Identification of a highly polymorphic tetranucleotide repeat locus (DXpS) at Xp and development of a DXpS/HUMARA biplex methylation-based PCR assay that enhances detection of X-chromosome inactivation

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INTRODUCTION

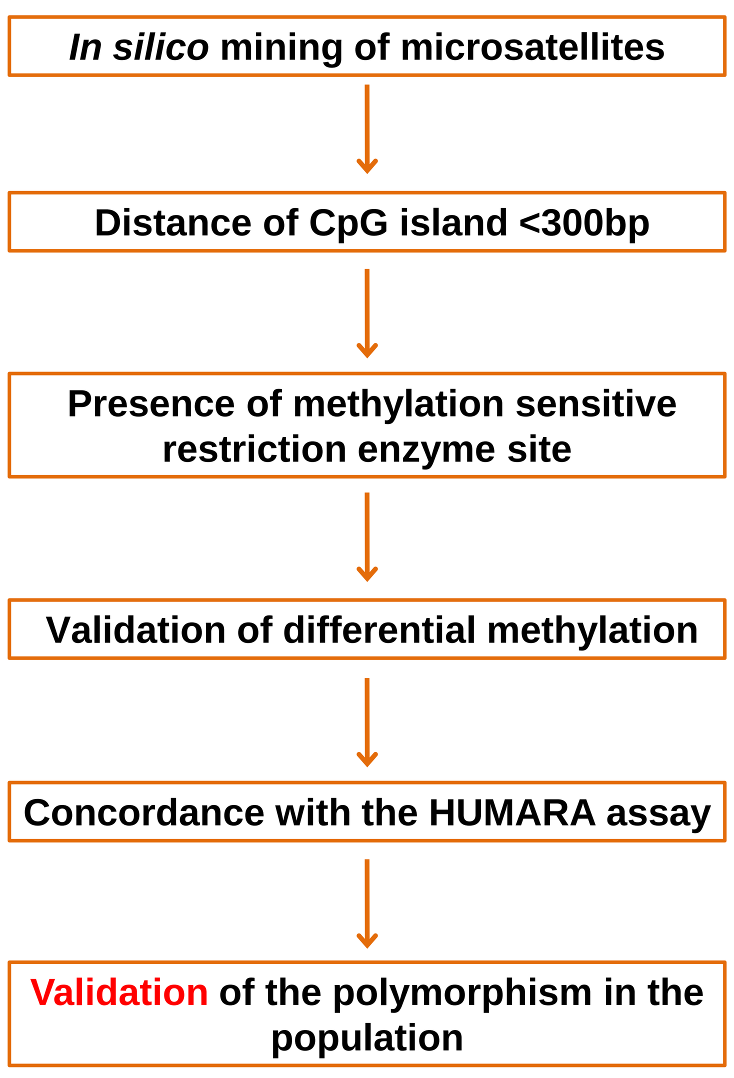
The methylation-based PCR assay in exon 1 of the human androgen receptor *AR* gene (HUMARA) is the gold standard indirect method for determination of activation status of human X chromosomes. The test is based on differential methylation between the active X chromosome (unmethylated) and inactive (methylated) of a CpG island in Xq12, close to a highly polymorphic $(CAG)_n$ microsatellite. HUMARA assay provides heterozygosity rates ~85% worldwide. This means that in a proportion of females the HUMARA assay is uninformative. Allele designation and quantification from trinucleotide repeats demand normalizing for minor (stutter) products differing from the original template by multiples of the repeat unit. Moreover, the HUMARA genotype is not neutral, being linked to Kennedy´s disease and has been implicated in breast cancer risk.[1]

OBJECTIVE

The aim was to verify the presence of microsatellites tetra- and pentanucleotides near differentially methylated CpG islands in promoter regions of the human X chromosome, and develop a methylation-based PCR assay that enhances detection of X-chromosome inactivation.

MATERIAL E METHODS

The identification of microsatellites was performed *in silico* with bioinformatics tools, and criteria developed by the group regarding the *in silico* prediction of informativeness. We analyzed promoter regions of genes that do not escape the inactivation of chromosome X,[2] containing CpG islands which had been validated *in vitro* [3,4].



