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A Rare Mutation in the Amelogenin Gene and Its Potential Investigative Ramifications*

REFERENCE: Roffey PE, Eckhoff CI, Kuhl JL. A rare mutation in the amelogenin gene and its potential investigative ramifications. J Forensic Sci 2000;45(5):1016–1019.

ABSTRACT: Over the past few years, the Australian forensic science community has adopted a common methodology and technology in the application of DNA profiling for investigative and forensic purposes. The ultimate objective of this initiative is the establishment of a national DNA database similar to that used in the UK. An integral part of this methodology is the use of "Profiler Plus," a nonaplex of STRs combined with amelogenin, a locus utilized for sex determination. This paper reports the results from a case where a mutation in the annealing region of the amelogenin primers appears to have resulted in the failure to amplify the amelogenin Y-homolog from a phenotypically normal male. The result was confirmed using two different primer sets that amplify different regions of the amelogenin gene. This situation suggests that the genetic determination of sex based on the amelogenin sequences from specimens of unknown origin, such as crime scene samples, should not be considered infallible.

KEYWORDS: forensic science, amelogenin, sex-typing, ampFℓSTR profiler plus, mutation, DYZ1, PCR, allelic dropout

There are many occasions in forensic investigations where the accurate determination of the sex of biological samples has valuable applications, both for investigative and evidentiary purposes. The value of sex typing is not with its discriminating power but rather with its ability to support other more discriminating evidence, particularly with its ability to provide testimony to the reliability of modern forensic DNA profiling systems. Sex tests based on the amelogenin gene have found a niche in many of the common DNA profiling systems that are used today. The AmpFℓSTR kits (PE Biosystems), the GenePrint kits (Promega), and the Forensic Science Service SGM (1) are examples of modern profiling systems that make use of this locus.

The amelogenin gene is a single copy gene with homologs on the X and Y chromosomes, AMGX (Xp22.1–Xp22.3), and AMGY (Yp11.2), respectively (2,3). The X and Y homologs are chromosome specific. Although homologous, they differ quite significantly in size and sequence (4), and consequently can be used as markers for their relevant chromosomes. Even though the X and Y alleles are quite different, there are regions that are sufficiently conserved to design primers that amplify both alleles in a single PCR amplification, but generate products that differ in size. The two most common sex tests (or variations of them) that are based on amelogenin have primer sets which delimit a 6 bp deletion on the X chromosome to produce fragments 106/112 bp or 212/218 bp (5), X/Y products, respectively. These tests have proven reliable and robust, and readily adapted for multiplexing, hence, their popularity in modern forensic profiling systems (e.g., 1,6–8).

Like all genetic loci, amelogenin is exposed to mutation. The disparity between the X and Y homologs is an obvious example of the effects of mutation. In this paper we report a case where a mutation in the Y homolog had the potential to cause the misidentification of the sex genotype of a phenotypically normal male individual, and discuss the potential associated investigative ramifications.

Materials and Methods

The Sample

The sample was a buccal swab taken from a phenotypically normal male Caucasian individual. The sample was extracted using the chelex extraction method (9).

Amelogenin Multiplex Typing Using the AmpFℓSTR Profiler Plus Kit

The extracted sample (~1 to 5 ng) was amplified with the AmpFℓSTR Profiler Plus kit (PE Biosystems, Victoria, Australia) in accordance with manufacturers instructions. Amplification products were analyzed using an ABI Prism 310 Genetic Analyzer. The separation was performed in a 50 μm capillary, 47 cm in length, loaded with POP-4 polymer using standard run parameters. Fragment size was calculated using the GeneScan Analysis 2.1 Software calibrated against known Genescan-500 (ROX) Internal Lane Size Standards. Sex genotype was determined from electropherograms by the presence or absence of 103 and 109 base peaks (X/Y respectively) and from comparison with a known female control. Female profiles are visualized as a single peak at 103 bases, whereas male profiles are visualized as two peaks of roughly equal quantity at 103 bases and 109 bases. These peaks correspond with the 106/112 base products described previously; this apparent imprecision is an intrinsic trait associated with the ABI Prism 310 Genetic Analyzer and has been well documented by the manufacturer.

Amelogenin Single Plex Typing

The extracted sample was also amplified using the AMXY primers described by Akane, et al. (1991) (10). The reaction con-

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* This work was supported by grants from the CSU-Small Granting Scheme, the Gilmore Centre, and the Biomedical Research Group (Charles Sturt University).

