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TECHNICAL NOTE

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Characterization of Deletions in the DYS385 Flanking Region and Null Alleles Associated with *AZFc* Microdeletions in Koreans

ABSTRACT: Eight DYS385 allele size discrepancies and six DYS448 null types were detected among 708 Korean men when results of three in-house multiplex short tandem repeat (STR) systems were compared. The systems included both ordinary and reduced size amplicons. Sequence analysis revealed deletion mutations at two sites upstream of the DYS385 core repeats and deletion of the entire DYS448 locus. At DYS385, allele size differences were one or two repeats and were dependent on the primer set used for polymerase chain reaction (PCR) amplification. Location of the primer target sequence in a flanking region of the STR, distal or proximal to the deletion, determined allele size. Two widely used commercial kits amplify DYS385 so as to include the mutable sites. Arrangement analysis of sequence tagged sites demonstrated that the deletion patterns at DYS448 (and DYS464) were associated with arrangements of the azoospermia factor c gene (*AZFc*). The DYS448 deletion appears relatively frequent in Asians.

KEYWORDS: forensic science, Y-chromosomal short tandem repeat, DYS385, DYS448, flanking region mutation, null allele, *AZFc* deletion, Koreans

Y-chromosome short tandem repeats (STRs) are amplified and analyzed by a polymerase chain reaction (PCR) multiplex assay using in-house PCR sets or commercial multiplex kits (1). Each PCR multiplex uses locus-specific primers that are designed to bind to conserved flanking sequences of an STR locus. However, mutations in flanking regions may interfere with the primer binding and bring about allele dropout (2). In addition, deletion or insertion mutations in flanking regions may cause errors in allele designation and discrepancies among different PCR sets or commercial kits if one is designed to amplify a locus along with the mutated flanking regions (3). Therefore, one should be aware of the effects of specific methods and reagents in investigations and database constructions or analyses.

In our laboratory, 708 Korean males, including individuals previously typed for 19 or 22 Y-STRs (4,5), were analyzed using three in-house multiplex PCR systems (4) which contain both ordinary and reduced size amplicons for DYS385. For two different primer pairs, eight samples showed discordant allele designation at DYS385. In addition, six samples displayed a null type at DYS448. Here, we study and discuss the molecular basis for these allele designation discrepancies and null alleles.

Materials and Methods

DNA Samples and Genotyping

Blood or buccal samples from 708 unrelated Korean males, including 355 males already typed for 22 Y-STRs (4) and 301 males typed for 19 Y-STRs (5), were analyzed. Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. A total of 9948 male DNA (Promega, Madison, WI) was used to calibrate allelic ladders. PCR and genotyping were carried out using three multiplexes according to our previous report (4).

PCR Amplification and Sequence Analysis for DYS385

Locus-discrimination primers for DYS385a (AC007379) and DYS385b (AC022486) were designed using the Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). These primers were used for the analysis of samples with allele designation discrepancies between the different primer pairs of multiplex I/II and multiplex III (Fig. 1). To compare our primer pairs with commercial systems, we used the Powerplex® Y (Promega) and the AmpFlSTR® Yfiler™ (Applied Biosystems, Foster City, CA) kit according to the manufacturer's recommendations.

Sequence analyses were performed on the flanking region of samples with discordant allele designation. Each PCR products was cloned using pGEM®-T Easy Vector System I (Promega) following the manufacturer's recommendation, and then sequenced on an ABI PRISM 310 Genetic Analyzer using a BigDye Terminator Cycle Sequencing v2.0 Ready Reaction kit (Applied Biosystems).

Multiplex PCR Assay to Characterize the Null Allele at DYS448

To detect rearrangements at the azoospermia factor c (*AZFc*) region, we amplified five *AZFc*-specific sequence tagged site (STS) markers, sY1161, sY1191, sY1201, sY1206, and sY1291, and a control gene pair ZFX/ZFY in samples with a null allele at DYS448. Primers for the five markers and the ZFX/ZFY gene were the same as used by Lin et al. (6). Multiplex PCR reactions were performed in a total volume of 10 μL containing 10 ng DNA, 2.0 U AmpliTaq Gold DNA polymerase (Applied

