fragment analyses protocols are adapted. Taken together, both saliva and hair appear to be excellent sources of DNA that can be used to advantage in clinical

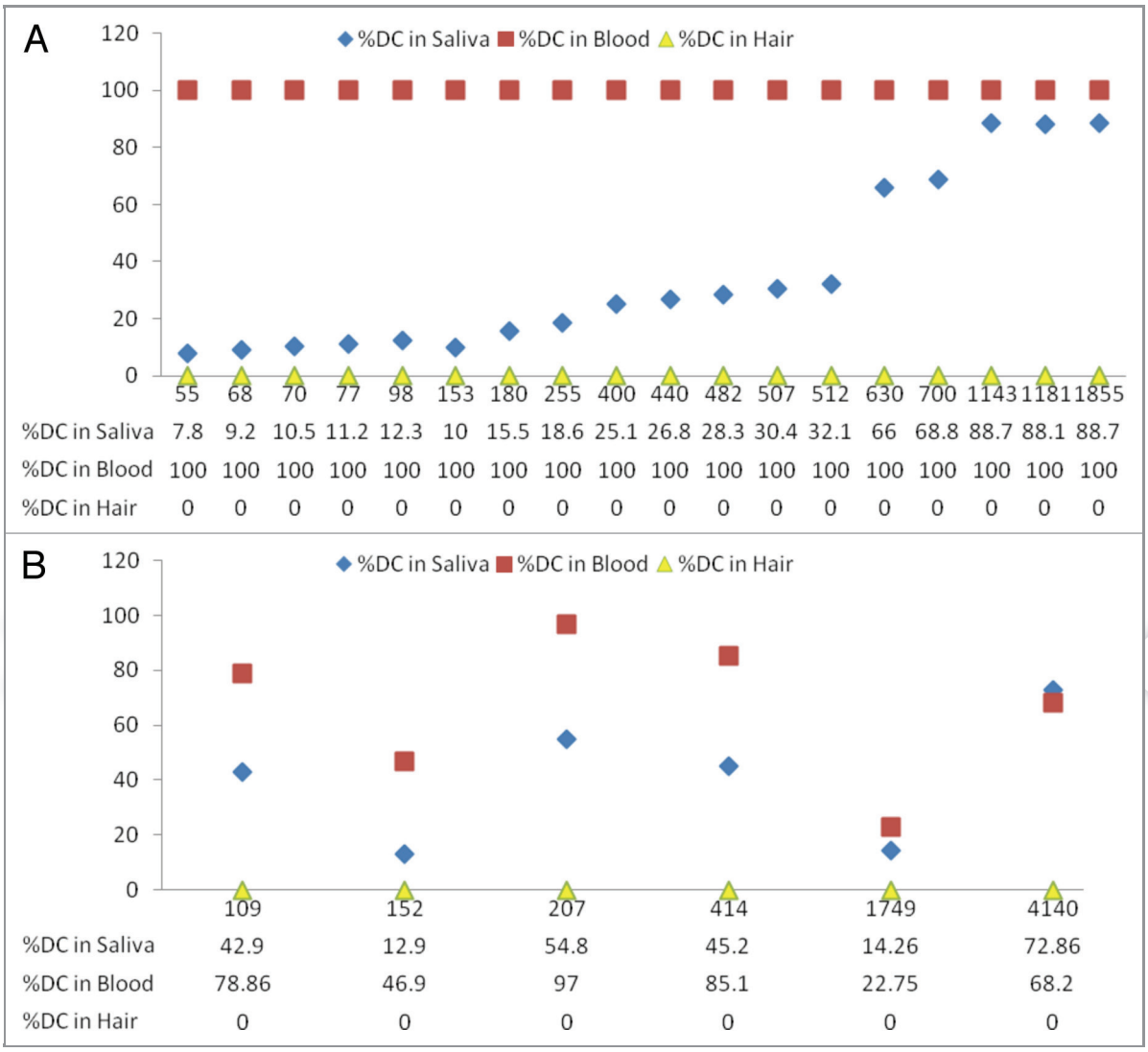

Figure 4. Relative donor chimerism (% DC) observed post-HSCT in saliva and hair in patients with (A) complete 100% DC and (B) in patients displaying mixed DC in blood.

settings for hematopoietic stem cell transplantation and particularly for situations involving pediatric patients or those with leucopenia.