Received 6 July 1999; and in revised form 15 Oct. 1999; accepted 22 Nov. 1999.

tained the following final concentration of components; 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 μM each primer, 200 μM dNTP's, 1 U Taq polymerase (Life Technologies, Victoria, Australia), 1 μL template DNA (~1–5 ng DNA), 25 μL total volume. The PCR amplification was performed as a hot-start procedure as previously described (11). After an initial denaturation at 90°C for 3 min, the amplification was performed as a biphasic process. The first phase consisted of 15 cycles of denaturation at 94°C for 45 s and annealing/extension at 70°C for 90 s. This was followed by 25 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 90 s and extension at 72°C for 60 s. A final extension was performed for 10 min at 72°C.

The amplification products, 977 bp and 788 bp (X/Y respectively) were separated by electrophoresis at constant voltage (100 V) through a 1.0% horizontal agarose gel submerged in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.8), stained with ethidium bromide (0.5 μg/mL) and visualized with UV light at 302 nm. The size of fragments was calculated according to the method of Priefer (12), by comparison with the fragments in the 1 Kb ladder (Life Technologies, Victoria, Australia) as molecular weight standards. Designation of genotype was also confirmed by visual comparison of profiles with known male and female controls. Male profiles were seen as two bands of roughly equal intensity at 977 and 788 bp, whereas female profiles had only one band at 977 bp.

DYZ1 Single Plex Typing

The DYZ1 primers described by Akane, et al. (1991) (10) were used to amplify a 1024 bp sequence on the DYZ1 locus. This amplification and electrophoretic analysis was performed using the same conditions as used for the 977/788 bp amelogenin sequences described above. Genotype was designated by the presence (or absence) of the 1024 bp band and through comparison with known male and female controls. Male samples exhibited the 1024 bp product whereas female samples failed to produce this product.

Results

Our results showed that the individual, although a phenotypically normal male, was genotyped as female using the AmpFℓSTR Profiler Plus kit (PE Biosystems), as demonstrated by the absence of the 109 bp peak but presence of the 103 bp peak (Fig. 1). In order to determine whether this anomaly was caused by primer mismatch due to a point mutation, or a more significant genetic alteration, we decided to assess sex genotype via two other popular PCR-based sex tests.

The 977/788 bp primer set, which amplifies a different region of the amelogenin gene, also typed the individual as female. This was demonstrated by the presence of the 977 bp (i.e., X-homolog product) alone (Fig. 2*a*). However, the other sex test, based on the DYZ1 repeat sequences (on the heterochromatic region of the long arm of the Y chromosome) clearly produced the characteristic 1024 bp fragment, demonstrating that the individual does carry a Y chromosome, and hence, was typed as male (Fig. 2*b*).

Discussion

As a step towards developing a National Criminal Identification DNA Database (NCIDD) similar to the UK database, the Aus-

