Validation of an Enhanced Version of a Single-Nucleotide Polymorphism-Based Noninvasive Prenatal Test for Detection of Fetal Aneuploidies

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Abstract

Objective: To validate an updated version (Version 2) of a single-nucleotide polymorphism (SNP)-based noninvasive prenatal test (NIPT) and to determine the likelihood of success when testing for fetal aneuploidies following a redraw.

Methods: Version 2 was analytically validated using 587 plasma samples with known genotype (184 trisomy 21, 37 trisomy 18, 15 trisomy 13, 9 monosomy X, 4 triploidy and 338 euploid). Sensitivity, specificity and no-call rate were calculated, and a fetal-fraction adjustment was applied to enable projection of these values in a commercial distribution. Likelihood of success of a second blood draw was computed based on fetal fraction and maternal weight from the first draw.

Results: Validation of this methodology yielded high sensitivities (≥99.4%) and specificities (100%) for all conditions tested with an observed no-call rate of 2.3%. The no-call threshold for sample calling was reduced to 2.8% fetal fraction. The redraw success rate was driven by higher initial fetal fractions and lower maternal weights, with the fetal fraction being the more significant variable.

Conclusions: The enhanced version of this SNP-based NIPT method showed a reduced no-call rate and a reduced fetal-fraction threshold for sample calling in comparison to the earlier version, while maintaining high sensitivity and specificity.

Introduction

Since the introduction of noninvasive prenatal testing (NIPT) for whole chromosome aneuploidies in 2011, its increasing uptake over traditional serum screening has led to a large reduction in the number of false-positive tests, and therefore in the number of women seeking confirmatory invasive testing procedures such as amniocentesis and chorionic villus sampling [1, 2]. Although there remain some identified benefits of traditional screening programs, such as standardized first-trimester sonography [3], the enhanced detection rates with NIPT, particularly for Down syndrome, have irrevocably changed the prenatal testing landscape [4].

Single-nucleotide polymorphism (SNP)-based technology uses targeted PCR amplification and sequencing of SNPs on specific chromosomes of interest rather than a quantitative or ‘counting’ method that involves whole genome or targeted sequencing and analysis of nonpolymorphic loci [5–12]. Analytical and clinical validation of
the SNP-based methodology demonstrated high levels of performance, with sensitivities of ≥96.0% and specificities of ≥99.9% observed for trisomies 13, 18 and 21 in analytical validation studies [13, 14]. Clinical validation of this methodology revealed a redraw rate of 5.4% for samples with a gestational age of ≥10 weeks [14]. To reduce the redraw rate observed in clinical practice, a number of improvements to the SNP-based NIPT methodology were implemented, and are described here. The likelihood of a successful call following a redraw based on initial fetal fraction and maternal weight was also determined for the modified version of this SNP-based method, Version 2 (V2).

Materials and Methods

Methodology Changes
A number of improvements were introduced in V2 of the SNP-based method. Removing poorly performing or superfluous primer pairs reduced the total number of primer pairs from 19,488 to 13,392. The algorithm was modified to account for the removal of the noted primer pairs. The PCR chemistry was optimized to enable a one-step massively multiplex PCR, as opposed to the previous two-step PCR, and to increase amplification uniformity among PCR targets.

Validation Study Cohort
A total of 587 clinical samples (184 trisomy 21, 37 trisomy 18, 15 trisomy 13, 9 monosomy X, 4 triploidy and 338 euploid) with a gestational age of ≥9 weeks were used to validate V2 of the SNP-based methodology (Table 1). Briefly, maternal blood samples were sent to a CLIA-accredited and CAP-certified laboratory where cell-free DNA was isolated from the plasma. Massively multiplexed PCR with next-generation sequencing was used to target SNPs on chromosomes of interest. Genomic DNA from the maternal blood sample, and optionally from a paternal buccal sample, was also analyzed. Fetal copy number was determined using a proprietary algorithm [9, 11–14].

The true copy number status of the chromosomes of interest was obtained prior to validation via at least one of the following methods: (1) verbal or written communication of invasive testing procedure results by the referring physician for research samples, (2) amplification and sequencing of target SNP loci by copy number analysis for placental biopsies obtained after elective termination, and (3) clinical follow-up confirmation for a high-risk Version 1 (V1) test result obtained from the referring clinician. Sample collection protocols were IRB-approved as appropriate. Gender truth was not available for all samples.

