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ORIGINAL ARTICLE

Null alleles of the X and Y chromosomal amelogenin gene in a Chinese population

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Abstract The use of amelogenin locus typing as a gender marker incorporated in short tandem repeat (STR) multiplexes is a common practice in sex typing. Mutations in the X or Y homologue of the amelogenin gene can be misleading and result in serious mistakes in forensic applications and prenatal diagnosis. In these present studies, the amelogenin gene of 8,087 unrelated male individuals from Chinese Han population was genotyped with Powerplex® 16 system. The samples that showed discordant results were taken for frequency calculation and further validated by re-amplification with different primer sets, Y-STR typing, and sequencing. Our results describe six amelogenin X-allele (*AMELX*) or amelogenin Y-allele (*AMELY*) null cases in these studied subjects with an overall prevalence of 0.074%. Further validation revealed point mutations in the amelogenin-priming sites associated with *AMELX* nulls (three cases, 0.037%) and deletions on the Y chromosome encompassing the *AMELY* and other Y-STR loci with three *AMELY* nulls (0.037%). These mutations and failure of the amplification of the *AMELX* and *AMELY* alleles have not been reported for the Chinese population. These and previous findings suggest that mutations in the amelogenin gene may result in amplification failure of the *AMELX* or *AMELY* allele, and an additional gender test for unambiguous sex determination may be needed.

Keywords Amelogenin gene (*AMEL)* · Null allele · Deletion · Variant · Chinese Han population

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Introduction

Homologues of the amelogenin (*AMEL*) gene, which codes for a protein of dental enamel, are located on Xp22.1-22.3 and Yp11.2 [1]. X-chromosome amelogenin gene (*AMELX*) and Y-chromosome amelogenin gene (*AMELY*) differ both in size and sequence and thus can be explored for sexing. The most commonly used test relies on the simultaneous PCR amplification of differently sized *AMELX* and *AMELY* fragments, flanking a 6-bp deletion of *AMELX*, resulting in 106 and 112 bp PCR products from the *AMELX* and *AMELY* gene, respectively [2]. Commercially available kits for genotyping short tandem repeats (STRs) usually contain the *AMEL* primers and have been widely used in DNA databasing, forensic casework, prenatal diagnoses, and transplantation monitoring. Several studies have reported mutations in the X or Y homologue of the *AMEL* gene, which could cause amplification failure of *AMELX* or *AMELY* and result in incorrect sex identification [3–12]. In the Chinese population, however, some scattered cases with *AMEL* gene mutations have been reported [5–7], although no systematic analysis of the frequency data based on large samples has been done. The present study describes the types and frequencies of *AMEL* gene variants in 8,087

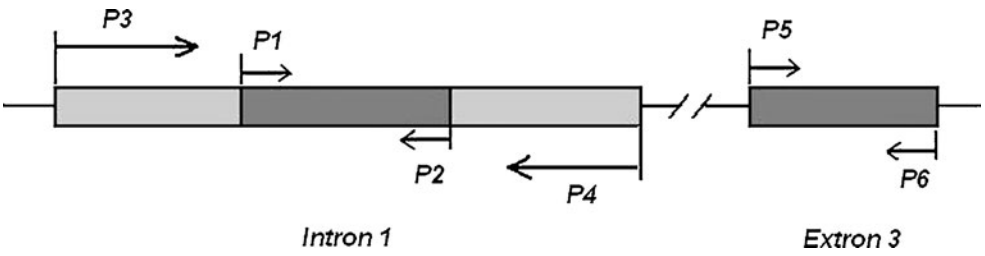

Fig. 1 The annealing sites of three alternative *AMEL* primer sets

unrelated male individuals from Chinese Han population and discusses the influence on sex determination.

Materials and methods

Samples

Blood samples deposited on qualitative filter papers from 8,087 unrelated phenotypically normal Han males in Guangdong Province, Southern China, were collected with an informed consent.

Amelogenin genotyping using multiplex STR kit

Genomic DNA was extracted from blood spots using the Chelex-100 method. DNA samples were amplified using PowerPlex® 16 system [13] (Promega Corporation, Madison, USA) containing the *AMEL* primers (106/112 bp) described by Krenke [14] et al. for gender determination. The amplified PCR products were separated by capillary electrophoresis on an ABI PRISM 3100 genetic analyzer (Applied Biosystems, USA) and analyzed with GeneMapper™ ID v3.2 software.