RESULTS

Here, we report on the identification of a highly polymorphic tetranucleotide repeat (named DXpS) mapping to within a CpG island on the Xp. This island is 191 bp upstream from the start of the repeat element, and contains sites for the *HhaI*, *HpaII* and *BstUI* methyl-sensitive restriction enzymes (Figure 1). We developed the DXpS and the HUMARA markers into a biplex methylation-based quantitative fluorescent PCR assay. For DXpS we observed twelve alleles with negligible stuttering (Figure 2). DXpS exhibited a heterozygosity rate of 87% (n = 60), matching that of HUMARA. The combined informativeness of the biplex assay was 98%. Random and nonrandom X-inactivation patterns inferred with DXpS in phenotypically normal females and haemophiliac females carrying a defective *F8* gene were highly concordant (r^2 = 0.96) with HUMARA patterns (Figures 3, 4 and 5).

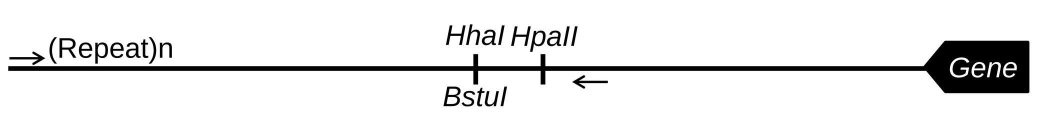


Figure 1. Schematic representation of the region containing the new polymorphic marker on Xp for assaying the methylation state of X chromosomes.

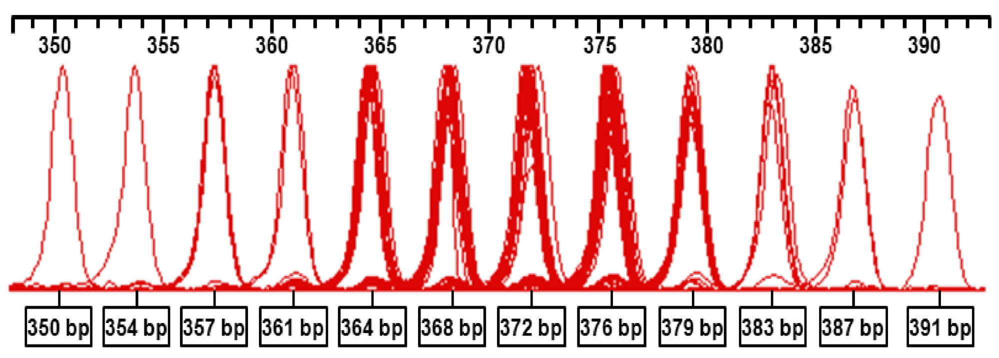


Figure 2. Allele distribution for the DXpS repeat locus. Eletropherogram of alleles observed in 60 unrelated females. The intensity of the red line tracing is indicative of the frequency of alleles. Allele names are the length in base pairs of each fluorescence peak. Allele span, the difference in length between the longest and the shortest allele per locus, is 41 bp.

