Consultations in Molecular Diagnostics

A Rare Mutation in the Primer Binding Region of the Amelogenin Gene Can Interfere with Gender Identification

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PCR amplification of part of the X-Y homologous amelogenin gene with a single primer pair has been used as a sex identification test because it generates different length products from the X and Y chromosomes. Using a commercially available kit that contains amelogenin primers, we report a single phenotypically normal Caucasian male out of 327 males tested to date that failed to show an X chromosome-specific PCR product. Using alternative amelogenin primers external to but encompassing the initial amplicon, an X chromosome-specific product was seen. Sequence analysis of this X-specific PCR product revealed a C to G mutation at the most 3' base of the initial reverse amelogenin PCR primer. An alternative reverse PCR primer with this most 3' base deleted showed X- and Y-specific products from the case study male. Rare mutations that result in a failure to amplify sex chromosome-specific products can result in incorrect gender identification. (*J Mol Diagn* 2004, 6:401–405)

Amelogenin is a protein of dental enamel that is present on the human X and Y chromosomes, and deletion of this gene causes X-linked amelogenesis imperfecta, a genetic disorder affecting enamel formation.^{1,2} Sequence differences between the X and Y homologues of the amelogenin gene have been used to differentiate males from females.^{1,3,4} Because both X- and Y-specific sequences can be amplified in a single reaction, amplification of the amelogenin gene offers the advantage of having an internal positive control since the X chromosome should always amplify. The two most commonly used amelogenin primer sets span a 6 base pair (bp) deletion on the X chromosome and generate fragments of 106/112 bp or 212/218 bp for X/Y products, respectively.⁴ Several companies manufacture amelogenin primer sets for

sex identification as well as multiplex short tandem repeat (STR) kits containing primers specific for the amelogenin gene which allows for individual as well as gender identification. The most widely used application of STR analysis is in forensics. However, DNA typing applications in pathology are gaining in popularity and include testing to monitor for bone marrow engraftment in patients undergoing allogeneic bone marrow transplants, evaluating the origin of tumors inadvertently transmitted by solid organ transplantation, and resolving sample mix-ups.^{5,6}

Several studies have reported mutations in the Y homologue of the amelogenin gene which can cause typing of males as females.^{7–10} Failure to amplify the Y homologue could result in dire consequences if used in cases of criminal investigation or for gender identification in the setting of prenatal diagnosis. In this study we report a male with a mutation in the annealing region of a commonly used amelogenin primer. This mutation resulted in the failure to amplify the X homologue.

Materials and Methods

Samples

DNA from the case study male, a normal male, and a normal female were extracted on the MagNA Pure Instrument (Roche Molecular Biochemicals, Mannheim, Germany) from 200 μ l of EDTA-anticoagulated whole blood using the MagNA Pure LC DNA Isolation Kit 1 (Roche Molecular Biochemicals) according to manufacturer's recommendations.

Amelogenin Typing Using GenePrint PowerPlex 16 and Sex Identification Systems

DNA samples were subjected to PCR using GenePrint PowerPlex 16 and GenePrint Sex Identification Systems

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Figure 1. Positions and sequences of oligonucleotide primers used for amelogenin PCR. Amel-A forward and Amel-A reverse 1 primers amplify a 106-bp X chromosome-specific product and a 112-bp Y chromosome-specific product. These primers are used in the PowerPlex 16 System. The Amel-A forward primer is fluorescent-labeled with 5' FAM (6-carboxyfluorescein). Amel-A reverse 1 and Amel-A reverse 2 are identical except Amel-A reverse 2 has the most 3' G removed. Amel-B forward and Amel-B reverse primers amplify a 212-bp X chromosome-specific product and a 218-bp Y chromosome-specific product, and these primers are used in the Sex Identification System. The five-base overlap (ATCAG) between the 5' end of the Amel-A reverse primer and the 3' end of the Amel-B primer is in **italics**.

