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Forensic implications of the presence of chimerism after Hematopoietic Stem Cell Transplantation.

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HIGHLIGHTS

- The coexistence of cells from two or more genetically different origins in an individual is called chimerism.
- Chimerism can occur naturally (errors in fertilization and embryogenesis) or artificially (associated with medical interventions, such as blood transfusion or transplants).
- After hematopoietic stem cell transplantation, cells of donor origin could be found not only in blood, but also other tissues of the recipient resulting in mixed genetic profiles (i.e. chimerism).
- Biological vestiges coming from chimeric individuals may present a challenge in forensic analyses.

ABSTRACT

Biological vestiges are used in forensic science to resolve a large number of cases by typing the genetic profile and identifying the individual to whom it belongs. However, chimeric persons that possess cells with two or more different DNA make these types of analyses difficult. This situation can occur naturally, by errors in the fertilization or early embryogenesis, or in an artificial way, for example after hematopoietic stem cell transplantation (HSCT), when host and donor cells coexist in the patient. In this paper, we will specially focus on the latter.

The vestiges from transplant patients represent a challenge from a forensic perspective since the interpretation of the genetic fingerprint can be misleading because of the presence of chimerism.

Due to the high number of transplant patients (and their increase over the years) and the existence of natural chimeras (probably many of them hidden), it is necessary to consider whether we are facing a possible

chimeric person or someone who has been a donor of hematopoietic stem cells in a forensic context.

In this review, the presence of donor bone marrow derived cells in some tissues of forensic interest will be discussed. Finally, to emphasize the importance of chimerism after HSCT in forensic genetics, some real-life cases will be examined.

KEYWORDS: Chimerism; Biological vestige; Hematopoietic Stem Cell Transplantation; Forensic Genetics; Human identification

1. INTRODUCTION

From a forensic perspective, genetic analysis of biological samples collected from a crime scene is an important tool, as it can be used to identify persons in order to solve a large number of crimes [1]. However, as it will be shown later, sometimes individual identification is hindered in people who have cells with different DNA, who are known as "chimeras". A chimera is an individual who possesses cells from two or more genetically different origins [2, 3, 4, 5], and when dealing with samples showing these characteristics it is easy to mistake them for DNA mixture evidence or cross-contamination.

In this review, we will analyze different natural situations in which chimerism can be found in human samples, as well as artificial chimerism caused in an allogeneic hematopoietic stem cell transplant patient in detail.

Finally, 4 real cases in which the presence of chimerism was a challenge from a forensic perspective are discussed.

2. CHIMERISM: NATURAL AND ARTIFICIAL

Natural chimerism occurs in very rare circumstances: fusion of zygotes, blood exchanges between fetuses inside the utero or between the fetus and the mother, double parental contribution, etc. However, artificial chimerism associated with medical intervention (such as HSCT or blood transfusion) is more common [2, 5].

2.1. Abnormal fertilization

At the time of fertilization, abnormal situations for the oocyte and/or sperm may occur. For example, in the case of diandric chimerism, two sperm cells (or a diploid sperm cell if any error occurs in the meiotic division) fertilize an oocyte (which in turn replicates its genetic material to compensate) or even a second polar body.

It may also arise from a sperm that fertilizes an empty ovum, and then replicates its genetic material and fuses itself with another fertilized oocyte, androgenetic chimerism. In this case, one of the genetic lines of the chimeric embryo would be uniparental with only paternal genetic contribution, which is known as androgenetic chimerism.

Another option is that the oocyte replicates its genetic material before being fertilized by one sperm, or that it retains a polar body, and then the lonely female genetic material replicates again. In this case, one of the cell lines of the chimeric zygote would only have maternal genetic material, which is known as digynic chimerism [3].

2.2. Abnormal early embryogenesis

In the post-zygotic tetragametic chimerism, the fertilization process is normal, but two embryos that are in contact are merged, which gives rise to tetraploid chimeras (fusion of 2 diploid embryos).

Embryos that have undergone abnormal fertilization can also be modified, in a process known as post-zygotic splitting. In this case, a chimeric embryo undergoes a series of cell divisions that form a mass with mixed genetic material that divides into two embryos [3].