Materials and Methods

Subjects. The study was performed on six healthy volunteers and 24 patients [6 chronic myeloid leukemia (CML), 3 acute lyphoid leukemia (ALL), 3 acute myeloid leukemia (AML), 9 severe aplastic anemia (SAA) and 3 thalassemia major] who underwent allogeneic sibling donor HSCT at the All India Institute of Medical Sciences, New Delhi (**Table 1**). The study volunteers were enrolled after their informed consent and as per the ethical guidelines for human experimentation laid down by the ethics committee of the All India Institute of Medical Sciences, New Delhi.

The patients were tested for chimerism at various time points post-transplant using a panel of STR/ VNTR markers as described in the following sections.

Collection of samples. *Peripheral blood.* Approximately 5 ml of blood was collected by venipuncture in K_2-EDTA tubes and mixed properly to avoid coagulation.

Saliva. Samples of saliva were collected from subjects after an hour of brushing their teeth (avoiding eating, drinking or smoking in the duration). After having rinsed their mouths briefly, 2 ml saliva was expelled into 50 ml sterile conical centrifuge tubes.

Hair. Six strands of hair with attached follicles/bulbs (determined by visual confirmation) were plucked from different areas of scalp of the subjects. The extra shaft regions of the hair were cleaved and the hair bulbs (~0.5 cm) were collected in a 2 ml Eppendorf tube containing 1 ml 1X PBS. The follicles were rinsed and washed carefully to remove any contaminating blood cells before processing for DNA extraction.

Extraction of DNA from blood. The genomic DNA was extracted from peripheral blood by the modified salting out method;[25] 5 ml of whole blood was mixed with 45 ml red cell lysis buffer (RCLB) (0.14 M ammonium chloride, 1 mM sodium

bicarbonate) and incubated for 20 min at room temperature. Following centrifugation at 2,000 rpm (900 g), the nuclear pellet was resuspended in 3 ml of nucleus buffer (0.4 M NaCl, 2 mM Na2EDTA, 10 mM TRIS-HCl, pH 8, and 0.4% SDS) and subjected to proteinase K (50 µg/ml) digestion at 42°C for 3–6 h. Proteins were precipitated by addition of 4 ml of 4 M ammonium acetate and removed by centrifugation at 3,000 rpm (2,000 g) for 30 min. The supernatant was extracted with chloroform and the DNA was precipitated with isopropanol. It was quantified spectrophotometrically, subjected to quality evaluation and processed for HLA typing.

Extraction of DNA from saliva. A slightly modified salting out procedure was used for isolation of DNA from saliva as described. The saliva sample was diluted with three volumes of PBS and vortexed for 3–5 sec. The tubes were centrifuged at 6,000 rpm in a microfuge for 5 min to pellet out nucleated cells. To this, 700 µL TNES (0.01 M Tris, 0.01 M Na2EDTA, 0.1 M NaCl, 2% SDS) and 50 µg/ml Proteinase K were added. The tubes were then incubated at 50°C overnight. After incubation, 500 µL (8 M ammonium acetate + 1 mM EDTA) and 500 µL chloroform were added and vortexed. The mix was microfuged at 10,000 rpm for 10 min to ensure separation of DNA and proteins into two distinct layers. The upper aqueous phase (containing the DNA) was transferred into a clean and sterile 2 ml Eppendorf tube containing 540 µL of isopropanol. The solutions were mixed gently by inverting the tubes gently and centrifuged at 10,000 rpm in a microfuge for 1 min so as to pellet the DNA. The supernatant was drained off and 2 ml of 70% ethanol was added and the tubes inverted several times to wash the DNA off from any remaining salts or contaminants. The pellet was air-dried of excess ethanol and re-suspended in 100 µl TE buffer (10 mM TRIS-HCl; 1 mM EDTA; pH 7.3).

