International Congress Series 1261 (2004) 293–295

www.ics-elsevier.com

Humara X-chromosome inactivation assay for the detection of female minor component in male/female mixed bloodstains

C. Robino*, S. Gino, C. Torre

Laboratory of Criminalistic Sciences, Department of Anatomy, Pharmacology and Legal Medicine, University of Turin, C.so M. D'Azeglio 52, 10126 Turin, Italy

Abstract

Analysis of mixed stains from forensic casework by means of standard PCR-based typing of autosomal short tandem repeats (STRs) becomes difficult when the minor component is present at less than one tenth of the concentration of the major component. The human androgen receptor (HUMARA) X-chromosome inactivation assay allows to detect even a small number of female cells in the presence of a high background of male cells in male/female mixed bloodstains. DNA cleavage by means of methyl-sensitive restriction enzyme (*Hha*I) is followed by typing of the HUMARA locus with the nested PCR technique: methylation of restriction sites on inactivated female X chromosomes allows the efficient amplification of low amounts of endonuclease-resistant female-derived target sequences. In this study, the method attained a 10^{-3} level of sensitivity in the detection of the female minor component.

Keywords: DNA mixture; Human androgen receptor gene (HUMARA); X-chromosome inactivation; DNA methylation; Nested PCR

1. Introduction

Mixtures of body fluids from different individuals are an integral component of forensic casework. It has been shown that, by standard PCR-based typing of autosomal STRs, it is usually difficult to detect the minor component of a mixed stain when it is present at approximately less than one tenth of the major component [1]. For male/female mixtures, identification of the male minor component can be improved through analysis of Y-specific polymorphic STR loci [2].

* Corresponding author. Tel.: +39-116707731; fax: +39-116707732.
E-mail address: carlo.robino@unito.it (C. Robino).

0531-5131/
doi:10.1016/S0531-5131(03)01782-5

Here, we report a simple test that allows a highly efficient detection of a small number of female cells in the presence of a high background of male cells. The human androgen receptor (HUMARA) gene contains a polymorphic CAG trinucleotide repeat in its first exon [3]; close to this polymorphism are *Hha*I restriction enzyme sites. The methylation status of the *Hha*I sites corresponds with the inactivation status of the X chromosome and thus allows for the detection between the active and inactive X chromosomes [4]. Methylation (inactivation) of X chromosome prevents *Hha*I from cutting next to the HUMARA polymorphism. Since male HUMARA sequences are always on an active X chromosome, they are prone to digestion by the methyl-sensitive *Hha*I, whereas digestion-resistant HUMARA genes on inactivated X chromosomes are protected from cleavage, allowing the subsequent typing of the female minor component of a mixture [5].

2. Material and methods

Blood samples from male and female donors of known HUMARA genotype were volumetrically mixed in proportions of $1:10^{-1}$, $1:10^{-2}$, $1:10^{-3}$ and $1:10^{-4}$, spotted on filter paper and dried. Stains were then extracted by means of spin columns (Macherey-Nagel, Düren, Germany); DNA was quantitated spectrophotometrically and 500 ng of DNA from each stain was digested overnight at 37 °C with 1 U of *Hha*I restriction endonuclease (New England Biolabs, Beverly, MA, USA) in a total volume of 25 μl.

HUMARA polymorphism was analyzed in the digested samples by means of nested PCR. Five microliters of the digested DNA was amplified in a 25-μl PCR reaction volume containing 2 X PCR Master Mix (Promega, Madison, WI, USA) and 0.5 μM of the following outer primers: 5′ -GGT AAG GGA AGT AGG TGG AAG ATT C-3′ ; 5′ -TCT GGG ACG CAA CCT CTC TC-3′ (Sigma-Genosys, Pampisford, UK). The cycle conditions were 94 °C for 7 min; 94 °C for 45 s, 55 °C for 30 s, 72 °C for 45 s, 28 cycles; 72 °C for 15 min final extension. One microliter of the PCR product was then used as template for amplification in a 25-μl PCR reaction volume containing 2 X PCR Master Mix (Promega) and 0.5 μM of the following inner primers: 5′ -(FAM) TCC AGA ATC TGT TCC AGA GCG TGC-3′ ; 5′ -GCT GTG AAG GTT GCT GTT CCT CAT-3′ (Sigma-Genosys). The cycle conditions were 95 °C for 8 min; 95° for 40 s; 63 °C for 40 s; 72 °C for 60 s; 32 cycles; 72 °C for 10 min final extension. Fluorescent PCR products were electrophoresed on ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