Amelogenin genotyping using single *AMEL* primer sets

Samples that showed amplification of only *AMEL-X* or *AMEL-Y* products were subjected to re-extraction and re-amplification with PowerPlex® 16 system and then identified. Further examinations were performed with three alternate single *AMEL* primer pairs [2, 15] (Fig. 1; Table 1).

The thermal cycle conditions were: 95°C for 2 min, and then 30 cycles at 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were separated by non-denaturing polyacrylamide gel electrophoresis (PAGE) (T=6%, C=3.3%) and subsequent silver staining.

DNA sequencing

The alleles which were absent with PowerPlex® 16 system while present with single *AMEL* primer pairs *P3/P4* were separated from the gel. DNA was recovered and re-amplified with the same PCR conditions as described above. All the gel-separated alleles were direct sequenced with BigDye™ Terminator v3.1 (Applied Biosystems).

Genotyping of *AMEL-Y* nulls with 17 Y-STR markers

Samples that failed the amplification of the *AMEL-Y* allele were also typed using a commercial AmpFlSTR® Yfiler kit (Applied Biosystems), which included 17 Y-STR loci. The amplified PCR products were separated by capillary electrophoresis on an ABI PRISM 3100 genetic analyzer (Applied Biosystems, USA) and analyzed with GeneMapper™ ID v3.2 software.

DYS458 amplification using alternative primer sets

DYS458 null samples were re-amplified with three alternate single DYS458 primers (Table 2) followed by non-denaturing PAGE and subsequent silver staining as described above.

Table 1 Three sets of primer pairs for *AMEL* genotyping

Target sequence	Primer name	Primer sequence	Product size (bp)
Intron 1	P1	5′-CCCTGGGCTCTGTAAAGAA-3′	*AMELX*, 106
	P2	5′-ATCAGAGCTTAAACTGGGAAGCTG-3′	*AMELY*, 112
Intron 1	P3	5′-ACCTCATCCTGGGCACCCTGG-3′	*AMELX*, 212
	P4	5′-AGGCTTGAGGCCAACCATCAG-3′	*AMELY*, 218
Extron 3	P5	5’-CCCTTTGAAGTGGTACCAGAGCA-3’	*AMELX*, 80
	P6	5’-GCATGCCTAATATTTTCAGGGAATA-3’	*AMELY*, 83

Table 2 Three alternative primer sets for DYS458

Primer sequence (5′-3′)	Product size (bp) (repeats)	Reference
5′-GCAACAGGAATGAAACTCCAAT-3′ 5′-GTTCTGGCATTACAAGCATGAG-3′	132–160 (13–20)	Schoske et al. [20]
5′-GGGTGGTGGAGGTTACTGTG-3′ 5′-CTAGAGGTTCCTGCCACCAC-3′	320 (16)	Kline et al. [21]
5′-GGTGGTGGAGGTTACTGTGA-3′ 5′-TTCCTGACCTTGTGATCCAG-3′	225–237 (14–17)	Ehler et al. [22]

Data analysis

Chi-square test was employed for pairwise population comparisons. Excel program and SPSS 16.0 for windows software were used for the analysis of data. The level of significance was 0.05 for all statistical tests.

Results and discussions

Allelic dropouts of the *AMEL* gene

For the 8,087 unrelated Chinese Han male individuals, 6 (samples 1–6) showed abnormal *AMEL* typing results using the PowerPlex® 16 kit (Fig. 2), with an overall prevalence of 0.074%. Among these discordant samples, three *AMELX* nulls (samples 1–3, 0.037%) and three *AMELY* nulls (samples 4–6, 0.037%) were observed. All loci of autosomal STR of these six samples were successfully amplified and genotyped.

