Rapid screening for Klinefelter syndrome with a simple high-resolution melting assay: a multicenter study

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Klinefelter syndrome (KS) is the set of symptoms that result from the presence of an extra X chromosome in males. Postnatal population-based KS screening will enable timely diagnosis of this common chromosomal disease, providing the opportunity for early intervention and therapy at the time point when they are most effective and may prevent later symptoms or complications. Therefore, through this study, we introduced a simple high-resolution melting (HRM) assay for KS screening and evaluated its clinical sensitivity and specificity in three medical centers using 1373 clinical blood samples. The HRM assay utilized a single primer pair to simultaneously amplify specific regions in zinc finger protein, X-linked (ZFX) and zinc finger protein, Y-linked (ZFY). In cases of KS, the ratios of ZFX/ZFY are altered compared to those in normal males. As a result, the specific melting profiles differ and can be differentiated during data analysis. This HRM assay displayed high analytical specificity over a wide range of template DNA amounts (5 ng–50 ng) and reproducibility, high resolution for detecting KS mosaicism, and high clinical sensitivity (100%) and specificity (98.1%). Moreover, the HRM assay was rapid (2 h per run), inexpensive (0.2 USD per sample), easy to perform and automatic, and compatible with both whole blood samples and dried blood spots. Therefore, this HRM assay is an ideal postnatal population-based KS screening tool that can be used for different age groups.

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INTRODUCTION

Klinefelter syndrome (KS), the set of symptoms that result from an extra X chromosome in males,1 is one of the most common chromosomal disorders, with a prevalence of 0.1%–0.2% of all live male births, 3%–4% of infertile males, and 10%–12% of azoospermic patients.2 The typical karyotype of KS patients is 47,XXY, which constitutes approximately 90% of cases. Other chromosomal abnormalities found in patients with KS include higher-grade aneuploidies, such as 48, XXXY, and mosaicsisms, such as 47, XXXY/46,XY, which constitute the remaining approximately 10% of cases.3

The complete spectrum of KS phenotypes is unclear. The classic phenotypes of KS include tall stature, small testes, sparse facial and body hair, signs of androgen deficiency, and azoospermia.4 However, KS patients may also suffer from a number of illnesses, including osteoporosis, metabolic syndrome, psychiatric illnesses, and even cancer.5–7 More recently, other phenotypes, including specific cognitive, behavioral, and psychosocial features, which may present in childhood, along with delayed development and/or language difficulties, were also shown to be related to KS, broadening our knowledge of this genetic syndrome.5–10 Notably, increasing evidence has shown that offering a wide range of treatments and interventions, such as testosterone replacement treatment, testicular sperm extraction, early speech and occupation therapy, educational assistance, and classroom interventions, at the appropriate age can improve the long-term quality in life of individuals with KS and alleviate later complications.11–14

Although the phenotypes observed in patients with KS are treatable, effective intervention and treatment rely on timely diagnosis. Unfortunately, most KS patients (an estimated 75%) do not receive an essential, timely diagnosis because it is frequently overlooked by health-care professionals and the public. Among the diagnosed cases, most are diagnosed in adulthood during infertility investigations.15–17 Because of this systemic lack of early diagnosis, most KS patients fail to receive the potential benefits of specific treatments and interventions and may suffer a wide spectrum of physiological and psychological difficulties.4 Thus, population-based KS screening is urgently needed for comprehensive diagnosis of KS.18,19 Such comprehensive diagnosis may also eliminate research bias and improve our understanding of KS.19

Traditional diagnostic methods, including karyotyping, fluorescence in situ hybridization,20 and quantitative polymerase chain
reaction (PCR), are accurate; however, they are also labor-intensive, expensive, and lacking inadequate capacity, which limits their utility for large-scale population-based KS screening. Noninvasive prenatal testing for various conditions has been widely integrated into prenatal care. However, the application of noninvasive prenatal KS screening remains controversial because of the mild phenotypic presentation of KS, unsatisfactory sensitivity and specificity of testing, maternal anxiety, and the complications of genetic counseling. Therefore, postnatal screening is preferred for comprehensive diagnosis of KS.