Extraction of DNA from hair. The protocol used for extraction of DNA from hair was similar to the one used for blood.[26] Six hair follicles were suspended in 450 µl RCLB for 20 min and microfuged at 8,000 rpm for 3 min. The pellet was washed again with 450 µl RCLB. It was finally resuspended in 300 µl of NLB followed by 20 µl 10% SDS and 10 µl proteinase K (10 mg/ml). The mix was incubated at 60°C for 3 h or at 37°C overnight. After incubation, 400 µl of ammonium acetate and 200 µl chloroform were added and vortexed. The tube was microfuged at 10,000 rpm for 10 min and the top aqueous phase of the supernatant was transferred to a new tube. The DNA in this aqueous phase was precipitated by adding 200 µl isopropanol, washed with 70% ethanol twice by centrifugation before dissolving in 20 µl TE buffer.

Evaluation of DNA quality. The DNA concentration and purity were assessed by measuring OD using a NanoDrop spectrophotometer (ND1000) at 230, 260, 280 and 320 nm (**Table 1**). The concentration was adjusted to the desired concentration by adding TE buffer. The DNA preparations were also assessed for their quality and integrity in 0.8% agarose gels.

HLA class I and class II typing for selection of donors. HLA class I (HLA-A, B) and class II alleles (HLA-DRB1, DRB3, DRB4, DRB5) were tested using different test procedures of commercially available Reverse Line Strip (RLS) hybridization (Invitrogen), PCR-SSP (BAG Healthcare GmbH/*INNO-TRAIN* Diagnostik GmbH/One Lambda Inc./Invitrogen) and reverse SSO by luminex (One Lambda Inc.) kits as per the manufacturer's instructions and basic guidelines as recommended by the 13th International Histocompatibility Workshop (13IHWC).

Chimerism-based monitoring of engraftment. DNA samples collected at pre- and post-transplant time points were amplified using customized primers for nine autosomal microsatellite loci, namely D19S253, D18S51, THO1, D8S1179, FES, D21S11, D3S3045, D16S5539 and D17S1290 as shown in **Table 2**. These repetitive markers were selected on the basis of their high informative indices standardized in our laboratory. The PCR reactions were set up in the presence of 2 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primer, 5 ng/µl DNA and 0.5 U/µl Taq DNA polymerase enzyme. A touchdown PCR cycling program was followed for amplification. It included 20 initial cycles of 94°C for 15 sec, 65°C for 30 sec touchdown with 0.5°C decrease at each step, followed by 20 cycles at 94°C for 15 sec, 55°C for 30 sec, 72°C for 30 sec, followed by a thermal delay at 72°C for 15 min.

The DNA samples were also simultaneously genotyped using AmpFlSTR® Identifiler amplification kit (Applied Biosystems) that amplifies 15 repeat markers, and examined in ABI 3130 ×l

Table 2. Primer sequences used for STR based chimerism monitoring

Marker	5′ primer	3′ primer	I-index (%)
D19S253	5′- (Fam) at aga cag aca gac gga ctg –3′	5′- ggg agt gga gat tac ccc t –3′	52
D18S51	5′- (Fam)ca aac ccg act acc agc aac –3′	5′- gag cca tgt tca tgc cac tg –3′	33
THO1	5′- gtg att ccc att ggc ctg ttc ctc –3′	5′- (Fam)gt ggg ctg aaa agc tcc cga tta t –3′	45
D8S1179	5′- (Fam)tt ttt gta ttt cat gtg tac att cg –3′	5′- cgt agc tat aat tag ttc att ttc –3′	39
FES	5′- ggg att tcc cta tgg att gg –3′	5′- (Fam)gc gaa aga atg aga cta cat –3′	42
D21S11	5′- gtt gta tta gtc aat gtt ctc c –3′	5′- (Fam)gt gag tca att ccc caa g –3′	33
D3S3045	5′-acc aaa tga gac agt ggc at-3′	5′- (Fam)atg agg acg gtt gac atc tg-3′	63
D16S5539	5′-gat ccc aag ctc ttc ctc tt-3′	5′-(Fam)acg ttt gtg tgt gca tct gt-3′	51
D17S1290	5′-gcc aac aga gca aga ctg tc-3′	5′-(Fam)cga aac agt taa atg gcc aa-3′	33

I-index (%) refers to informative index and indicates percent patient-donor pairs that possessed different fingerprints for the marker tested.

