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Maternal X chromosome copy number variations are associated with discordant fetal sex chromosome aneuploidies detected by noninvasive prenatal testing

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ABSTRACT

Background: The sensitivity and specificity of noninvasive prenatal testing (NIPT) for detection of sex chromosome aneuploidies (SCAs) compared to common autosomal trisomies are significantly lower. We speculated that in addition to altered maternal X chromosome karyotype, maternal X chromosome copy number variations (CNVs) may also contribute to discordant NIPT SCA results.
Methods: Clinical NIPT was performed for pregnant women at a single hospital. Copy number variation sequencing (CNV-Seq) was used to identify and quantitate the copy number of maternal X chromosome CNVs for each positive SCA pregnancy.
Results: Two out of 25 SCA positive NIPT samples had slightly abnormal ChrX/ChrY z-scores and were referred for invasive test confirmation. However, fetal karyotypes were found to be normal. CNV-Seq analysis of the maternal white blood cell DNA archived from the original two NIPT blood samples identified small CNVs spanning the *STS* gene, which is associated with X-linked ichthyosis. Correcting for the altered plasma levels of X chromosome DNA caused by the two CNVs and, taking into consideration the phenotypic consequences for X-linked disease, both fetuses were diagnosed as normal.
Conclusions: Maternal DNA sequencing is recommended for all positive NIPT SCA results to avoid unnecessary referral for invasive testing and also to evaluate the risk to the fetus of X-linked disease.

1. Introduction

Sex chromosome aneuploidies (SCAs) are commonly associated with genetic disease of the newborn and involve either whole chromosome numerical changes, partial deletions and duplications or mosaicism [1]. The most frequently occurring SCAs are 45,X (Turner syndrome), XXX (Triple X syndrome), XXY (Klinefelter syndrome) and XYY (Jacob syndrome) although other numerical variations can occasionally arise [2–5]. During pregnancy, approximately 99% of all 45,X conceptions spontaneously abort [2]. Conversely, the vast majority of the other SCA conceptions do not usually show any obvious clinical symptoms or abnormalities by ultrasound scan. Thus their detection is usually co-incidental in situations where the fetus is tested for other genetic diseases, such as Down Syndrome.

Noninvasive prenatal testing (NIPT) by massively parallel sequencing of the circulating cell free DNA in the maternal plasma, which comprises a mixture of fetal and maternal DNA, is now a standard screening test for detection of common fetal aneuploidies. In several large prospective studies the sensitivity and specificity for trisomy 21, 18 and 13 detection have proven to be very high, approaching 100% in some studies [6,7]. In contrast, the specificity for SCA detection is consistently lower [6–8], leading to higher false positive rates. To improve the accuracy of NIPT, identification of the causes of false positive SCAs is fundamental. In limited studies conducted to date, fetoplacental mosaicism seen occasionally for autosomal trisomies [9], has also been reported as a biological cause of discordant SCAs [10,11]. In genetic studies, we have also demonstrated that maternal X chromosome aneuploidy and mosaicism contribute to approximately 8.6% of discordant SCA NIPT results [12].

We further speculated that more subtle abnormalities of the two maternal X chromosomes such as copy number variations (CNVs) may also be important causative genetic factors of false positive SCA NIPT results. Here we demonstrate for the first time that small CNVs on the maternal X chromosome are sufficient to alter the normal circulating maternal chromosome X DNA fraction, and potentially cause a misdiagnosis of the fetus by NIPT.

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2. Methods

2.1. Non-invasive prenatal testing

The research study was approved by the Ethics Committee of the Beijing Hospital (approval number 201302108). Patients provided written informed consent and their NIPT samples were de-identified. Clinical NIPT for detection of common aneuploidies involving chromosomes (Chr) 21, 18, 13, X and Y was performed as previously described [6]. Fetal aneuploidies were identified by chromosome z-scores [13]. The normal range for Chr21, Chr18 and Chr13 was defined as $-3 < z < 3$. For female pregnancies, the normal range for ChrX and ChrY was $-3 < z < 3$ whereas for male pregnancies, the normal range for ChrX and ChrY was $z < -3$ and $z > 3$, respectively. The percentage cell free fetal (cff) DNA in male pregnancies was calculated on the basis of the relative proportion of Y chromosome reads, as previously described [14].

2.2. Copy number variation sequencing

Copy number variation sequencing with a resolution of approximately 0.1 Mb for genomic DNA [12,15] was used to determine the maternal karyotype. White blood cells (WBCs) frozen from the original NIPT blood samples were used as the source of maternal DNA for sequencing. In brief, DNA libraries were generated from fragmented WBC genomic DNA and subjected to massively parallel sequencing on the Illumina HiSeq 2500 platform. Approximately 3.5 million uniquely mapped 36 bp sequencing reads generated from test and normal reference samples were compared to derive 24-chromosome CNV plots of each maternal white blood cell (WBC) DNA sample.