In this study, we introduced a simple high-resolution melting (HRM) assay for postnatal population-based KS screening and evaluated its clinical sensitivity and specificity in three medical centers, using 1373 clinical blood samples. We also investigated the compatibility of this assay with dried blood spot (DBS) samples.

**MATERIALS AND METHODS**

**Principle of KS screening by HRM**

The principle behind KS screening by HRM is based on the results of a previous study and is illustrated in Figure 1. Briefly, zinc finger protein, X-linked (ZFX) and zinc finger protein, Y-linked (ZFY) are paralogous genes located on the X and Y chromosomes, respectively. The DNA sequences of these two genes are highly similar. We designed a primer pair based on the identical regions of these two genes to amplify ZFX and ZFY simultaneously (Figure 1a and b). The two amplicons differ at some nucleotides; thus, they can form heterozygous dsDNA structures that have a specific melting profile during HRM. When the ratios of ZFX/ZFY differ (e.g., 47,XXX vs 46,XY), the specific melting profiles also differ (Figure 1c). To delineate KS cases, the relative signal difference (RSD) values were plotted using a 46,XY reference sample as a baseline. Then, the cases with RSD values below an appropriate cut-off value were scored as KS cases (Figure 1d).

**Determining the optimal amount of DNA template for KS screening**

We first determined the optimal amount of DNA template to achieve the highest analytical specificity. First, 46,XY and 47,XXX DNA samples (n = 12 each; the sample size was determined by statistical analysis) were collected from the Molecular Diagnostics Laboratory of Xiamen Maternal and Child Health Hospital, Xiamen, China. Then, these two groups of DNA samples were analyzed using the HRM assay with 50, 25, 10, or 5 ng of DNA template. The amount of template at which the highest analytical specificity was achieved was used in subsequent analyses.

**Cut-off value for KS screening**

To determine the cut-off value for KS screening, we first evaluated the RSD range of normal samples. For the analysis, 96 normal 46,XY DNA samples were used in the HRM assay on 3 consecutive days, and the RSD range was determined. Next, mixed samples containing 10%, 20%, 30%, 40%, or 50% 47,XXX DNA (n = 12 each) were prepared by diluting 47,XXX DNA samples with a 46,XY reference DNA sample to mimic 46,XY/47,XXX mosaicism. The mixed samples were then analyzed with the HRM assay, and the RSD range was determined. A cut-off value was established and adjusted by analyzing the RSD ranges for the 46,XY samples and 46,XY/47,XXX mosaics. The DNA samples used in this study were collected from the Molecular Diagnostics Laboratory of Xiamen Maternal and Child Health Hospital.

**Multicenter validation**

To better evaluate the clinical sensitivity and specificity by detecting additional KS cases in the multicenter study, instead of population-based recruitment, we recruited patients with oligozoospermia (i.e., sperm concentration <15 × 10^6 ml⁻¹) or azoospermia who received karyotyping and Y chromosomal microdeletion testing at Xiamen Maternal and Child Health Hospital, Xiamen; Obstetrics and Gynecology Hospital Affiliated with Nanjing Medical University, Nanjing; and the Women's Hospital at the School of Medicine of Zhejiang University, Hangzhou, China. We used the same DNA samples which were used for Y chromosomal microdeletion for our KS screening. At each center, DNA samples were numbered by a technologist who did not know the corresponding karyotype, and a second technologist who performed and analyzed the HRM assays knew only the sample number. Then, the results of the HRM screening and karyotyping were analyzed by a third technologist.

A total of 1373 samples from the three hospitals were screened with the HRM assay. We used −6.00 as a cut-off value in this study; if the RSD value of a sample was smaller than −6.00, the sample was classified as “high risk” for KS; other samples were categorized as “low risk” for KS.

**HRM testing using DBS**

To investigate the potential for using the HRM assay for newborn KS screening, we conducted preliminary tests to determine if DNA extracted from DBS samples could be used for the HRM assay. Sixty DBS samples from male births were collected from the Newborn Screening Center of Xiamen Maternal and Child Health Hospital. For each sample, DNA was extracted from 5 punches (3 mm diameter) of DBS. First, we evaluated whether the concentration of DBS-DNA was sufficient for HRM testing. Second, we compared the RSD values of DBS-DNA to those of whole blood samples to evaluate the effect of sample source on the HRM assay.