sequencer following user instructions. The thermal cycling conditions included 20 initial cycles of 94°C for 15 sec, 65°C for 30 sec followed by 20 cycles at 94°C for 15 sec, 55°C for 30 sec, 72°C for 30 sec followed by a thermal delay at 72°C for 40 min. Amplified fragment analysis was performed using Gene Mapper® Software Version 4 (Applied Biosystems) and results documented as percent donor chimerism using mathematical calculations as suggested.[27]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Tran SD, Pillemer SR, Dutra A, Barrett AJ, Brownstein MJ, Key S, et al. Differentiation of human bone marrow-derived cells into buccal epithelial cells in vivo: a molecular analytical study. Lancet 2003; 361:1084-8; PMID:12672312; http://dx.doi.org/10.1016/S0140-6736(03)12894-2
2. Beckett SM, Laughton SJ, Pozza LD, McCowage GB, Marshall G, Cohn RJ, et al. Buccal swabs and treated cards: methodological considerations for molecular epidemiologic studies examining pediatric populations. Am J Epidemiol 2008; 167:1260-7; PMID:18326872; http://dx.doi.org/10.1093/aje/kwn012
3. Iakovlev AG. [Isolation of DNA from epithelial cells of the internal portion of the cheek]. Biull Eksp Biol Med 1988; 105:67-8; PMID:3337885
4. Trevilatto PC, Line SR. Use of buccal epithelial cells for PCR amplification of large DNA fragments. J Forensic Odontostomatol 2000; 18:6-9; PMID:11324090
5. Lum A, Le Marchand L. A simple mouthwash method for obtaining genomic DNA in molecular epidemiological studies. Cancer Epidemiol Biomarkers Prev 1998; 7:719-24; PMID:9718225
6. de Vries HG, Collèe JM, van Veldhuizen MH, Achterhof L, Smit Sibinga CT, Scheffer H, et al. Validation of the determination of deltaF508 mutations of the cystic fibrosis gene in over 11 000 mouthwashes. Hum Genet 1996; 97:334-6; PMID:8786076; http://dx.doi.org/10.1007/BF02185766
7. Rivero ER, Neves AC, Silva-Valenzuela MG, Sousa SO, Nunes FD. Simple salting-out method for DNA extraction from formalin-fixed, paraffin-embedded tissues. Pathol Res Pract 2006; 202:523-9; PMID:16723190; http://dx.doi.org/10.1016/j.prp.2006.02.007
8. Wurmb-Schwark N, Malyusz V, Fremdt H, Koch C, Simeoni E, Schwark T. Fast and simple DNA extraction from saliva or sperm cells obtained from the skin or isolated from swabs. Leg Med 2006; 8:177-81; http://dx.doi.org/10.1016/j.legalmed.2005.11.004
9. Ehli EA, Lengyel-Nelson T, Hudziak JJ, Davies GE. Using a commercially available DNA extraction kit to obtain high quality human genomic DNA suitable for PCR and genotyping from 11-year-old saliva saturated cotton spit wads. BMC Res Notes 2008; 1:133; PMID:19102763; http://dx.doi.org/10.1186/1756-0500-1-133
10. Walsh DJ, Corey AC, Cotton RW, Forman L, Herrin GL, Jr., Word CJ, et al. Isolation of deoxyribonucleic acid (DNA) from saliva and forensic science samples containing saliva. J Forensic Sci 1992; 37:387-95; PMID:1500889
11. Freeman B, Smith N, Curtis C, Huckett L, Mill J, Craig IW. DNA from buccal swabs recruited by mail: evaluation of storage effects on long-term stability and suitability for multiplex polymerase chain reaction genotyping. Behav Genet 2003; 33:67-72; PMID:12645823; http://dx.doi.org/10.1023/A:1021055617738
12. Terasaki P, Chia D, Sugich L. Saliva as DNA source for HLA typing. Hum Immunol 1998; 59:597-8; PMID:9757916; http://dx.doi.org/10.1016/S0198-8859(98)00057-3
13. Cook DJ, Li TC, Bolwell BJ. HLA class II DNA typing and subsequent transplantation of an aplastic anemia patient using hair follicles as a DNA source. Bone Marrow Transplant 1994; 14:463-5; PMID:7994274