Samples were excluded for reasons including known multiple gestation or vanishing twin pregnancy, known egg donor or surrogate pregnancy, insufficient plasma volume, evidence of confined placental mosaicism, contamination quality control failure, and fetal or maternal partial deletions or duplications of chromosomes 13, 18, 21, X and Y. Samples were not excluded based on fetal fraction. Samples with ≤7% fetal fraction were resequenced at a higher depth of read to ensure a sufficient number of sequencing reads to make the correct copy number call. Samples that met quality control metrics were run at an average depth of read of 4.3 million reads per sample. Samples that passed quality control and qualified for resequencing at a higher depth of read were run at an average of 11.5 million reads per sample.

Data Analysis
Sensitivity and specificity for each condition were calculated separately; positive samples at one chromosome were treated as negative controls for those chromosomes that were disomic. Samples that received a no-call were not included in sensitivity and specificity calculations. The no-call rate was calculated for samples with a gestational age of ≥10 weeks, weighted to a 2% aneuploidy rate [14].

Fetal-fraction adjustment was applied to project the sensitivity, specificity and no-call rate in a commercial cohort. Observed values were projected to a known fetal-fraction distribution as measured using commercial data from V1 of the SNP-based NIPT method. The relationship between fetal fraction as estimated in V1 and V2 was calculated from 511 samples that were run using both methodologies; the relationship was found to be a bijective function. This relationship was used to convert the commercial fetal-fraction distribution from V1 into an equivalent fetal-fraction distribution for V2 of this methodology.

Likelihood of Redraw Success
Likelihood of a successful call following redraw was calculated based on fetal fraction and maternal weight at first draw. First, the conditional distribution of fetal fraction for a second blood draw was computed based on the observed fetal fraction and maternal weight of the first draw, using 3,135 cases from V1 that had undergone a redraw. These cases were put into bins according to fetal fraction and maternal weight of the first draw, using 3,135 cases from V1 that had undergone a redraw. These cases were put into bins according to fetal fraction and maternal weight of the first draw. The mean and standard deviation of the log-transformed fetal fraction of the second draw was computed for each bin. Thus, a parametric distribution for the second fetal fraction was available as a function of the fetal fraction and maternal weight of the first draw. Then, using 3,700 V2 commercial cases, the probability of a successful call based on

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<th>Table 1. Patient demographics of the validation cohort</th>
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¹ At estimated date of delivery.
fetal fraction (for the redraw) was determined. Cases were put into bins according to fetal fraction, and the successful call rate was observed in each bin. The probability of success following a redraw was then computed by combining these two models as follows:

\[ P(\text{success} | f_1, w) = \int P(\text{success} | f_2) P(f_2 | f_1, w) \, df_2, \]

where \( f_1 \) is the fetal fraction at first draw, \( f_2 \) the fetal fraction at second draw (predicted) and \( w \) maternal weight.

Results

To validate the SNP-based NIPT approach following methodology changes, a cohort of 587 clinical samples of known genotype were used. Of these, 8 (1.4%) were excluded because they failed quality control. Analysis was carried out on the remaining 579 cases. Calculated sensitivities and specificities for each condition are shown in table 2. The observed sensitivity for trisomy 21 was 99.4%. After fetal-fraction adjustment, the predicted sensitivity for trisomy 21 in a commercial distribution was 98.6%, with a standard deviation of 3.1% and 3-sigma confidence bounds of 90.9 and 100%. The observed sensitivity for trisomy 13, trisomy 18 and monosomy X was 100%; adjustment to the fetal-fraction distribution was not applied to these conditions as it would not have had any effect. There were no incorrect gender calls.

The observed no-call rate in the validation cohort was 2.3%. The no-call rate was 2.2% (6/279) for euploid samples and 10.6% (25/235) for aneuploid samples. The observed no-call rate was 7.3% (13/177) for trisomy 21, 25.0% (9/36) for trisomy 18 and 6.7% (1/15) for trisomy 13 cases; the observed no-call rates for trisomy 18 and trisomy 21 were significantly higher than the no-call rate for euploid samples (\( p < 0.001 \) and \( p = 0.007 \), respectively). Following fetal-fraction adjustment, the overall no-call rate was projected to be 3.8%, with a projected no-call rate of 3.7% for euploid samples and 10.4% for aneuploid samples.