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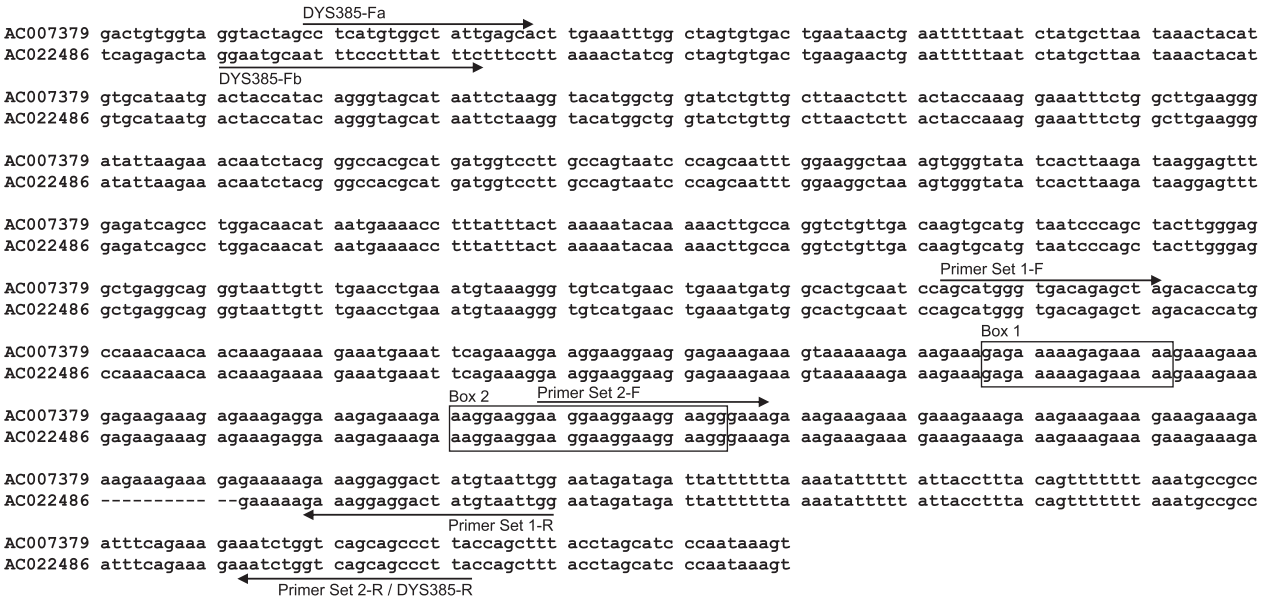

FIG. 1—*Sequence structure comparison of DYS385a (AC007379) and DYS385b (AC022486). Arrows indicate amplification primers used in the present study. Boxed sequences illustrate the two deletion sites. One is an 8 bp (GAGAAAAA) deletion and the other is a 4 bp (AAGG) deletion.*

Biosystems), 1X PCR buffer I (Applied Biosystems), 200 μM each dNTP, and appropriate concentrations of primers. Primer concentrations were adjusted empirically to balance PCR yields. Thermal cycling conditions were as follows: 95°C for 11 min; 35 cycles of 94°C for 30 sec, 61°C for 45 sec, 72°C for 1 min; and a final extension of 72°C for 10 min. The amplification reaction was analyzed by agarose gel electrophoresis stained with ethidium bromide to observe amplified STS markers.

Results and Discussion

Deletions in the DYS385 Flanking Region

In the present study, DYS385 amplicon primers of multiplex I/II (primer set 1) and multiplex III (primer set 2) encompass different flanking regions (Fig. 1), and accordingly, they display different allelic size designations when deletion or insertion mutations occur in one of these flanking regions. Allele designation repeat number discrepancies between the primer sets were observed in eight (1.13%) of 708 individuals (Fig. 2). Among these, three individuals showed a two-repeat unit difference and five individuals showed a one-repeat difference. However, PCR analysis using locus-discrimination primers for DYS385 revealed that these variations are not linked to a specific locus (DYS385a or DYS385b).

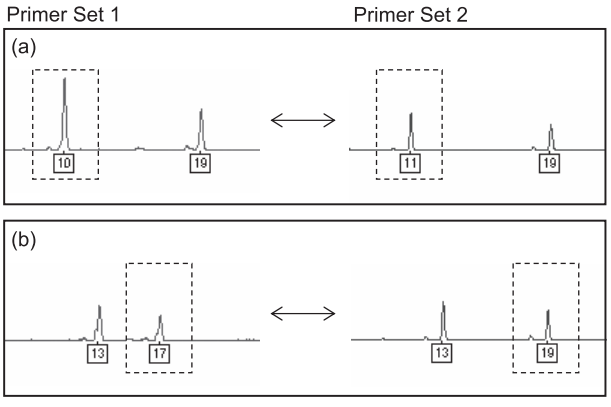

FIG. 2—*(a) Representative electropherogram showing DYS385 allele designation discrepancies of one repeat between multiplex I/II using primer set 1 and multiplex III using primer set 2. (b) Representative electropherogram showing DYS385 allele designation discrepancies of two repeats between multiplex I/II using primer set 1 and multiplex III using primer set 2.*