2.3. Microchimerism

At certain times during pregnancy, blood exchanges may occur between embryos within the uterus, or between the fetus and the mother. After feto-maternal cell trafficking, some of the fetus cells can remain in the mother's blood for decades, or even settle in solid tissues [6, 7].

2.4. Artificial chimerism

Although it is very likely that many cases of natural chimerism exist that are unknown due to the lack of characteristic phenotypes, artificial chimerism is much more frequent. Artificial chimerism is associated with medical interventions, such as blood transfusion or transplants, due to the coexistence of cells from recipient and donor in a single person [2, 5]. The following sections will explain the type of chimerism that occurs after hematopoietic stem cell transplantation (HSCT) and how it is analyzed, as well as the forensic implications of the presence of two DNA profiles in the same tissue.

3. HEMATOPOIETIC STEM CELL TRANSPLANTATION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a medical procedure in which cells of the hematologic and immune systems of a patient are replaced by healthy hematopoietic stem cells (HSCs) from a donor. These HSCs can come from three different sources: bone marrow, mobilized

peripheral stem cells or placental blood of the umbilical cord [8, 9, 10, 11]. The main clinical indication is hematologic malignancies (as leukemia), but HSCT can also be used to treat non-malignant disorders, both congenital and acquired diseases of the hematopoietic system (as some forms of bone marrow failures, red cell disorders, immunodeficiencies, etc.) [8, 9, 10].

The transplant can fail due to relapse of the malignancy. In addition, it has a significant risk of mortality because of severe graft-versus-host disease (GVHD) and infections due to immune suppression [8, 12]. However, despite this risk, the use of this clinical approach has increased as a result of improvements in donor selection (the compatibility between donor and recipient can be assayed by the human leukocyte antigen-HLA-system), the use of immunosuppression to prevent GVHD, advances in drugs against infection agents, advances in conditioning regimens and better supportive care [8, 9, 12].

3.1 Transplant patient and its clinical monitoring by human identification markers analysis

In allogeneic HSCT, healthy hematopoietic stem cells are extracted from a different donor and then infused into the patient, after a conditioning regimen [8, 9]. This fact results in the coexistence of host origin cells and non-host cells with different genomes (except in syngeneic HSCT, when donor cells come from an identical twin), i.e., chimerism [13].

The study of the proportion of donor-host chimerism after the transplantation is an important step as it provides information about the quality of the graft (rejection or GVHD), as well as the presence of minimal residual disease and the possible relapse or recurrence of malignant cells. This way, the most appropriate course of treatment for the patient can be determined [4, 14].

After HSCT, the recipient becomes a chimera, since they possess cells with different genetic origins in their organism. The presence of both donor and recipient cells in a tissue is called mixed chimerism, while complete chimerism is the situation where all cells correspond to donor DNA. When a successful graft is undergone, all of the patient's hematopoietic stem cells and malignant cells are eliminated, resulting in complete chimerism in blood and bone marrow. Therefore, the presence of mixed chimerism in these tissues when treating malignant diseases is usually associated to disease relapse. With non-malignant disorders, the HSCT conditioning is less aggressive, so the presence of mixed chimerism is quite frequent and it reflects an immunotolerance status between both hematopoietic systems [4, 5, 15].

For chimerism monitoring, certain polymorphic regions of the genome are examined, making it possible to distinguish donor and recipient alleles. The loci analyzed must vary between individuals, must be easy to characterize and interpret, must not be under selective pressure and must have a low mutation ratio [16, 17].

Nowadays, the analysis of short tandem repeats (STRs) by polymerase chain reaction (PCR) followed by separation of the amplified sequences by capillary electrophoresis is the most commonly used procedure for chimerism quantification.

However, single nucleotide polymorphisms (SNP) and insertion/deletion polymorphisms (InDel) are also used, since their mutation rate is smaller than the mutation rate of STRs and may provide additional information in some cases where STRs assays present sensibility issues.