(Promega Corporation, Madison, WI) according to the manufacturer's recommendations. Briefly, each PowerPlex 16 PCR reaction contained 1.25 μ l Promega 10X Gold ST[®]R Buffer, 1.25 μ l PowerPlex 16 10X primer pair mix, 2 units AmpliTaq Gold DNA Polymerase, $\sim$ 1 ng DNA, and sterile water to a final volume of 12.5 μ l. Each GenePrint Sex Identification PCR reaction contained 2.5 μ l Promega 10X Gold ST[®]R Buffer, 2.5 μ l amelogenin 10X primer pair (TMR), 4 units AmpliTaq Gold DNA polymerase, $\sim$ 1 ng DNA, and sterile water to a final volume of 25 μ l. PCR was performed in a MJ Research PTC-225 DNA Engine Tetrad (Waltham, MA) using the following conditions adapted from Promega's recommendations: 95°C for 11 minutes; 96°C for 1 minute; 10 cycles of: 94°C for 30 seconds, ramp 68 seconds to 60°C and hold for 30 seconds; ramp 50 seconds to 70°C and hold for 45 seconds; 22 cycles of: 90°C for 30 seconds, ramp 60 seconds to 60°C and hold for 30 seconds; ramp 50 seconds to 70°C and hold for 45 seconds; 60°C for 30 minutes; and 4°C hold. One μ l of PCR product was mixed with 12.5 μ l of deionized formamide and 0.5 μ l of ILS600 ladder (Promega Corporation) as an internal lane size standard. Reaction products were detected using an ABI PRISM 310 Genetic Analyzer (3-second injection, 15 kV, GS STR POP4 (1 ml) A module, 60°C, 30-minute run time) with a 47 cm $\times$ 50 μ m capillary and Performance Optimized Polymer 4 (Applied Biosystems, Foster City, CA). Allele designations for the PowerPlex 16 System were determined using the PowerTyper 16 Macro (Promega Corporation). GeneScan and Genotyper softwares (Applied Biosystems) were used to size PCR products generated from the GenePrint Sex Identification System.

Sequence Analysis

Amel-B forward and Amel-B reverse primers were purchased from Gibco BRL (Rockville, MD). These primers generate the 212/218-bp fragments for X/Y, respectively, found in the GenePrint Sex Identification System. Primer sequences are listed in Figure 1 and were kindly pro-

vided by Promega Corporation. These primers were not fluorescently labeled and were used for sequencing. Each 50- μ l PCR reaction contained 1 μ l MagNA Pure DNA ($\sim$ 50 to 100 ng), 5 μ l Promega 10X Gold ST[®]R buffer, 8 units AmpliTaq Gold DNA Polymerase (Applied Biosystems), and final primer concentrations of 0.5 μ mol/L Amel-B forward and 0.5 μ mol/L Amel-B reverse. Thermocycling was performed as described above. PCR products from the case study male and a normal male were ligated into the PCR 2.1 vector and transformed into One Shot TOP10 competent *E. coli* provided in a TA cloning kit (Invitrogen, Carlsbad, CA) following manufacturer's recommendations. Individual colonies (10 from the case study male and 10 from the normal male) were picked and placed individually and directly into 50 μ l of master mix containing 1.5 units AmpliTaq Gold DNA Polymerase (Applied Biosystems), and final reagent concentrations of 0.2 mmol/L of each dNTP, 0.2 μ mol/L Amel-B forward primer, 0.2 μ mol/L Amel-B reverse primer, 1.5 mmol/L MgCl₂, and 1X PCR buffer (Applied Biosystems). Thermocycling was performed as described above. Twenty μ l of the resultant PCR products was electrophoresed on a 2.5% NuSieve 3:1 (BioWhittaker Molecular Applications, Rockland, ME) agarose gel. Bands were visualized by ethidium bromide staining of the gel. The remaining 30- μ l of PCR product from two X-specific case study male clones and two X-specific normal male clones were purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). Purified PCR products were subjected to DNA sequencing using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems) followed by analysis on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with a 50-cm array and Performance Optimized Polymer 6 according to the manufacturer's recommendations. Forward and reverse strands were sequenced using Amel-B forward and Amel-B reverse primers, respectively.

Allele-Specific PCR

Amel-A primers (Gibco BRL) generate the 106-bp X-specific and 112-bp Y-specific products found in the PowerPlex 16 System. Primer sequences were provided by Promega Corporation and are listed in Figure 1. The Amel-A forward primer was labeled with 5' FAM (6-carboxyfluorescein). Amel-A reverse 1 is identical to the reverse primer found in the PowerPlex 16 System. Amel-A reverse 2 is identical to Amel-A reverse 1 except the terminal 3' base (G) was removed. Each 50 μ l PCR reaction contained 1 μ l MagNA Pure DNA ($\sim$ 50 to 100 ng), 25 μ l 2X TaqPCR master mix (Qiagen) and 0.5 μ mol/L Amel-A forward primer and 0.5 μ mol/L Amel-A reverse 1 or 2 primers. PCR was performed using one cycle of 95°C for 3 minutes followed by 49 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 15 seconds with a final 10-minute extension at 72°C and a 4°C hold. Approximately 0.01 μ l of the PCR products were mixed with 12 μ l of deionized formamide and 0.5 μ l of GeneScan 400 HD Rox size standard (Applied Biosystems). Mixtures were injected onto an ABI Prism 310

Figure 2. Amelogenin PCR using the PowerPlex 16 and Sex Identification Systems. DNA from a normal male (**A** and **D**), a normal female (**B** and **E**), and the case study male (**C** and **F**) were subjected to PCR using the PowerPlex 16 (**A** to **C**) and Sex Identification (**D** to **F**) Systems. XY designations for the PowerPlex 16 System were determined using the PowerTyper 16 Macro. GeneScan and Genotyper softwares were used to size the X (~210 bp) and Y (~216 bp) PCR products generated from the Sex Identification System. **Horizontal axes** represent PCR product sizes in bp. **Vertical axes** denote fluorescent intensity in relative units.