**46,XY reference DNA samples**

A total of 100 normal 46,XY DNA samples were collected from the Molecular Diagnostics Laboratory of Xiamen Maternal and Child Health Hospital. These samples were diluted to 20 ng µl⁻¹ and mixed in equal amounts to prepare a 46,XY reference DNA sample. The 46,XY reference DNA sample was used as a standard to determine RSD values and was diluted as needed.

**DNA extraction and quantification**

The DNA samples used in this study were extracted using the QIAamp DNA mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. DNA extracted from whole blood was eluted in 200 µl of Elution buffer (Qiagen), whereas DNA extracted from DBS was eluted in 80 µl of Elution buffer. The DNA concentration was determined by measuring the absorbance at 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA, USA).

**HRM assay**

PCR amplification and the HRM assay were performed on a LightCycler 480 II Thermocycler (Roche Applied Science GmbH, Mannheim, Germany). Each 25 µl reaction contained 10 mmol l⁻¹ Tris-HCl (pH8.3), 50 mmol l⁻¹ KCl, 1 U TaqHS (Takara, Dalian, China), 2.5 mmol l⁻¹ Mg²⁺, 2 µmol l⁻¹ SYTO™ Green Fluorescent Nucleic Acid Stain (Molecular Probes, Eugene, OR, USA), 0.2 µmol l⁻¹ of each deoxynucleoside triphosphate, and 0.2 µmol l⁻¹ of each forward and reverse primer. The primers used were 5’-GAAACCTGCTGGCAGAAA-3’ and 5’-CAGCTTGGCTCCCTCCTC-3’. The reaction conditions were as follows: 95°C for 3 min followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. The HRM analysis began with a denaturation step at 95°C for 1 min and a renaturation step at 40°C for 1 min, followed by melting (70°C–85°C, with a 0.03°C s⁻¹ ramp rate), and 20 fluorescence acquisitions per °C were collected.

HRM data were analyzed as fluorescence versus temperature graphs using Gene Scanning software, version 1.5.0 (Roche Applied...
Science GmbH). The melting curve analysis comprised four steps: (1) data normalization by selecting the linear regions of the melting curves before (70°C–79°C) and after (82°C–84°C) DNA dissociation; (2) data adjustment by shifting the temperature axes of the normalized melting curves; (3) plotting the RSD value versus temperature using the 46,XY reference sample as a baseline (Figure 1d); and (4) collection of the RSD value at a given temperature (80°C).

**Statistical analyses**

A normality test was conducted to determine if a set of RSD values followed a normal distribution. When the RSD value data set was nonnormally distributed, a nonparametric method, Kruskal–Wallis analysis of variance (ANOVA), was used to test whether samples originated from the same distribution. All statistical analyses were performed using OriginPro 8.0 software (OriginLab Corp., Northampton, MA, USA).

**Ethics statement**

The samples used in this study were remainders from previous tests, and no additional sampling was performed. Except for the karyotypes, identifying information, including the names and ages of the patients, were withheld from the study group. Therefore, no written informed consent was required. The study protocol was approved by the Research Ethics Committees of Xiamen Maternal and Child Health Hospital, Obstetrics and Gynecology Hospital Affiliated to Nanjing Medical University, and Women’s Hospital, School of Medicine, Zhejiang University.

**RESULTS**

**The optimal amount of DNA template for KS screening**

As shown in Figure 2, the RSD values of the 46,XY and 47,XXY samples were segregated into distinct groups for all tested DNA template amounts. The RSD values in the same group were not normally distributed (normality test). For samples with identical karyotypes, there was no significant difference in the RSD values at the four tested DNA template amounts ($P = 0.88$ for 46,XY and $P = 0.22$ for 47,XXY, Kruskal–Wallis ANOVA). For samples with different karyotypes, significant differences were found for all DNA template amounts ($P < 10^{-4}$, Kruskal–Wallis ANOVA; Figure 2a). Therefore, the analytical specificities of the HRM assays were similar when 5–50 ng of DNA template was used.