3. Results

Our results showed that, after *Hha*I digestion and nested PCR of the HUMARA locus, female-specific allele peaks could be clearly seen for mixed bloodstains with male/female ratios of $1:10^{-1}$, $1:10^{-2}$ and $1:10^{-3}$. No female-specific PCR products were obtained from mixtures with ratios of $1:10^{-4}$. Due to incomplete digestion of active X chromosomes, male-specific allele peaks were present in the electropherograms even at less extreme male/female DNA ratios ($1:10^{-1}$). Since the amount of input DNA digested by *Hha*I was 500 ng and one fifth of the digestion volume was subsequently used for nested

PCR, the HUMARA X-chromosome inactivation assay was capable to detect as little as 0.1 ng of female DNA.

4. Discussion

The HUMARA X-chromosome inactivation assay has been recently reported as a highly sensitive method for the detection of microchimerism in sex-mismatched stem cell transplantation from male donors to female recipients [5]. When applied to mock forensic samples (mixed bloodstains), the method displayed a 10^{-3} sensitivity with an observed detection limit—in terms of total female DNA present in the mixture—of 0.1 ng.

Since HUMARA is an extremely polymorphic marker with high power of discrimination, chances of finding a mixture from non-informative individuals (that is, a homozygous woman carrying the same allele of the male contributor) are low [6]. However, the constant presence of male-specific HUMARA allele peaks resulting from partial digestion of active X chromosomes can interfere with the definition of the genotype of the female contributor.

Despite these limitations, HUMARA X-chromosome inactivation assay was shown to be a hundred-fold more efficient than standard autosomal STRs in detecting the female minor component in male/female mixtures and may be applied to forensic case work in selected circumstances. Future characterization of other X-STRs amenable to methylation-specific PCR will also improve the efficacy of this method for identity testing purposes.

References

[1] J.E. Lygo, P.E. Johnson, D.J. Holdaway, S. Woodroffe, J.P. Whitaker, T.M. Clayton, et al., The validation of short tandem repeat (STR) loci for use in forensic casework, Int. J. Leg. Med. 107 (2) (1994) 77–89.

[2] M. Prinz, K. Boll, H. Baum, B. Shaler, Multiplexing of Y chromosome specific STRs and performance for mixed samples, Forensic Sci. Int. 85 (3) (1997) 209–218.

[3] W.D. Tilley, M. Marcelli, J.D. Wilson, M.J. McPhaul, Characterization and expression of a cDNA encoding the human androgen receptor, Proc. Natl. Acad. Sci. U. S. A. 86 (1) (1989) 327–331.

[4] R.C. Allen, H.Y. Zoghbi, A.B. Moseley, H.M. Rosenblatt, J.W. Belmont, Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation, Am. J. Hum. Genet. 51 (6) (1992) 1229–1239.

[5] A. Yamane, M. Karasawa, T. Maehara, N. Tsukamoto, Y. Nojima, X chromosome methylation-based chimerism assay for sex-mismatched hematopoietic stem cell transplantation, Bone Marrow Transplant. 28 (10) (2001) 969–973.

[6] D. Desmarais, Y. Zhong, R. Chakraborty, C. Perreault, L. Busque, Development of a highly polymorphic STR marker for identity testing purposes at the human androgen receptor gene (HUMARA), J. Forensic Sci. 43 (5) (1998) 1046–1049.