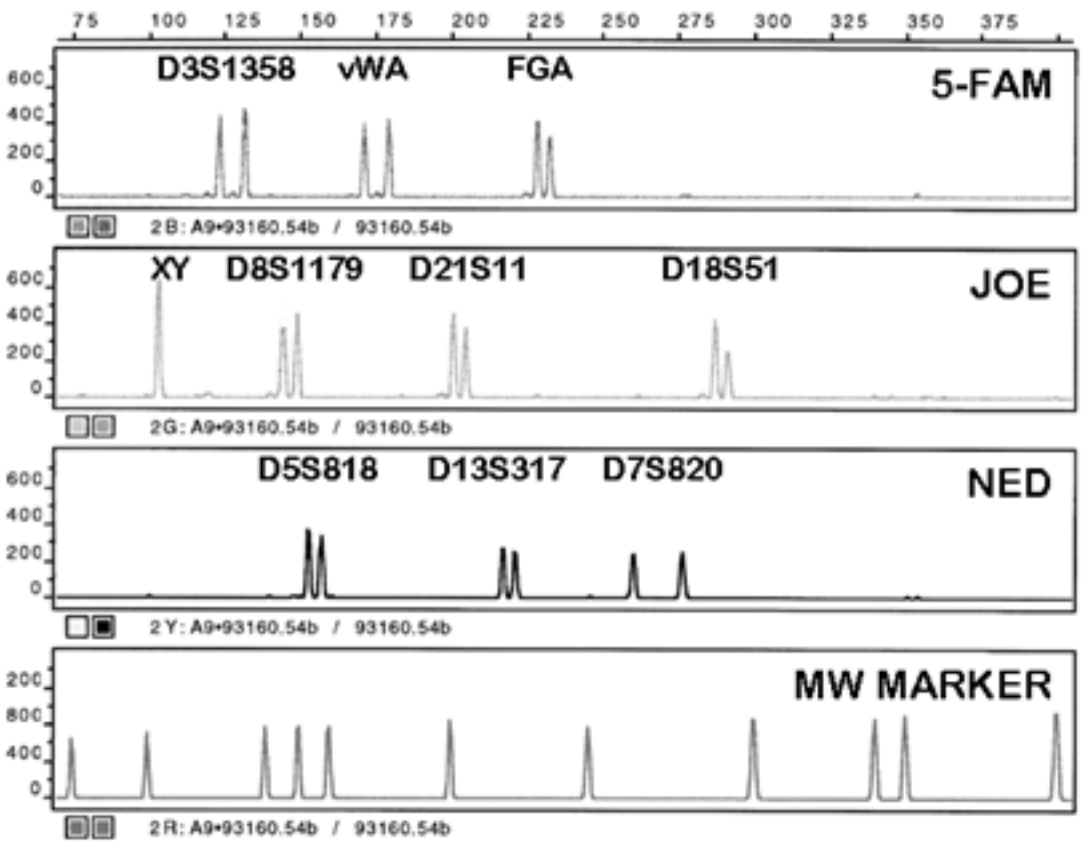

FIG. 1—*Electrophoretogram of the buccal sample amplified with the AmpFℓSTR Profiler Plus kit. The positions of the X and Y amelogenin amplification products (106 bp and 112 bp, respectively) are shown.*

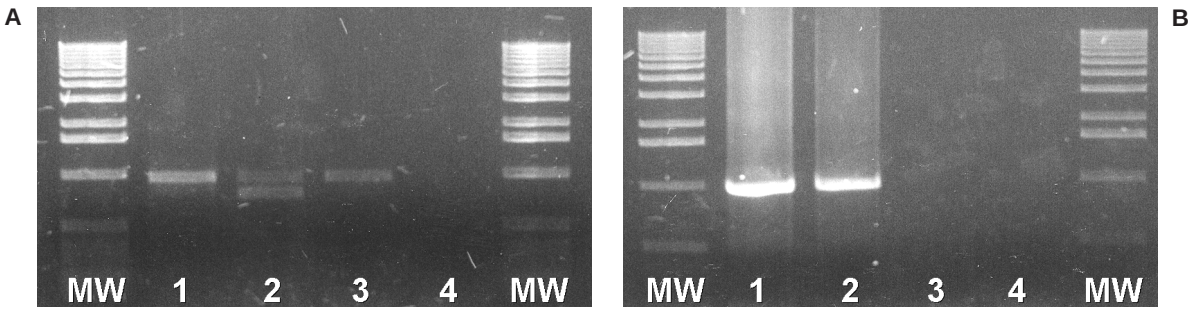

FIG. 2—*Buccal sample amplified with the* (A) *AMXY primers (977/788 bp X/Y products), and* (B) *DYZ1 primers (1024 bp Y product). Lane 1, buccal swab; Lane 2, male control (2 ng); Lane 3, female control (2 ng); Lane 4, negative control; MW, molecular weight marker (1 Kilobase ladder).*