14. Hong YC, Liu HM, Chen PS, Chen YJ, Lyou JY, Hu HY, et al. Hair follicle: a reliable source of recipient origin after allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant 2007; 40:871-4; PMID:17704789; http://dx.doi.org/10.1038/sj.bmt.1705823
15. Rovó A, Meyer-Monard S, Heim D, Arber C, Passweg JR, Gratwohl A, et al. No evidence of plasticity in hair follicles of recipients after allogeneic hematopoietic stem cell transplantation. Exp Hematol 2005; 33:909-11; PMID:16038783; http://dx.doi.org/10.1016/j.exphem.2005.04.010
16. Seifert B, Passweg JR, Heim D, Rovó A, Meyer-Monard S, Buechner S, et al. Complete remission of alopecia universalis after allogeneic hematopoietic stem cell transplantation. Blood 2005; 105:426-7; PMID:15073031; http://dx.doi.org/10.1182/blood-2004-01-0136
17. Jacewicz R, Lewandowski K, Rupa-Matysek J, Jedrzejczyk M, Brzezinski PM, Dobosz T, et al. Donor-derived DNA in hair follicles of recipients after allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant 2010; 45:1638-44; PMID:20173789; http://dx.doi.org/10.1038/bmt.2010.27
18. Thiede C, Prange-Krex G, Freiberg-Richter J, Bornhäuser M, Ehninger G. Buccal swabs but not mouthwash samples can be used to obtain pretransplant DNA fingerprints from recipients of allogeneic bone marrow transplants. Bone Marrow Transplant 2000; 25:575-7; PMID:10713640; http://dx.doi.org/10.1038/sj.bmt.1702170
19. Quinque D, Kittler R, Kayser M, Stoneking M, Nasidze I. Evaluation of saliva as a source of human DNA for population and association studies. Anal Biochem 2006; 353:272-7; PMID:16620753; http://dx.doi.org/10.1016/j.ab.2006.03.021
20. Lombaert IMA, Brunsting JF, Wierenga PK, Faber H, Stokman MA, Kok T, et al. Rescue of salivary gland function after stem cell transplantation in irradiated glands. PLoS One 2008; 3:e2063; PMID:18446241; http://dx.doi.org/10.1371/journal.pone.0002063
21. Badulli C, Sbarsi I, Di Giorgio D, Mantovani M, Maserati R, Barbarini G, et al. A new approach to safely type for HLA the HIV infected people eligible to abacavir therapy: saliva or buccal swab as reliable DNA sources. Clin Chim Acta 2011; 412:1995-8; PMID:21767530; http://dx.doi.org/10.1016/j.cca.2011.07.003
22. Themeli M, Waterhouse M, Finke J, Spyridonidis A. DNA chimerism and its consequences after allogeneic hematopoietic cell transplantation. Chimerism 2011; 2:25-8; PMID:21547035
23. Kuehnle I, Goodell MA. The therapeutic potential of stem cells from adults. BMJ 2002; 325:372-6; PMID:12183311; http://dx.doi.org/10.1136/bmj.325.7360.372
24. Imanguli MM, Atkinson JC, Harvey KE, Hoehn GT, Ryu OH, Wu T, et al. Changes in salivary proteome following allogeneic hematopoietic stem cell transplantation. Exp Hematol 2007; 35:184-92; PMID:17258067; http://dx.doi.org/10.1016/j.exphem.2006.10.009
25. Kaur G, Sarkar N, Bhatnagar S, Kumar S, Rapthap CC, Bhan MK, et al. Pediatric celiac disease in India is associated with multiple DR3-DQ2 haplotypes. Hum Immunol 2002; 63:677-82; PMID:12121676; http://dx.doi.org/10.1016/S0198-8859(02)00413-5
26. Aidar M, Line SRP. A simple and cost-effective protocol for DNA isolation from buccal epithelial cells. Braz Dent J 2007; 18:148-52; PMID:17982556; http://dx.doi.org/10.1590/S0103-64402007000200012
27. Nollet F, Billiet J, Selleslag D, Criel A. Standardisation of multiplex fluorescent short tandem repeat analysis for chimerism testing. Bone Marrow Transplant 2001; 28:511-8; PMID:11593326; http://dx.doi.org/10.1038/sj.bmt.1703162