AMEL typing of discordant samples with three sets of alternate single primers

One possible explanation for these observed *AMEL* allelic dropouts could be point mutation(s) within the annealing region of the primers that led to amplification failure. To determine if this was a problem, *AMEL* was amplified using alternative primers. The *P1/P2* amplification system exhibited the same phenomenon in samples 1–6 as did the commercial kits, the absence of the *AMELX* or *AMELY* amplicons. Using alternative *AMEL P3/P4* and *P5/P6* primers, X-chromosome-specific product was seen in samples 1–3, while the Y-specific amplicons in samples

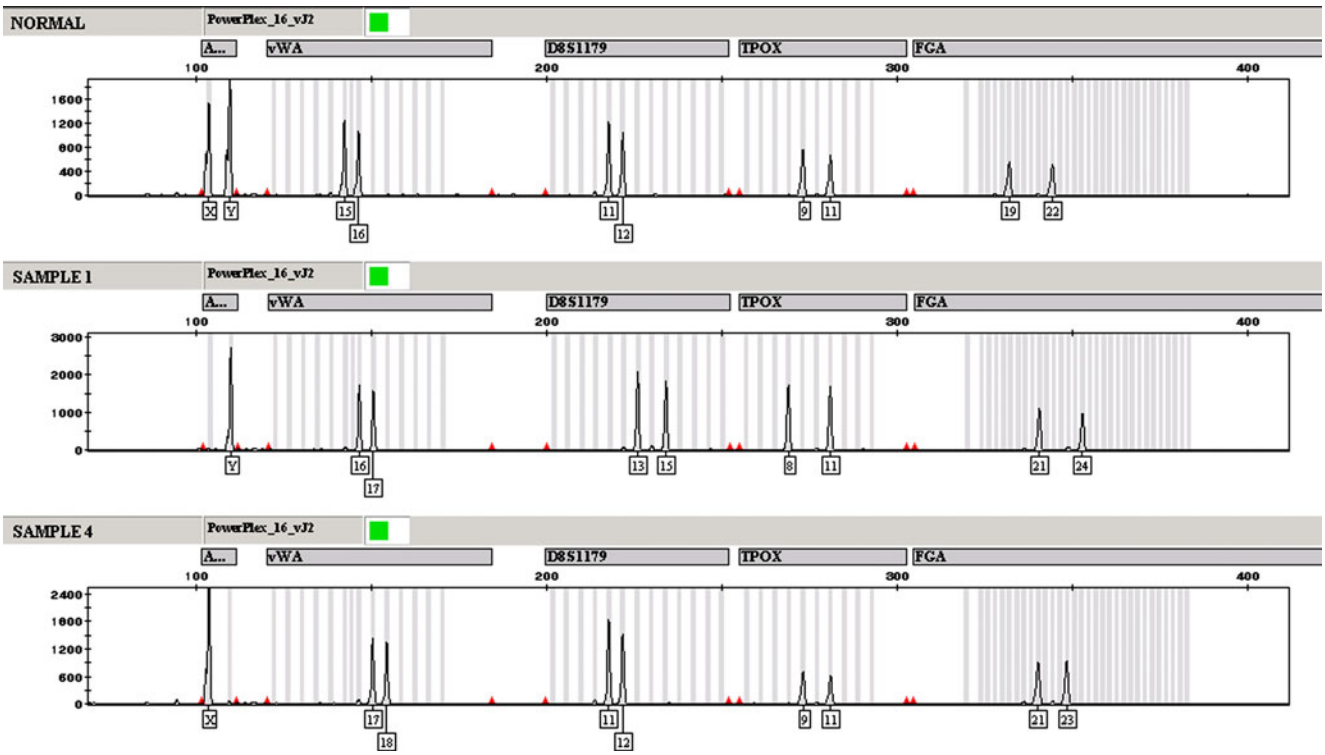

Fig. 2 Typical electrophoretograms of normal and abnormal *AMEL*-typing samples (**a**) normal male sample; (**b**) phenotypically normal male sample with *AMELX* allelic dropout; (**c**) phenotypically normal male sample with *AMELY* allelic dropout

Table 3 *AMEL* genotyping results of six discordant male samples with PowerPlex® 16 kit and three sets of alternate primers

Sample name	PowerPlex® 16	P1/P2	P3/P4	P5/P6
Normal male	X/Y	X/Y	X/Y	X/Y
Sample 1	–/Y	–/Y	X/Y	X/Y
Sample 2	–/Y	–/Y	X/Y	X/Y
Sample 3	–/Y	–/Y	X/Y	X/Y
Sample 4	X/–	X/–	X/–	X/–
Sample 5	X/–	X/–	X/–	X/–
Sample 6	X/–	X/–	X/–	X/–

– no specific amplicon

4–6 were still absent (Table 3). These three samples were confirmed as *AMELY* null males because the PCR products of *AMELY* failed to amplify when using any primer sets.