Moreover, some authors have shown that the use of quantitative real-time PCR (qPCR) and Droplet digital PCR (ddPCR) to study chimerism provides additional information in some cases with a small minor chimerism component; the sensitivity of commonly used techniques is about 3–5 %, while these procedures are able to detect between 0.1 - 0.5 % [18, 19].

Chimerism analysis should be carried out with efficient techniques in terms of power of discrimination, cost and time. Therefore, it is very important for laboratories to submit to external quality schemes and standards [12]. Nowadays, some heterogeneity exists in the techniques used from laboratory to laboratory, which makes it difficult to compare and exchange data between them [20]. In order to follow the same guidelines, there are certain recommendations aimed at improving and facilitating the process. Clark *et al.* [12] published several recommendations for laboratories, including collection techniques, storage, sample analysis, results interpretation and report elaboration. Regarding the analysis of chimerism, the recommendations are focused in the used of STR markers as it is the most used and standardized technique [12].

Regarding analysis kits, the EuroChimerism consortium currently aims to standardize and harmonize the technical approaches of European laboratories in the quantitative analysis of chimerism after allogeneic HSCT. For this purpose, a commercial kit of 16 STRs has been developed, which has proved to be more appropriate than other forensic kits in terms of efficiency in the analysis of chimerism (sensitivity, reproducibility, precision, etc.) [20].

4. FORENSIC IMPLICATIONS OF HEMATOPOIETIC STEM CELL TRANSPLANTATION

In 2011 a total of 35,660 HSCTs were reported in Europe, of which 14,549 were allogeneic transplants [21]; while in 2014 a total of 40,829 transplants were reported, with 16,946 allogeneic transplants [22]. Due to the high number of transplant patients and their increase over the years, it is important to consider the characteristics of their biological samples from a forensic point of view.

To carry out forensic analyses, biological traces are recovered from crime scenes. The basis of forensic genetics is that each person possesses a unique DNA sequence, so the forensic analysis of biological samples by obtaining their DNA profile is a useful tool to identify people [1, 16, 17]. However, individual identification by standard markers can be erroneous in the presence of

chimerism. Therefore, some biological vestiges from patients who have undergone a hematopoietic stem cell transplant may not be reliable sources for personal identification or paternity testing [1, 2, 16, 23]. For example, as explained above, blood cells are replaced after transplantation by cells from a donor, rendering conventional DNA analysis unsuitable for identification of the recipient.

In the past, it was thought that, after HSCT, donor cells were limited to hematological tissues (blood and bone marrow), and that the rest of tissues preserved cells with recipient origin. Nowadays, it is known that donor cells appear in several non-hematologic tissues [23, 24, 25].

The possible role of standing antigen-presenting cells, the plasticity of stem cells, cell fusion, and the fact that non-hematological cells contained within the donor bone marrow compartment are transferred in allogeneic recipient are some of the mechanisms that could be responsible for the appearance of the donor's genetic profile in other tissues [26, 27, 28, 29].

In the following section, the genetic profile found in different vestiges of forensic interest in patients who have undergone an allogeneic hematopoietic stem cell transplant is analyzed in detail. Figure 1 represents a summary of the process that should be followed from a legal medical perspective.

4.1 Genetic profile of different vestiges in the transplant patient

Blood

After saliva, blood is the most common source found in crime scenes due to the presence of violence in many crimes, which can result in wounds, either in the victim or the aggressor. In addition, blood can be easily localized because of its color [1, 16].

As explained above, after HSCT, blood cells of the patients are replaced by donor blood cells, so it is expected to find a donor profile in hematological tissues. If the transplant was successfully carried out, the collected blood sample will show a complete chimerism or complete donor profile; but in some cases (malignancy relapse, inefficient conditioning, etc.), it is also possible to find samples which present a mixture of donor and recipient DNA, i.e., mixed chimerism [4, 5]. In these situations, the genetic profile found in blood traces would not correspond to genetic profiles in data banks (usually obtained using buccal swabs).

Sperm

Sperm is an important biological evidence, especially in cases with a sexual component. Some studies have found a complete recipient profile in sperm [13, 23], which may be due to the fact that spermatogenesis is a highly conserved process [23].