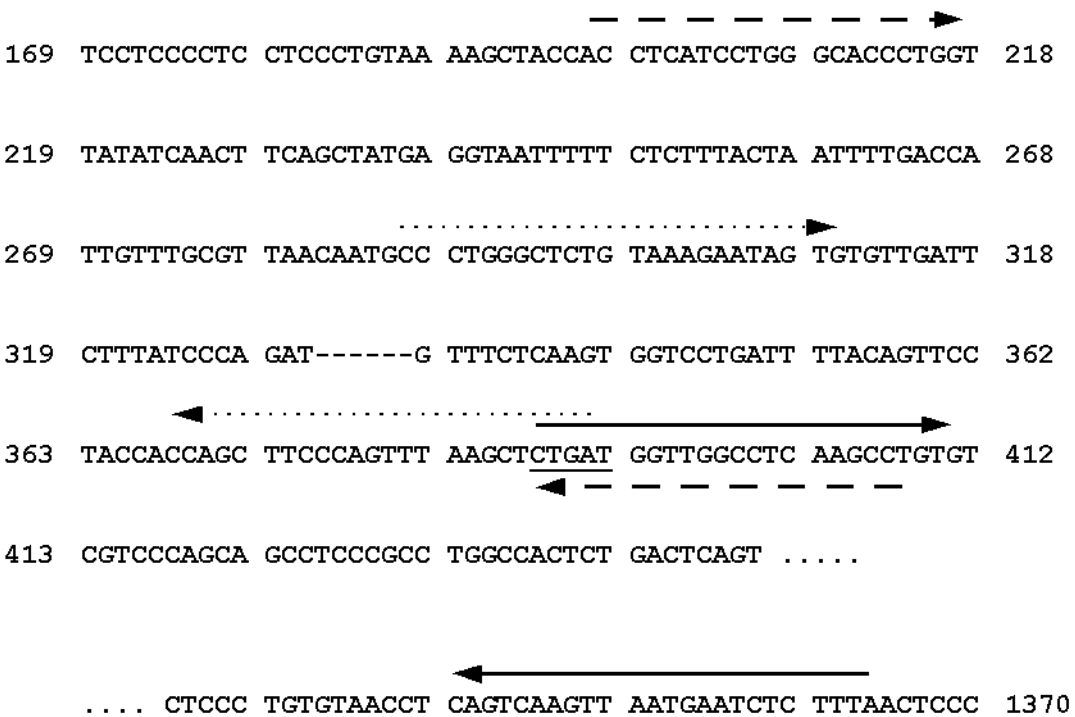

FIG. 3—*Annealing sites of primer sets of the three popular amelogenin tests in the amelogenin X homolog sequence as described by Nakahori, et al, 1991 (Genebank Accession Number M55418 J04777). Arrows indicate the position of primer sets; unbroken arrows, 977/788 bp primers; broken arrows, 212/218 bp primers; dotted arrows, 112/106 bp primers. The highlighted nucleotides are those common to the overlapping primers. The position of the 6 bp deletion on the X-homolog is shown (-------).*

tralian forensic science community has adopted the "Profiler Plus" kit (PE Biosystems), an STR nonaplex combined with amelogenin, as a standard methodology in the application of DNA profiling for investigative and forensic purposes. In constructing a Caucasian population database for the Northern Territory in Australia using this kit (which uses the 106/112 amelogenin primer set), the gender of a single individual could have been mistakenly identified.

In this case, it is likely that all of the popular multiplex profiling systems that incorporate an amelogenin-based sex test would have the potential to mistakenly identify the sex of this individual. All three of the commonly used primer sets (106/112 bp, 212/218 bp, 977/788 bp, X/Y, respectively) have one primer binding region of their pair that has at least a 5 bp region that overlaps with each of the other tests (Fig. 3). It is conceivable that a single point mutation in this overlapping region could be sufficient to cause the amplification dropout of the Y allele. However, the same result could also occur from a number of other genetic rearrangements, such as an insertion or deletion in the overlapping region, a small deletion or inversion spanning across the common primer binding sequences, or a more significant deletion of one or more of the primer binding regions of each primer pair.

It is well known that some polymorphic loci that are used for forensic profiling purposes, on occasions, can be affected by mutations that cause allelic dropout (e.g., 13–16). The AmpFℓSTR Profiler Plus system is a classic example of one of the current systems that has taken action to reduce the effects of known mutations that cause primer mismatch through the use of degenerate primers (PE Biosystems, personal communication). In any case,

with STR profiling, the worst implications of these mutations would be a full allelic dropout that results in a false homozygous genotype. Such mutations usually remain undetected and have little effect on the significance of a match, so long as the mutation is inherited throughout the somatic and sex cells of the individual and the same primer set is used on both unknown and reference samples. However, when measured genotypes are indicative of phenotypic traits, such as gender, these discrepancies could have quite dramatic effects. For example, it could greatly affect the perceived reliability of the forensic test as it is presented in a court of law, or more important, it could potentially influence or alter the progress of an active criminal investigation. In such instances where the progress of an investigation is greatly dependent upon the accuracy of gender determination via PCR-based DNA analyses, the verification of the gender of specimens from an independent locus may be worth considering.

Further testing on this individual using other Y-chromosome STRs, sequencing of the Y homolog and family studies is proposed.

Acknowledgments

The authors gratefully acknowledge the CSU-Small Granting Scheme, the Gilmore Centre, and the Biomedical Research Group (Charles Sturt University) for supporting this work. We also thank our professional colleagues for their critical review of this manuscript.

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