The differences in the mean and median RSD values between the 46,XY and 47,XXY samples were also calculated for all tested DNA template amounts. We used 25 ng of DNA template in subsequent studies because this amount showed the largest differences in the mean and median (Figure 2b).

**Cut-off value for KS screening**

The RSD values of 96 normal samples were not normally distributed (normality test). As shown in Figure 3a, the HRM results obtained on 3 consecutive days showed high reproducibility ($P = 0.81$, Kruskal–Wallis ANOVA). Evaluation of results collected over 3 days (288 total evaluated data points) showed that the RSD range was $-3.43$–6.29.

Analyzing the RSD range of mosaic samples showed that the HRM assay exhibited high resolution for KS screening (Figure 3b). When the lower limit of the normal samples (−3.43) was used as a cut-off value, the analytical sensitivity was 100% for samples containing more than 30% 47,XXY DNA. The cut-off value can be adjusted according to the expected resolution, sensitivity, and specificity (Figure 3c). Finally, we used $-6.00$ as a cut-off value for KS screening in the subsequent multicenter validation study. Theoretically, mosaicsisms containing more than 50% 47,XXY cells are detectable at this level of resolution.

**Multicenter validation**

We analyzed 1373 blinded clinical samples obtained from three independent clinical laboratories. As shown in Figure 4 and Table 1, according to the screening, 106 and 1267 samples were classified as “high risk” and “low risk” for KS, respectively. Comparison to the...
Table 1: Multicenter validation of the high-resolution melting assay for Klinefelter syndrome screening

| KS diagnosis by karyotyping | Total (n) | Clinical specificity, KS (n)/total (n) |
|-----------------------------|----------|---------------------------------------|
| High risk for KS (n)        | 104      | 106                                   |
| Low risk for KS (n)         | 0        | 1267                                  |
| Total (n)                   | 104      | 1269                                  |

KS: Klinefelter syndrome

Testing of DNA from DBS

As shown in Figure 5a, the mean concentration of DNA obtained from DBS was 8.0 ng μl⁻¹ (range: 3.7–14.0 ng μl⁻¹). When evaluating the RSD values of the DBS samples aggregated as a group, we assumed their corresponding karyotypes were all 46,XY (Figure 5b). The RSD values of the DBS samples were compared to those of the 288 results collected from 46,XY whole blood samples using the cut-off value. Statistical analysis revealed no significant difference in the RSD values obtained for 46,XY DNA samples extracted from DBS and whole blood (P = 0.74, Kruskal–Wallis analysis of variance [ANOVA]).

DISCUSSION

An ideal population-based screening method for a genetic disease should be highly sensitive and specific, high capacity, rapid, inexpensive, and easy to perform and automatic. Based on these criteria, HRM is one of the best screening methods used for the screening of various point mutation-based monogenic disorders.²⁶,²⁷ In this study, we report, for the first time, the evaluation of a HRM assay for postnatal screening of the highly prevalent chromosomal disorder, KS.

Recently, several real-time PCR-based methods have been developed for KS screening. However, these methods have some limitations that must be overcome before being used in clinical practice. For example, Mehta et al.²⁸ reported a methylation-specific PCR-based assay detecting the methylation status of the X chromosome inactive-specific transcript promoter. This method showed 100% diagnostic sensitivity and specificity in a validation study with a small sample size. However, the cost and workload were too high due to the need for bisulfate conversion of template DNA, and the total turnaround time was approximately 9 h. Moreover, the conversion efficiency must be monitored to avoid false-negative results. Campos-Acevedo et al.²⁹ reported another method based on the quantification of short stature homeobox (SHOX), vesicle-associated membrane protein 7 (VAMP7), and sex-determining region Y (SRY). However, this assay requires four hydrolysis probes and three reactions per sample, which leads to high cost and low test capacity. Although 1000 DBS samples were examined using this method, the clinical sensitivity and specificity are unknown because no strict karyotype validation was reported. We also developed a hydrolysis probe-based melting method to simultaneously detect KS and Y chromosomal microdeletions.³⁰ However, the diagnostic sensitivity and specificity of this method for KS were not satisfactory.
Compared with these three real-time PCR-based methods, the present HRM assay, in which a simple pair of primers with fluorescent dye in a single tube could differentiate KS, dramatically minimizes the cost (approximately 0.2 USD per reaction) and allows for relatively easy scalability (testing at least 96 samples per run). The overall turnaround time from DNA extraction to result in export was 2 h. In addition, the simple nature of HRM allows for easy automation of the assay, which can provide sufficient capacity for large-scale testing, meeting the demands of postnatal population-based screening.