3. Results

3.1. Assessment of clinical NIPT results for sex chromosome aneuploidies

Clinical NIPT was performed for pregnant women attending the Obstetrics and Gynecology Department at Beijing Hospital. Following the expansion of the NIPT service to include testing and reporting for ChrX and ChrY, the z-scores of the first 25 SCA positive samples called by automated analysis were manually re-evaluated as a quality control measure. Two samples SCA001 (male fetus) and SCA002 (female fetus) with ChrX/Y z-scores of −1.78/48.24 and −2.97/−0.23, respectively, raised suspicion. Both these ChrX/Y z-scores fell outside the boundaries for plots of a typical group of confirmed 46,XY male ($n = 100$) and 46,XX female ($n = 100$) fetuses (Fig. 1A), suggesting the presence of minor fetal ChrX abnormalities. Both women were referred for amniocentesis and confirmatory invasive prenatal diagnosis. Fetal karyotyping by examination of 20 metaphase amniocytes revealed that the fetuses from the SCA001 and SCA002 pregnancies had normal karyotypes of 46,XY and 46,XX, respectively.

3.2. Investigation of the basis of the discordant NIPT SCA results

To investigate a possible genetic cause of these two discordant NIPT results, we further analyzed the maternal WBCs stored from the original NIPT blood samples by CNV-Seq, which we have previously shown to be reliable, accurate, quantitative and rapid for detecting low levels of X chromosome mosaicism [12] and small CNVs associated with chromosome disease syndromes [15]. In the first sample SCA001, CNV-Seq detected four copies of a 1.69 Mb Xp22.31 segment (Fig. 1B). Taking into account the duplicated maternal ChrX sequences in the maternal plasma, we calculated that the effective circulating ChrX DNA fraction would increase by 1.8% (Supplementary Fig. 1A), thus artificially increasing the fetal ChrX z-score relative to the ChrY z-score. In the second sample SCA002, CNV-Seq detected a 1.69 Mb deletion event in the same Xp22.31 region (Fig. 1B). Applying similar calculations (Supplementary Fig. 1A), we deduced that the lost 1.69 Mb of sequence from one of the maternal X chromosomes would decrease the effective circulating maternal ChrX fraction by 0.94% and artificially decrease the fetal ChrX z-score relative to the ChrY z-score.

4. Discussion

The findings revealed from detailed examination of these two discordant SCA NIPT cases by CNV-Seq analysis highlight the potential of CNVs in the X chromosomes of pregnant women to cause a false positive result. Thus, from previous genetic studies, altered maternal X chromosome karyotype [12,16] and small ChrX CNVs as identified here, now appear to be the most significant genetic causes of false positive SCAs. CNV-Seq proved to be a very valuable tool for accurately deciphering the original NIPT test results for these two SCA positive plasma samples and thus its future application at the clinical level will ultimately help to reduce the incidence of NIPT false positive SCAs. Further, this approach should reduce the number of NIPT patients unnecessarily referred for follow-up amniocentesis and karyotyping when a potential SCA is detected. Based on these two maternal X chromosome CNV cases, together with previous reports of women with X chromosome mosaicism [12,16], larger prospective NIPT studies are now warranted to determine the relative contribution of maternal chromosome X genetic anomalies as well as fetoplacental mosaicism to the overall incidence of false positive fetal SCAs.

While maternal X chromosome analysis by CNV-Seq will prove a useful test to clarify some fetal diagnoses involving SCAs, a deeper understanding of the maternal X chromosomes as highlighted here, will no doubt raise ethical issues for reporting the potential risk of X-linked disease in the fetus. In the two case studies, the maternal Xp22.31 duplication and the Xp22.31 deletion CNVs co-incidentally encompassed the region encoding the steroid sulfatase gene *STS*. It is well known that deletions of *STS* sequences cause X-linked ichthyosis, however, duplications of this region are benign [17]. In the SCA001 pregnancy involving a male fetus, the plasma Xp22.31 copy number (CN) of 3.89 was 2.75% lower than the maternal Xp22.31 duplication CN of 4.0 (Fig. 1B). Based on a fetal DNA fraction of 10.43% calculated from the maternal CNV (Supplementary Fig. 1B), we were able to deduce that the mother actually carried two X chromosomes with the Xp22.31 duplication (total of 4 copies) and the male fetus inherited one copy of the Xp22.31 duplication (Fig. 1C). Nonetheless, the male fetus was not at risk for X-linked ichthyosis. Similarly, for the SCA002 pregnancy involving a female fetus, the plasma Xp22.31 deletion CN of 1.16 was 16% higher than the maternal Xp22.31 deletion CN of 1.0 (Fig. 1B) and based on a fetal DNA fraction of 16% calculated from the maternal CNV (Supplementary Fig. 1B), this was consistent with fetal inheritance of two normal X chromosomes (Fig. 1C). Thus in this pregnancy there was also no risk of the fetus carrying X-linked ichthyosis. Based on the lessons of these two cases, following the identification of a maternal CNV associated with a known X-linked disease in a male pregnancy by NIPT, the testing laboratory should also detail the risk to the fetus on the final NIPT chromosome report.

With the development of new sequencing based NIPT technologies for the detection of pathogenic fetal microdeletions and duplications [18], our findings suggest that interpretation of the genetic status of a fetus for CNV could in some instances be confounded by the presence of maternal CNVs across the genome. While the clinical significance of many CNVs remains unknown, the fetal diagnosis of de novo CNVs or those parentally inherited will be difficult in the absence of a detailed comparative CNV map of the maternal chromosomes. CNV maps can be derived using high-density oligonucleotide or SNP arrays which are designed specifically for pathogenic CNV analysis [19]. However, CNV-Seq which was used in this study may be more suitable for this purpose because the method not only displays a high chromosome resolution of ~0.1 Mb, but can also accurately quantitate the CNV [15] allowing precise adjustment of fetal NIPT CNV z-scores and thus a more accurate