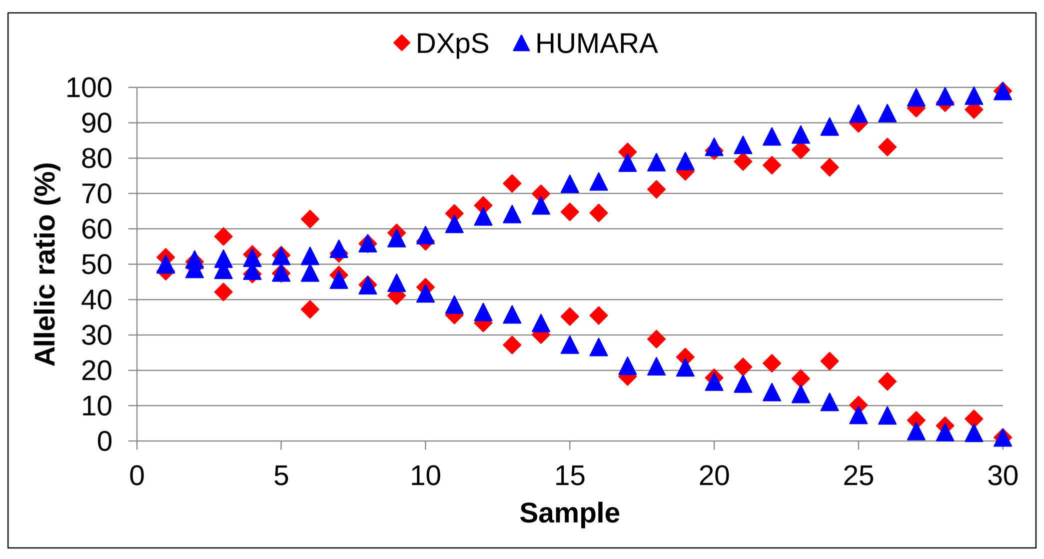


Figure 3. Random and nonrandom X-inactivation allele ratios are concordant between the tetranucleotide DXpS repeat and the HUMARA $(CAG)_n$ loci.

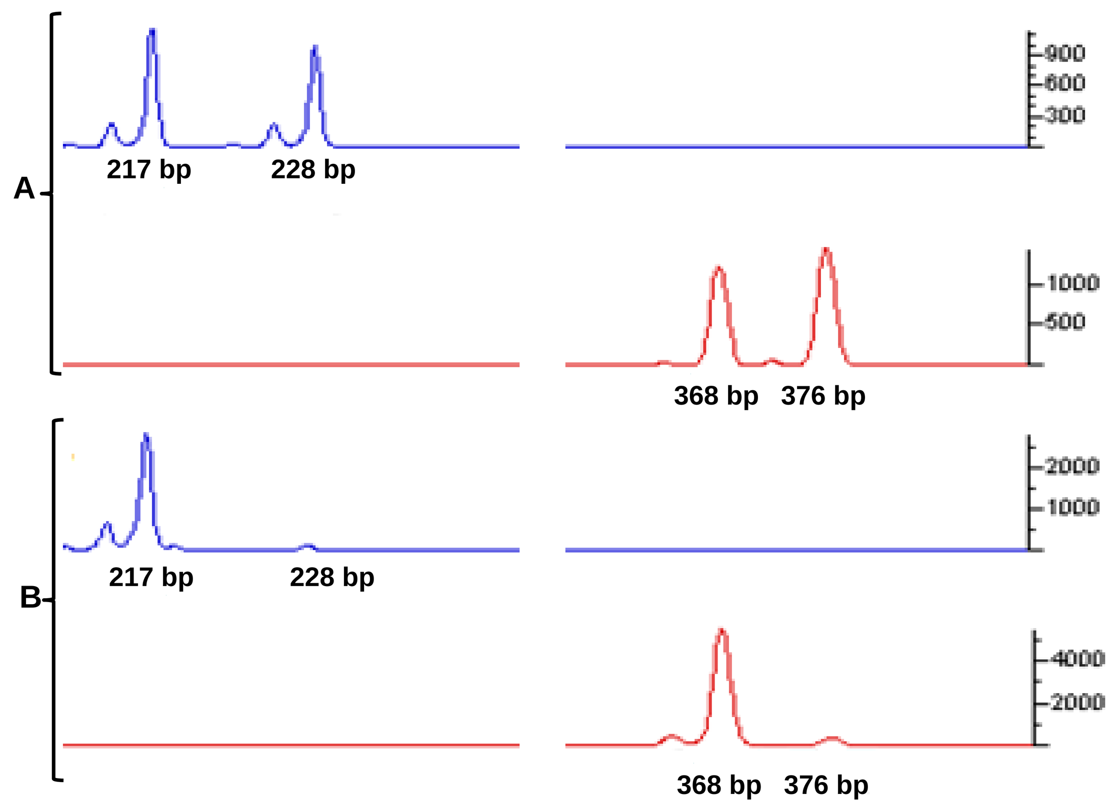


Figure 4. Inactivation pattern of the HUMARA and DXpS loci in a woman with haemophilia A due to skewed X-chromosome inactivation of the normal-*F8* bearing homolog (A) PCR amplification with biplex before *HpaII* digestion. (B) PCR amplification with biplex after *HpaII* digestion. The alleles are represented in base pairs (bp).