The no-call threshold for V2 was 2.8% fetal fraction. This was lower than the 3.8% fetal-fraction threshold in V1 of the methodology (equivalent to 3.6% using V2; see fig. 1) [13]. The no-call threshold was set at the point below which the algorithm stopped producing the desired accuracy; this point was at a fetal fraction approximately 0.8% lower than in the previous version. This lower no-call threshold would be expected to result in a corresponding reduction in the redraw rate observed in a clinical setting.
For patients who would require a redraw with V2, the likelihood of a successful call following a redraw was determined. The likelihood of success was calculated based on fetal fraction (1.5–5.5%) and maternal weight (100–175 vs. 175–250 lb) at the time of the initial blood draw. Overall, higher initial fetal fraction and a lower maternal weight were found to increase the likelihood of success following a redraw (fig. 2). The rate of redraw success at the highest fetal fraction of 5–6% was at least twice that observed at the lowest fetal fraction of 1–2% for both groups. Maternal weight appeared to have a greater effect on the likelihood of success at lower fetal fractions, with the difference in success rate between the two groups decreasing with increasing fetal fraction. At the highest fetal fraction of 5–6%, the likelihood of obtaining a successful call upon a redraw was close to 100%; maternal weight had no effect.

**Discussion**

This study showed that V2 of the SNP-based NIPT methodology maintained high levels of sensitivity and specificity, and it demonstrated that changes to the methodology did not adversely affect test performance. V2 had a lower projected no-call rate than that observed in the prior version; current experience using V2 in commercial cohorts supports this projection with a 3.8% no-call rate for >200,000 samples received between September 2015 and February 2016. The observed no-call rate for aneuploid samples was found to be higher than that for euploid samples (10.6 vs. 2.2%), which was consistent with previous findings, and confirmed that samples with a fetal fraction that was too low to generate a high-confidence result carried an increased risk for fetal aneuploidy [13].

Of the trisomy cases, the highest no-call rate was observed for trisomy 18 cases, which have been shown to have a smaller placental volume [15]. This is hypothesized to cause a reduction in fetal fraction, and in turn increase the likelihood of a no-call result.

Importantly, validation of V2 of the SNP-based methodology found that the desired accuracy could be achieved at the reduced fetal-fraction limit of 2.8%. This lower no-call threshold was expected to increase the fraction of samples with calls and lead to a concomitant reduction in the redraw rate, which is consistent with the lower no-call rate demonstrated here. Low fetal fraction has previously been shown to be the primary reason a patient is requested to submit an additional blood sample [14].

For those patients for whom a redraw is still necessary with V2, the likelihood of success following a redraw was determined. A higher initial fetal fraction was found to strongly correlate with the probability of a successful call upon redraw; this was unsurprising, given that the ability to detect fetal aneuploidies is highly dependent on fetal fraction, which increases with gestational age. Thus, a second blood draw at a later gestational age may result in a higher fetal fraction than observed at initial draw. The likelihood of redraw success was also shown to be inversely correlated with maternal weight, which in turn is known to be inversely correlated with fetal fraction [14].

The ability to predict the likelihood of a successful call following redraw is clinically relevant for both doctors and patients. Cases that receive a no-call upon initial draw due to low fetal fraction have been shown to have high rates of aneuploidy [13]. Knowing the probability of success following a redraw allows the doctor and patient to assess whether other testing methods, such as amniocentesis, are preferable to a redraw with NIPT. This is particularly relevant for cases with a late gestational age. Women with higher maternal weight and a very low fetal fraction upon first draw (1–2%) have a <45% chance of success upon redraw, and this should be taken into consideration when deciding the next best steps for the patient.

One limitation of this study was that the samples in the validation study cohort had a higher overall fetal fraction than that observed in a commercial cohort. This is because it is challenging to recreate appropriate validation cohorts, given that methods for collecting affected samples often...
In conclusion, we have developed an enhanced version of an SNP-based methodology that has a lower no-call threshold, consistent with our observation of a lower redraw rate in a clinical setting. We have also shown that higher fetal fraction and lower maternal weight at first redraw correlate with the ability of this method to make a successful call following redraw. This information may prove beneficial in a clinical setting and allow doctors and patients to make a more informed choice regarding next steps upon an initial no-call result with the SNP-based NIPT method.

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Disclosure Statement

All authors are employees of Natera Inc. and hold stock/options to hold stock in the company.

References