Amplification with a single primer pair in our HRM assay preserved the original ZFX/ZFY ratio with high fidelity, and the results demonstrated that this strategy was not only low cost and high capacity but also high reproducibility (Figure 3a), resolution (Figure 3b), and clinical sensitivity and specificity (Figure 4 and Table 1). Based on the cut-off value set in this study, 46,XY/47,XY mosaicisms containing more than 50% 47,XXY cells are detectable, which means that the HRM assay identified cases with a ≥1.5-fold change in the ZFX/ZFY ratio. Thus, forms of KS with higher-grade aneuploidies (e.g., 48,XXXXY and 49,XXXXXY) with a higher ZFX/ZFY ratio should be readily detectable. In clinical practice, the cut-off value can be adjusted or optimized according to retrospective analysis and the expected resolution, sensitivity, and specificity.

However, it must be noted that chromosomal abnormalities other than KS in which the ZFX/ZFY ratio is altered may lead to false-positive results. As in our multicenter study, a 45,X[26]/46,XY[39] mosaicism and 46,XY[37]/46,XX[16] mosaicism were falsely designated as “high risk” for KS. The ZFX/ZFY ratio changes in these two samples were 1.67- and 1.86-fold, respectively. However, such cases may benefit from a false-positive result, as they would likely lead to early diagnosis of chromosomal abnormalities since all positive screening results should be confirmed by a diagnostic method (e.g., karyotyping). In contrast, specific KS cases with no ZFX/ZFY ratio changes (e.g., 48,XXXXY and 48,XXXX/46,XY mosaicism) cannot be identified by our HRM assay and will result in a false-negative result. However, such cases are rare. Similarly, as shown in our clinical study, a rare chromosomal structural abnormality with a ZFX/ZFY ratio of 1 also cannot be detected. However, this does not compromise the clinical sensitivity of KS screening.

Interestingly, we detected no other forms of KS except for 47,XXY in our clinical study. Therefore, we hypothesize that the prevalence of other forms of KS in southern China are not as high as those published for other areas. In fact, all 156 postnatal KS cases diagnosed by karyotyping at Xiamen Maternal and Child Health Hospital in the past 3 years were 47,XXY (unpublished data). However, the prevalence of this chromosomal disease should be further examined after implementing this population-based screening.

DBS and whole blood are common clinical DNA resources that are used for genetic screening of different age groups. However, the amount of DNA that can be extracted from DBS is limited. Our HRM assay displayed high analytical specificity when as little as 5 ng of template DNA was used (Figure 2). Interestingly, the amount of DNA extracted from all 60 DBS samples used in our study met the optimal DNA template amount (25 ng) for the HRM assay (Figure 5). Moreover, there was no significant difference in the HRM results obtained with DNA extracted from DBS and whole blood. Therefore, our HRM assay can be used for screening of different age groups.

CONCLUSIONS
We developed a high-resolution melting assay and evaluated its clinical capability for KS screening. This is the first multicenter, blinded study for KS screening. The assay was shown to be highly sensitive (100%) and specific (98.1%), of high capacity, rapid, inexpensive, and easy to perform and automate, and it is compatible with both whole blood samples and dried blood spot. Therefore, the high-resolution melting assay is an ideal KS screening tool for different age groups.

AUTHOR CONTRIBUTIONS
DMF and YLZ participated in study design, data acquisition, analysis, and interpretation. JZ, PH, ZFX, SML, JH, and ZMX participated in data acquisition, analysis, and interpretation. QWG conceived of the study, participated in its design and coordination, and drafted the manuscript. All authors have read and approved the final version of the manuscript and agreed with the order of presentation of the authors.

COMPETING INTERESTS
The study was not influenced by any authority, institute, or company, and all authors declared no competing interests.

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