Sequence analysis of these eight samples revealed deletion mutations at two sites in the upstream flanking region of the DYS385 core repeat units, $(GAAA)_n$. One deletion was an 8 bp deletion of GAGAAAAA and the other was a 4 bp deletion of AAGG; these were found in blocks of $(GAGAAAAA)_2$ and $(AAGG)_6$, respectively (Fig. 1). Therefore, primer set 1 resulted in two or one repeat differences from those of primer set 2 by amplifying the two deletion sites along with the DYS385 core repeat unit. The Powerplex® Y and AmpFlSTR® Yfiler™ kits produced the identical genotyping results as primer set 1 of the present study. Without simultaneous use of two primer pairs, these flanking region variations could not be observed because the DYS385 core repeat unit is a tetramer.

Considering the ISFG recommendations for Y-STRs (7), which encourage indicating variations only in integral core repeats, primer set 2 may be preferable to primer set 1 in that it reflects only the variations in DYS385 core repeats. Moreover, primer set 2 may be very useful in developing size-reduced STR products because its amplicon size is small. However, many databases have been constructed already using commercial multiplex kits like Powerplex® Y and AmpFlSTR® Yfiler™ kits. These kits produce identical genotyping results with primer set 1 of the present study. In addition, considering Füredi et al. (3), the PCR product amplified by primer set 2 includes a rare thymidine deletion downstream from the core repeat unit. None of the 708 sampled Koreans showed this deletion. Therefore, selection of primer pairs for criminal investigations or for storing DNA profiles in national DNA databases should be carried out with much more consideration of the purpose of analysis and the possible presence of mutations in flanking sequences.

Null Alleles Associated with AZFc Microdeletions

Six samples showed a null allele at DYS448. Primers were designed for larger PCR fragments of DYS448, but failed to produce amplicons in the six samples (data not shown). This supported

that the null allele resulted from deletion of the entire DYS448 locus. Accordingly, we further characterized the DYS448 null allele in Koreans using PCR assay for five STS markers around the DYS448 locus.

DYS448 is located within the azoospermia factor c gene (*AZFc)* in the distal euchromatic part of the Y chromosome long arm, Yq11.223 (8–10). *AZFc* consists almost entirely of very long direct and inverted repeats (Fig. 3*a*). Therefore, it is prone to partial deletions or duplications by rearrangements (10). A multilocus STR known to be highly informative, DYS464, also lies within the r1-r4 ampliconic repeats of *AZFc* (Fig. 3*a*). From a multiplex PCR assay for the six DYS448 null allele samples using the five STS marker set, which included sY1161, sY1191, sY1291, sY1206, and sY1201 (6), three samples were found to have a b1/b3 deletion (Fig. 3*b*, lanes 3–5) and the other three were found to have a polymorphic 50f2/C deletion (Fig. 3*b*, lanes 6–8). Inferring from the alignment of direct and inverted repeats in *AZFc*, we expected that the three samples carrying a b1/b3 deletion would carry a simultaneous two-copy deletion at DYS464 (i.e., r1 and r2). Genotyping results confirmed this hypothesis, with two peaks of similar height at DYS464; one carried 11-17 haplotype and two carried 14-16 haplotype. In a recent report (11,12), it was suggested that the *AZFc* marker DYS464 should not be used for commercial typing because of the possibility of interpretive problems related to inadvertent diagnosis of male infertility and because of its characteristics as a multi-locus STR. On the other hand, the other three samples with a 50f2/C deletion presented the same DYS464 haplotype of 11-12-17, implying a possible relationship between the deletion pattern in *AZFc* region and a specific Y-haplotype. Meanwhile, the 50f2/C deletion has long been considered to be a very valuable marker for Uralic migration (13).

Notably, the DYS448 null allele has been observed in Japanese (10 of 1079 males), in Nepalese men (3 of 769), in three ethnic groups (Malays, Chinese, and Indians) living in Malaysia (3 of 980), in Kalmyk (7 of 99), in Mexican (1 of 326), in Spanish (1 of 247), and in three racial groups studied in Yfiler™ haplotype database by Applied Biosystems (2 each of 330 Asians, 985 African Americans, and 1276 Caucasians) (14–19). The relatively high frequencies of the DYS448 null allele in Asians suggest giving careful