Hair follicles

Hair is another biological vestige usually found in a crime scene. Although hairs recovered from crime scenes in some cases are in the telogen phase (containing little, if any, nuclear DNA), complete recipient profiles can be found in hair follicles [13, 23, 25].

It is important to note that hair follicles can currently be used as a biological sample to obtain the profile of the recipient (after HSCT) of patients from whom the genetic profile was not taken before transplantation when they later require monitoring through the analysis of chimerism.

Nail

Nails can also be important evidence in some criminal cases [30] and in the identification of decomposing corpses [31].

After chimerism studies, a mixed profile has been found in nails, with amplification of both donor and recipient profile [32, 33, 34].

At this point it is important to highlight the similarities between nail and hair, since they share many characteristics: both have an ectodermal origin since they are appendages of the epidermis, both present keratin, in many diseases both tissues are affected at the same time and, moreover, the niche of the adult nail stem cells is a structure analogous to the hair bulge (niche of the hair stem cells) [35].

Skin

Due to the natural flaking of the skin and to their constant replacement, epithelial cells are constantly being deposited on the floor or adhered to any surface with which the person has had contact [16], making them an interesting vestige in forensic science. In criminal contexts, it is common to find objects for which their origin or the person or persons who might have manipulated them must be determined [36].

In 2002, Körbling *et al.* [37] studied skin biopsy samples from patients who had undergone an allogeneic peripheral-blood stem cell transplantation, with special interest in females who received a transplant from a male donor. By using immunofluorescence techniques against cytokeratin and XY-FISH, they found epithelial donor-derived cells (XY-positives and cytokeratin-positive) in the layer of Malpighi of the skin [37]. Moreover, in 2018, Sanz-Piña *et al*. obtained the genetic profile of epithelial cell samples isolated from cutaneous biopsies of allogeneic HSCT patients by the analysis of 15 STR markers, finding a mixed profile as well as a positive correlation, without statistical significance, between the time elapsed from the transplant to the biopsy and the percent donor chimerism in epithelial cells of the epidermis [34].

Oral mucosa

Saliva is the largest source of DNA found in crime scenes (drinking receptacles, cigarette butts, etc.) [1]. Epithelial cells from the oral mucosa from patients who have undergone a bone marrow

transplantation show mixed chimerism [13, 23, 25]. In 2003, Tran *et al.* [38] demonstrated how stem cells derived from bone marrow, possibly hematopoietic stem cells, migrated from the marrow to the cheek, with some differentiating into epithelial cells [38].

For monitoring chimerism, the collection of buccal mucosa samples using a swab used to be the traditional method used to obtain the reference profile if no recipient samples had been taken before HSCT. However, in spite of the mixed profile found in saliva samples, since the donor type is usually known (or could be established using a blood sample), it is normally easy to separate donor and recipient profiles with a clinical purpose. Moreover, when swabs are taken after rinsing the mouth several times, the amount of donor cells is negligible even in patients with full donor chimerism.

Urine

Urine from transplant patients shows mixed chimerism in patients with no leukocyturia too. Therefore, it is hypothesized that donor transplanted HSCs differentiate into epithelial cells in the urinary tract [13].

Other vestiges or tissues

In addition of samples with forensic interest, other tissues have been also studied, showing how bone marrow donor-derived stem cells are able to differentiate in multiple cell lineages. In recipients of HSCT, bone-marrow-derived cells have been also found in the gastrointestinal epithelium, vascular endothelium, hepatic, muscular, pulmonary and neural tissue [37, 39, 40].

5. EXAMPLES OF REAL-LIFE CASES

To emphasize the importance of chimerism after HSCT in forensic genetics, three case examples reported in the published literature will be examined in this section. In addition, one case involving natural chimerism will be also discussed.