Sequencing analysis of the PCR products of *AMELX* alleles with *P3/P4* primer pair

Re-amplification with *P3/P4* primers, which encompass the initial amplicon by *AMEL* primers incorporated in a commercial kit, resulted in X-specific bands in these three *AMELX* nulls. Direct sequencing of the resulted *AMELX P3/P4* amplicon revealed three types of point mutations at the *AMEL* forward primer-binding sites of Powerplex® 16 kit: (1) G-to-A mutation at the thirteenth to the 3′ base of primer (sample 1); (2) A-to-G mutation at the second to the 3′ base (sample 2); 3) Y (G+A) heterozygous at both of the two sites mentioned above (sample 3). To our knowledge, the first type of mutation of this *AMELX* gene has not been described, while the second type is identical to that reported by Gong et al. [5]. Based on a case study of a Chinese group, Shadrach et al. [4] investigated a sample of 327 American males and found a C-to-G transversion at the most 3′ base of the reverse primer-binding site in one *AMELX* null sample, which was different from the results reported here. The same C-to-G transversion was also found in a single individual among 5,534 Polish males [16], and only one Caucasian male fetus out of 43,000 samples underwent prenatal screening of aneuploidies [17]. In this study, the observed *AMELX* null frequency was 0.037%.

It is difficult to detect *AMELX* dropout in females, unless simultaneous mutations occur in two X chromosomes, which is very rare. Failure to amplify one X chromosome would not lead to incorrect sex identification. However, if used quantitatively to determine certain sex chromosome aneuploidies (e.g., XXY), failure to amplify the X homologue could lead to misleading determination of X copy number and subsequent incorrect characterization of sex chromosome abnormalities. Its occurrence can also impact the expected X and Y amplicon ratios in a mixture in forensic casework.

Y-STR genotyping of three samples with *AMELY* allelic dropout

Genotyping of 17 Y-STR markers (Fig. 3) revealed the absence of the DYS458 locus both in samples 4 and 5, which has been reported previously [9–12]. Using three alternative single DY458 primer sets, the specific DY458 amplicons in samples 4 and 5 were still absent (Table 3), suggesting a deletion of DY458 region rather than a point mutation at primer sites. In addition, the DYS456 locus was also absent in sample 5, indicating a deletion of at least 3.60 Mb of the Yp11.2 region. Sample 6 only amplified successfully for DYS456, DYS458, DYS19, and DYS393 out of 17 Y-STR loci (Fig. 4), which implies a deletion polymorphism spanning a major part of the Y chromosome from Yp11.2 on the short arm up to some region on the long arm, although the exact size and nature of the deletion (single-point mutations at a number of positions or a single large deletion) need further investigation.

Deletion of the sequences on theYp11.2 region has been observed in males showing different haplotypes and haplogroups [7–12]. Lattanzi et al. [9] described a large interstitial deletion spanning approximately 2.5 Mb of the Y short arm encompassing the *AMELY* locus in infertile males and one amniotic liquid sample for prenatal diagnosis by performing pulsed-field gel electrophoresis, followed by fluorescence in situ hybridization and sequence-tagged sites (STS) marker analysis. Chang et al. [7] reported a distinct Y-STR haplotype characterized by a large Yp11.2 (DYS458-MSY1-AMEL-Y) deletion of at least 1.13 Mb in 18 males (14 Indians, 4 Malays). Recently, Takayama et al. [12] revealed three deleted regions of approximately 2.51 Mb,

Fig. 3 Sequence comparisons of normal and three *AMELX*-negative samples; (*gray highlight*) binding site of primer pair *P3/P4*; (*straight line*) binding site of primer pair used in PowerPlex® 16 system (*straight line*); Y=A+G