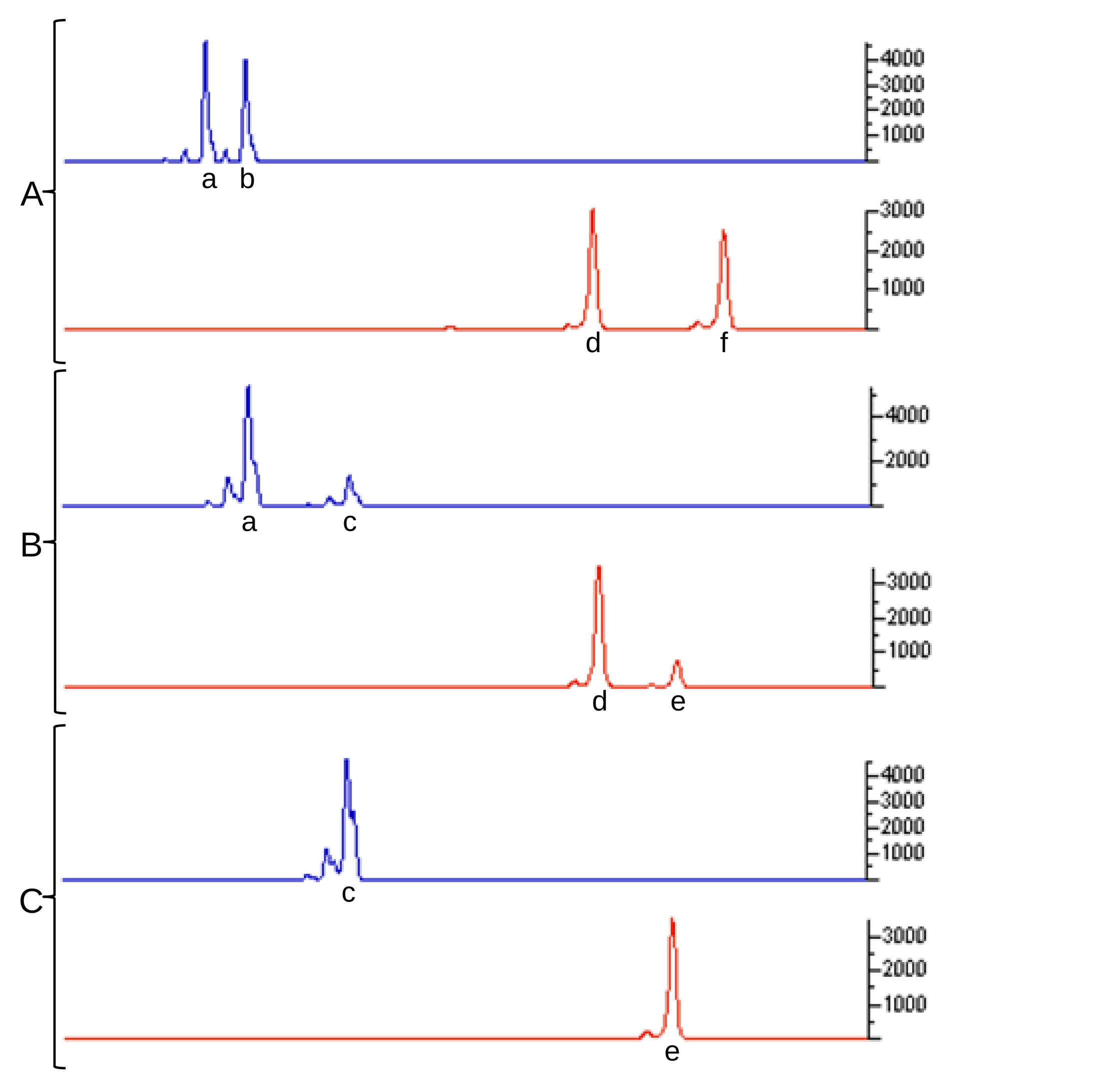


Figure 5. Demonstration that the differential methylation in both DXpS and HUMARA markers identify the same chromosome. Nuclear family genotyped with the biplex assay. Mother (A) and father (C) before digestion with *HpaII*, and daughter (B) after digestion with *HpaII*. Note that the paternal chromosome is preferentially active.

CONCLUSION

DXpS represents a notable advancement in detecting X-chromosome inactivation due to the observed high rate of heterozygosity, negligible occurrence of stutters, concordance with HUMARA, and the apparent neutrality of allelic variants.

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