Case 1

In November 2000, several forensic samples from a woman who was suspected to be the victim of sexual abuse while she was unconscious were analyzed. A request was made for the genetic profiles found on these samples to be compared to those in the National DNA Database in order to find a suspect. Besides semen (which seemed to support the allegations of abuse), a mixture of genetic profiles was found in samples from external vaginal swabs and corporal fluids on her underwear. However, a mixture of different genetic profiles was also found when a sample of buccal mucosa was taken, prompting further investigation of blood and hair samples, which turned up two distinct DNA profiles, albeit with many shared alleles. After researching the victim's medical history, it was discovered that she had undergone a bone marrow transplant when she was a child to treat

leukemia. The donor had been her brother, which explained why the profiles found in blood and hair shared so many alleles. Finally, the profile found in the samples from the vagina and underwear was not used for inclusion in the National DNA database since it was a mixed profile and showed similarities with the male profile that appeared in the victim's blood (presumably her brother's), who had already been excluded as a suspect in the investigation [41].

In this situation, in which the transplant evolved correctly, a complete profile of the donor was found in blood samples, and a complete profile of the recipient in hair samples, while the rest of samples showed a mixture of profiles, underlining the importance of analyzing several vestiges in criminal cases involving HSCT patients.

Case 2

After a fire in a family house, two corpses were found completely burned, which were assumed to belong to two children, a boy and a girl, who could not escape it. Nevertheless, individual identification was required. Due to the severity of the burns there were no characteristic features, making it necessary to obtain a genetic profile, so as to compare it with those of their alleged parents. The genetic profile of the boy's blood (obtained from cardiac chambers) was typified with no issues, but the girl turned out to have undergone a bone marrow transplantation 5 years before to treat myelogenous leukemia, with an unknown donor. Therefore, samples from blood and several tissues of the girl were collected for analysis. Several of the girl's tissues (uterine muscle, costal cartilage, mucous membrane of the urinary bladder...) showed alleles belonging to the donor (whose profile was obtained from blood samples). After several tests, it was determined that the girl was indeed the alleged parents' daughter [42].

Again, this case shows the importance of knowing the medical history of the individuals involved in identification though genetic profiling.

Case 3

To carry out a paternity study involving two dizygotic twins, blood samples and mouth swabs were taken from the children, the alleged father, and the mother. The mother stated that she had undergone a bone marrow transplant to treat sickle cell disease in the past. The alleged father turned out not to be the biological father of the children since many discrepancies between their genetic profiles were found. When the mother's samples were analyzed, different genetic profiles were found, none of them consistent with those of the children either, shedding doubt on her being the actual mother. Moreover, the bone marrow donor had been her sister, making the results more difficult to be interpreted.

Hair root samples from the alleged mother were then collected for analysis; in this case, a genetic profile consistent with those of the children was found, establishing her as the biological mother

[43].

As the authors of that study remarked, in cases in which it is known in advance that a person recipient of HSCT is involved, the analysis cannot be based on the standard tissues used in paternity tests. It is very important to take samples from other tissues, especially hair follicles, where a complete profile of the recipient appears [43].

Case 4

After suffering from focal sclerosing glomerulonephritis and renal failure, a 52-year-old woman needed to undergo a kidney transplant, for which histocompatibility tests were done on her family. However, after these tests, it was found that two of her three children had no coinciding maternal haplotype. Additional tests were performed on the woman, resulting in the discovery of a tetragametic chimera with tissues showing 4 HLA haplotypes, except in blood, where only 2 haplotypes were found. After STR analysis, 4 alleles were also found in several loci of a large number of chromosomes. Maternity was however confirmed by comparing the children's haplotypes with those of their maternal grandparents [44].

This was a rare case of tetragametic chimerism of an XX/XX female, as she showed no phenotypic characteristics and was fertile. This condition had not been discovered before because only one of the cell lines was present in blood. The most probable cause for the woman's tetragametic chimerism is the fusion of two normal fertilized embryos (each with a different HLA haplotype) [44].

As the article authors indicate, other molecular studies should be considered in cases of chimerism or suspected chimerism, as it can be underdiagnosed or misinterpreted in terms of maternity or paternity [44]. Sometimes, after performing histocompatibility studies for transplants, some inconsistences in the HLA-ABO system are found between parents and children, which are assumed as misattributed paternity or maternity. However, HLA-ABO typing, despite providing a high accuracy (<97%), is not enough to confirm paternity in several cases. In addition, there are some genetic phenomena that can explain these incongruences, chimerism being one of them [45]. The fact that the use of *in vitro* fertilization implies a higher probability of conceiving twins, which in turn increases the likelihood of fusion due to the close contact, or the probability of double fertilizations, must also be taken into consideration [44]. Thus, it is likely that the number of people who have been born with some type of chimerism is greater than thought.