Genetic Analyzer (3-second injection, 15 kV, GS STR POP4 (1 ml) D module, 60°C, 20-minute run time) and data were analyzed with Genescan and Genotyper softwares.

Results

Figure 1 shows the positions and sequences of the amelogenin primers used in the current study. Amelogenin primer sequences in the PowerPlex 16 (Amel-A forward and Amel-A reverse 1 primers) and GenePrint Sex Identification (Amel-B forward and Amel-B reverse primers) Systems were kindly provided by Promega Corporation. Using the PowerPlex16 System, expected PCR product sizes for X and Y are 106 bp and 112 bp, respectively. Expected PCR product sizes in the GenePrint Sex Identification System are 212 bp for X and 218 bp for Y. The X/Y PCR products generated using the PowerPlex 16 primers are within the X/Y PCR products generated using the GenePrint Sex Identification primers. The 5' end of the Amel-A forward primer is 69 bp downstream from the 3' end of the Amel-B forward primer. There is a 5-base overlap between the 5' end of Amel-A reverse 1 and 2 primers and the 3' end of the Amel-B reverse primer. Amel-A reverse 2 primer is identical to the Amel-A reverse 1 primer except the most 3' base (G) has been removed.

Figure 2 shows amelogenin PCR results using the PowerPlex 16 and Sex Identification Systems from a normal male, a normal female, and the case study. The case study was a Caucasian male who underwent an allogeneic bone marrow transplant for acute myelogenous leukemia. Review of electronic medical records revealed that this male had no phenotypic abnormalities and that he had two normal children. At the time of bone marrow transplantation, a bone marrow biopsy was performed and was negative for leukemia. Cytogenetics on the bone

marrow sample showed a normal male karyotype (46, XY). Pre-transplant recipient (and donor) DNAs were submitted to the Molecular Genetic Pathology Laboratory at the Cleveland Clinic Foundation for STR analysis using the PowerPlex 16 System to identify informative loci that could be used to monitor engraftment in post-transplant specimens.⁶

Using the PowerPlex 16 System, the amelogenin primers generate an X-specific product (~104 bp) in the normal male (Figure 2A) and normal female (Figure 2B) but not in the case study male (Figure 2C). A Y-specific product (~110 bp) is present in the normal male (Figure 2A) and case study male (Figure 2C) but not in the female (Figure 2B). Using the Sex Identification System, a ~210-bp X-specific product is seen in all three samples (Figure 2, D to F) while a ~216-bp Y-specific product is seen in the two male samples only (Figure 2, D and F). Since an X chromosome was present in a bone marrow biopsy from the case study male by cytogenetics and an X-specific PCR product was observed using the primers in the Sex Identification System, this suggested the X homologue contained a mutation, most likely involving a PCR primer-binding region specific for the primers in the PowerPlex 16 System. It is not clear why the PowerPlex 16 and Sex Identification Systems generate X/Y product sizes of 104/110 bp (PowerPlex 16) and 210/216 bp (Sex Identification System) rather than the expected sizes of 106/112 bp (PowerPlex 16) and 212/218 bp (Sex Identification System). X/Y sizes of 103/109 bp rather than 106/112 bp have been reported previously.⁹ The size differences were attributed to imprecision of the ABI310 Genetic Analyzer.⁹ It is also possible that the fluorescent label on one of the primers affects migration.

To investigate whether there was a mutation on the X chromosome in the case study, male, non-fluorescent primers (Amel-B forward and Amel-B reverse) used in the Sex Identification System were synthesized. DNA from the case study male as well as DNA from a normal male and female was subjected to PCR using these primers. Following agarose gel electrophoresis of the resultant PCR products, an ~212-bp X-specific band was seen in all three samples and an ~218-bp Y-specific band was seen in the two male samples (data not shown). The X-specific bands from the case study male, a normal male, and a normal female were gel-purified and sequenced. While the sequence from the female was clean and easily readable, the sequences from the two male samples became difficult to read at the site of the 6-bp deletion of the X homologue (data not shown). This difficult to read sequence may have resulted from contamination of X sequence with Y sequence when the X-specific band was excised from the gel and/or heteroduplex formation between X and Y amplicons.¹¹

To circumvent this problem and to obtain "pure X" sequence, the PCR products generated with Amel-B forward and Amel-B reverse primers from the case study male and a normal male were subcloned into a TA cloning kit. Two pure "X clones" from the case study male and two pure "X clones" from the normal male were sequenced using Amel-B forward and Amel-B reverse primers. The two case study male clones both showed a C to

Figure 3. Sequence analysis of the forward strand of the X chromosome from the case study male (**top**) and a normal male (**bottom**). The **arrows** show a C to G mutation in the case study male. The complement of the Amel-A reverse 1 primer sequence is outlined.