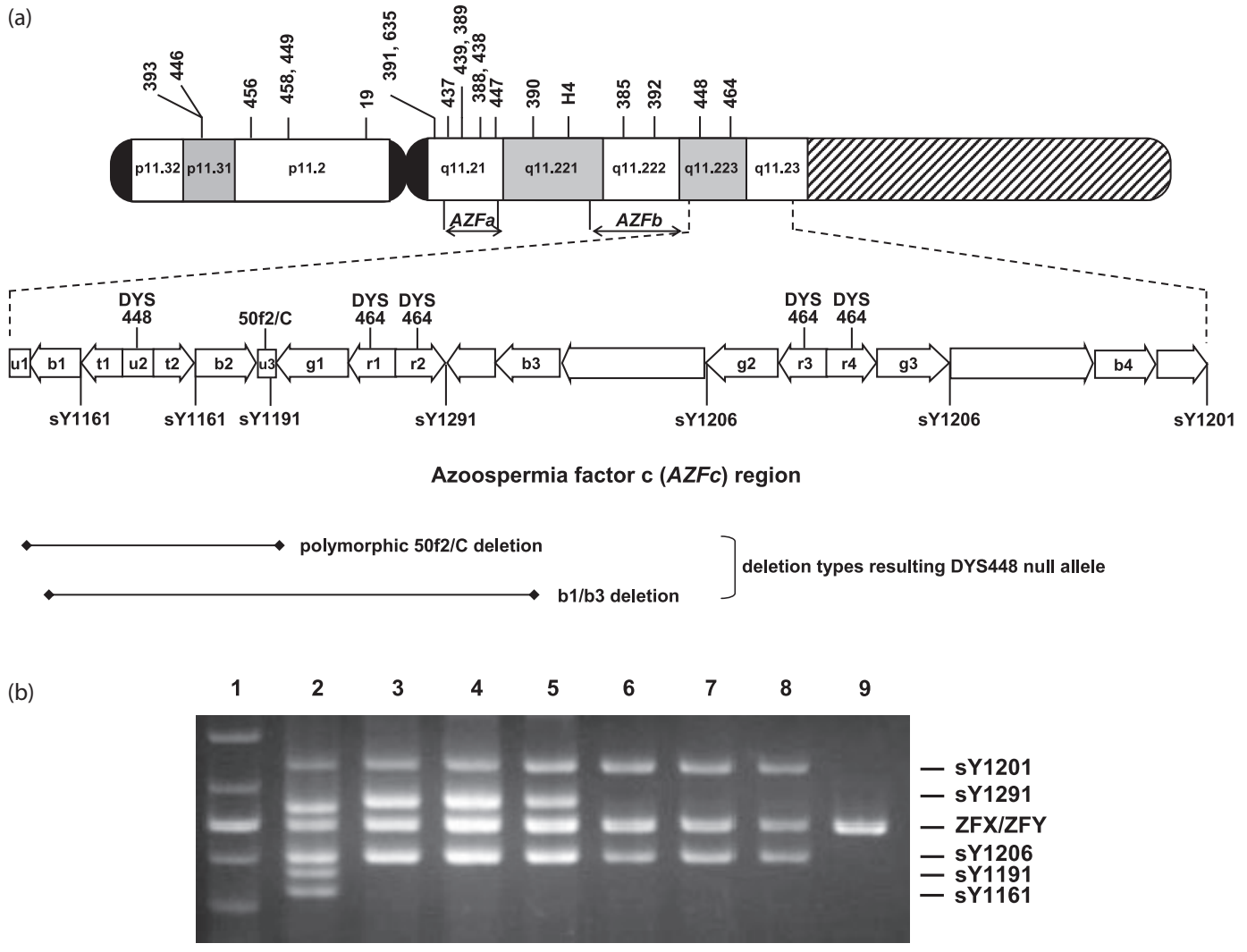

FIG. 3—*(a) Y chromosome ideogram showing the approximate positions of AZFa, AZFb, AZFc, and the 22 Y-STR markers. The locations of DYS448, DYS464, and a series of STSs are indicated on the AZFc amplicon structure. (b) Multiplex PCR assay of AZFc markers. Lane 1, 100 bp ladder size marker; lane 2, a positive control without a null allele at DYS448; lanes 3–8, six samples with a null allele at DYS448; lane 9, a female control.*

consideration to the use of DYS448 for commercial genotyping and further database construction in Asians.

Conclusion

At DYS385, two deletion mutations were observed in the upstream flanking region of the core repeat units. Deletions of DYS448 and DYS464 were observed in association with *AZFc* rearrangement. Importantly, a DYS448 null allele was more frequent in Asians than any other population. These findings will provide useful information in forensic practice for improved interpretation of Y-STR data and database construction.

Acknowledgments

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