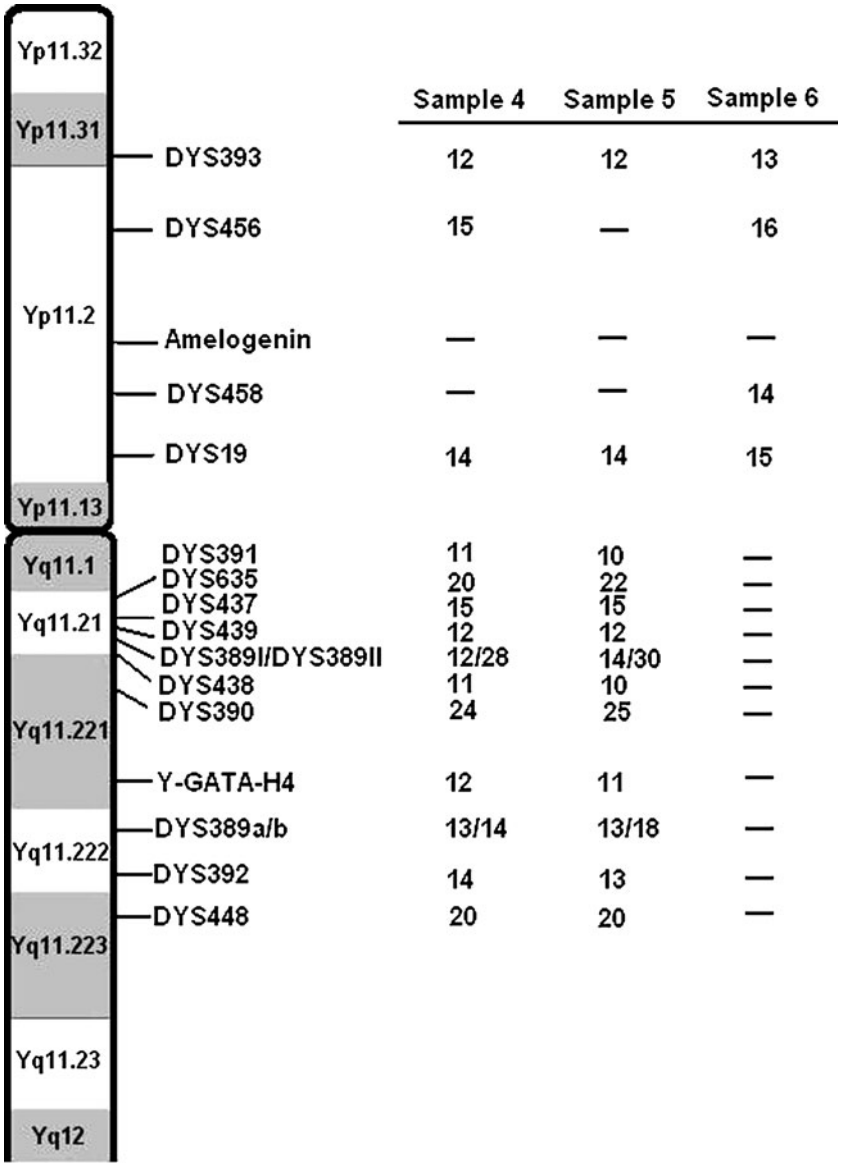

Fig. 4 Typing results of the *AMELY* gene and 17 Y-STR loci for three samples with *AMELY* allele dropout, (*straight line*) no specific amplicon

25 kb, and 834 b in Yp11.2 in one Japanese male (0.2%, n=500) using a total of 60 loci from Y-STRs, STSs, and newly designed primer sets.

The frequency of *AMELY* dropouts presented here is 0.037%, which is similar with that in a previous study based on 541 Chinese male samples (p>0.05) [7]. Moreover, we compared our results with published *AMELY* deletion frequencies for other populations from Indian [10, 18], Australian [3], and Malaysian [10] population. The statistically significant differences (p<0.05) were both

Table 4 Deletions observed at *AMELY* in different population groups

No.	Population	No. of nulls/individuals studied	Frequency (%)	95% confidence limits	p value[a]
1	Chinese (current study)	3/8,087	0.037	0–0.00079	–
2	Chinese [7]	0/541	0	0–0	1.000
3	Malaysian Malays [10]	2/334	0.599	0–0.01426	0.003
4	Malaysian Indians [10]	10/315	3.175	0.01238–0.05111	0.000
5	Indians (general) [18]	10/4,257	0.235	0.00089–0.00380	0.003
6	Austrians [3]	5/28,182	0.018	0–0.00033	0.543

[a] P versus no. 1

found between these studied populations versus the Indian population and versus the Malaysian population (Table 4). Even though the frequency of the *AMEL* deletion is low, considering the relevance of sex identification in prenatal diagnosis of sex chromosome-linked recessive diseases, forensic criminal investigations, and the consequences of gender misidentification, conclusions about gender should not be drawn based solely on the *AMEL* typing. Additional gender tests such as SRY and/or Y-STR testing should be considered for unambiguous gender identification [19].

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