G mutation at the most 3' base of the Amel-A reverse 1 primer. Figure 3 shows sequencing results from one of the clones from the case study male (top) and from one of the clones from the normal male (bottom).

Because correct base-pairing at the 3' end of PCR primers is the basis of allele-specific PCR,¹² we hypothesized that the X chromosome would amplify from the case study male using the Amel-A reverse 1 primer with the 3' G deleted (ie, Amel-A reverse 2 primer). Figure 4 shows PCR results from the case study male, a normal male, and a normal female using Amel-A forward (FAM-labeled) primer and two Amel-A reverse primers. Using Amel-A forward and Amel A reverse-1 primers, an X-specific product is seen in the normal male (Figure 4A) and normal female (Figure 4B) but not in the case study male (Figure 4C). A Y-specific product is seen in the case study male (Figure 4C) and normal male (Figure 4A). These are the expected results since these are the same primers used in the PowerPlex 16 System which failed to give an X product in the case study male (Figure 1C). Again, X/Y product sizes were slightly different from the expected sizes of 106/112 bp. Using Amel-A forward and

Figure 4. Amelogenin and allele-specific PCR. DNA from a normal male (**A** and **D**), a normal female (**B** and **E**), and the case study male (**C** and **F**) were subjected to PCR using the Amel-A forward (FAM-labeled) and Amel-A reverse 1 (**A** to **C**) and Amel-A reverse 2 (**D** to **F**) primers. GeneScan and Genotyper softwares were used to size the X (~103 bp) and Y (~109 bp) PCR products. **Horizontal axes** denote PCR product sizes in bp while **vertical axes** denote fluorescent intensity in relative units.

Amel-A reverse 2 primers, X- and Y-specific products are seen in the normal male (Figure 4D) and case study male (Figure 4F) while the normal female (Figure 4E) only shows X. These are expected results since the most 3'G of the Amel-A reverse 1 primer which resulted in allelic dropout of the X chromosome in the case study male has been removed. The peaks in Figure 4 appear split and most likely resulted from the 3' nucleotide addition of adenine by *Taq*DNA polymerase.¹³

Discussion

In this study we report a phenotypically normal Caucasian male with a C to G transversion in intron 1 of the amelogenin gene. Because the alteration was intronic and did not create or alter splice site acceptor or donor sites, it is not expected to lead to any functional consequences. The alteration was rare and was seen only in one individual out of 327 sequential males tested, generating an allele frequency of 0.3%. This is consistent with the alteration being a mutation rather than a polymorphism. In females, one cannot differentiate amplification of both X chromosomes from amplification of a single X chromosome so the possibility that the mutation occurred in females cannot be excluded.

The mutation was located at the most 3' base of a commonly used reverse amelogenin primer and led to allelic dropout of the X chromosome. It is well known that correct-base pairing at the 3' end of PCR primers is the principle of allele-specific PCR and forms the basis for the amplification refractory mutation system (ARMS).¹² To confirm that the primer binding site mutation was responsible for the allelic dropout of the X homologue, a new reverse primer that lacked the most 3' base of the initial amelogenin reverse primer was synthesized. Using this new reverse primer, the X homologue amplified in the case study male.

To our knowledge, this mutation and the failure to amplify the X homologue of the amelogenin gene has not been reported previously. Failure to amplify sex chromosome-specific products can result in incorrect gender identification. Several previous studies, however, have reported failures in amplifying the Y homologue due to Y chromosome deletions.^{7,8} Roffey et al⁹ reported failure to amplify the Y homologue from a normal male and attributed this failure to a mutation in the annealing region of the amelogenin primer because alternate amelogenin primers produced a Y product. Although the frequency of failing to amplify the Y chromosome is low [0.02% (6 of 29,432) in Austrian males,⁷ 1.85% (5 of 270) in Indian males,⁸ 8% (2 of 24) in Sri Lankan males¹⁰], the consequences of failing to amplify the Y chromosome could be dramatic in forensic/rape cases, in identifying human remains from mass disasters, and for gender identification in the setting of prenatal diagnosis of X-linked recessive diseases. It has been suggested that multiple Y chromosome markers be analyzed in cases where sex identification is critical.⁷ Failure to amplify the X chromosome would not be expected to lead to such consequences. However, if used quantitatively to determine

certain sex chromosome aneuploidies (for instance, XXY),⁴ failure to amplify the X homologue could lead to incorrect determinations of X copy number and subsequent incorrect characterization of sex chromosome abnormalities.

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