6. CONCLUSIONS

Biological samples from HSCT recipients can prove a challenge for legal-medical experts. Therefore, when evidence is gathered for a biological paternity test or in the context of a criminal

investigation in which the involvement of an HSCT donor or recipient is suspected, from an expert witness perspective, it is of the utmost importance to consider which genetic profile may appear in any of the different vestiges.

As explained throughout this work, new cases of naturally-born chimeras occasionally occur, and the increase of *in vitro* fertilization favors the probability of abnormalities in fecundation or the fusion of zygotes. Therefore, it is important to also consider this kind of chimerism either clinically or in forensic investigations.

Conflict of Interest: Elena Sanz-Piña, Ana Santurtún and María T. Zarrabeitia declare that they have no conflict of interest.

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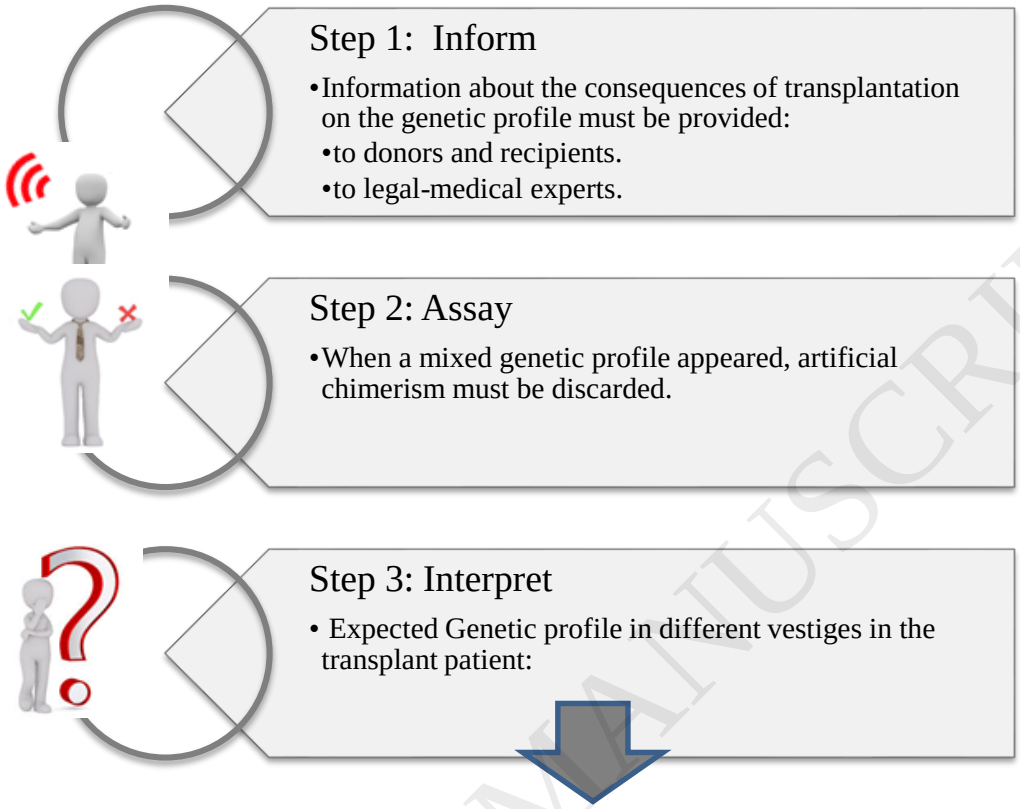
FIGURES

Figure 1: Steps to be followed when dealing with a HSCT patient (donor or recipient) in a forensic context.



Sample	***Expected genetic profile***
Blood	Donor profile
Sperm	Complete recipient profile
Hair follicles	Complete recipient profile
Nail	Mixed chimerism
Skin	Mixed chimerism
Oral mucosa	Mixed chimerism
Urine	Mixed chimerism