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False positive cell free DNA screening for microdeletions due to non-pathogenic copy number variants

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Title: **False Positive Cell Free DNA Screening for Microdeletions Due to Non-Pathogenic Copy Number Variants**

Short Title: False positive cfDNA due to alternate CNVs

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What's Already Known About This Topic?: Aneuploidy screening using cell free DNA (cfDNA) has recently been expanded to include selected microdeletions. However, validation has been limited and the real-world positive-predictive value is unknown.

What Does This Study Add?: Here we describe three cases of false positive cfDNA microdeletions due to small, non-pathogenic copy number variants near the region of interest. These findings elucidate one mechanism of false-positive results with this screening test and suggest that additional validation is necessary before wider use in clinical practice.

Abstract

Objective: Fetal aneuploidy screening using cell free DNA (cfDNA) has recently been expanded by some laboratories to cover selected microduplications/microdeletions. While validation testing has reported high sensitivity and specificity for these disorders, the real-world positive predictive value of cfDNA is largely unknown.

Methods: Confirmatory cytogenomic microarray analysis was performed on three consecutive amniotic fluid samples with positive cfDNA screening results for microdeletion syndromes.

Results: All three tests were false positives, likely due to small copy number variants near the critical regions for the disorders reported by cfDNA.

Conclusion: Copy number variants other than those related to microduplication/microdeletion syndromes may cause false positive cfDNA results, reaffirms the necessity of follow-up testing to confirm cfDNA screening, and underscores the need for additional evaluation before implementing these tests in practice.

Main Text

The use of aneuploidy screening using fetal cell-free DNA has recently been introduced as a method to identify chromosomal aneuploidies in fetuses from maternal plasma samples[1,2]. The high sensitivity and specificity of cfDNA screening for common aneuploidies has made this a very attractive screening test for pregnant women. Within the last year, commercially-available cfDNA testing as performed in some laboratories has been extended to cover a number of microduplication/microdeletion syndromes, most of which are very rare[3]. The sensitivity and specificity of the assays for these regions is high in laboratory validation studies[3,4], but the positive predictive value, which has been reported only for the detection of the relatively common 22q11.22 microdeletion (DiGeorge syndrome), is low in clinical testing[5]. In this report, we describe the first three cases of positive cfDNA microduplication/microdeletion results subsequently sent to our laboratory for confirmatory microarray following amniocentesis.

In all three cases, the cfDNA result was false positive due to likely benign copy number variants near the critical genomic region for the disorder in question. Our findings may disclose a common mechanism for the low positive predictive value of such tests. These results also underscore the need for additional validation before routine use in practice as well as the necessity for confirmatory diagnostic testing after a positive cfDNA result.

Case 1:

Amniotic fluid from a 42 year old female at 17 weeks, 6 days gestation was sent for single nucleotide polymorphism (SNP) array testing due to a high likelihood of DiGeorge syndrome on cfDNA testing (quoted a priori risk 1/2000, post-test risk 1/19). The cfDNA test had been performed at 9 weeks, 2 days gestation and revealed a male fetus with a fetal cell-free DNA fraction of 8.4%. On array, using an Illumina CytoSNP 850k platform with hg19 genome build, a 0.270 Mb interstitial deletion was found at chromosomal positions 22q11.22(22,307,381-22,573,637), which is downstream of the DiGeorge critical region and is not associated with any known phenotypic consequences (Figure 1A). No deletions or duplications were identified in the DiGeorge critical region (Figure 1A).

Case 2:

Amniotic fluid from a 31 year old female at 16 weeks, 4 days gestation was sent for SNP array testing due to a high likelihood of DiGeorge syndrome on cfDNA testing (quoted a priori risk 1/2000, post-test risk 1/19). The cfDNA test had been performed at 12 weeks, 4 days gestation and revealed a male fetus with a fetal cell-free DNA fraction of 7.3%. On array, a 0.240 Mb interstitial duplication was identified at chromosomal positions 22q11.22(22,314,463-22,555,078), which is similar to the region deleted in case 1 and is likewise not associated with known phenotypic consequences (Figure 1A). No deletions or duplications were identified in the DiGeorge critical region (Figure 1A).

Case 3:

Amniotic fluid from a 34 year old female at 16 weeks, 0 days gestation was sent for SNP array testing due to a high likelihood of Angelman syndrome on cfDNA testing (quoted a priori risk 1/12,000, post-test risk 1/26). The cfDNA test had been performed at 11 weeks 4 days gestation and revealed a female fetus with a fetal cell-free DNA fraction of 7.5%. On array, a small 0.026 Mb interstitial deletion was identified at chromosomal position 15q11.2(25,089,832-25,116,221) within intron 1 of the *SNRPN* gene, a deletion not associated with aberrant imprinting in Prader-Willi syndrome and with no change on the *UBE3A* gene related to Angelman syndrome (Figure 1B). Follow up parental testing revealed that the deletion was maternally inherited (Figure 1B). Testing of the amniotic fluid sample by Southern blot revealed normal imprinting for both alleles, confirming the absence of Angelman syndrome in the fetus (Figure 1C). We note that for all three cases our results are limited in that we have not yet obtained clinical follow-up postnatally. However, we sought to report our results immediately, as physicians are currently encountering such cfDNA testing with minimal guidance for incorporation into practice.

In laboratory validation testing, largely involving mixed samples of normal and abnormal genomic DNA, cfDNA for the identification of microduplications/microdeletions has a high sensitivity and specificity[4]. The number of false positive cases in the recently published laboratory validation series for the cfDNA assay used in our three patients ranged from 0 of 422 (Angelman syndrome) to 3 out of 397 (DiGeorge syndrome). With additional sequencing of positive samples, a false positive rate as low as 1 out of 396 (0.25%) has been reported for DiGeorge syndrome[4]. However, a recent report from real-world clinical testing of more than 21,000 women found that the false positive rate for 22q11.22 deletion is much higher, as the positive predictive value in that study was only 18%[5]. No real-world clinical data has

been published to describe false positive rates for testing of the 15q11.2 region, nor other regions analyzed on commercially available testing such as 1q36 and 5p deletions.

The difference between the reported specificity of the assay from laboratory-only validation studies and that seen in clinical testing may be that none or very few of the control samples used in the laboratory-only validation studies harbored non-pathogenic microduplications or microdeletions near or in the critical regions for these disorders. The non-pathogenic 22q11.22 copy loss seen in case 1, for example, is seen in <0.05% of the population (DGV database, http://dgv.tcag.ca), and microduplications in that region as identified in case 2 are seen in <0.2% of the population per the DGV. The 26 kb microdeletion in *SNRPN* is likewise rare; less than 0.01% of the population has been identified with similar or larger deletions per the DGV.

Our results suggest that such likely benign microduplications/microdeletions may cause false positives for clinical syndromes using cfDNA. As cfDNA is a screening test, when reporting a positive result laboratories performing this test note that risk for a given clinical syndrome is greatly increased but the syndrome is not definitively present. Our results suggest that the methodology used by the testing laboratory is sufficient to detect small CNVs near a clinically relevant region, thus avoiding the more clinically concerning situation of a false negative test, but are perhaps not accurate enough to determine the precise genomic coordinates of the CNV and thus pathogenicity. We would encourage laboratory providers to report the precise genomic coordinates examined by their testing for each microduplication/microdeletion syndrome. Based on population studies, the likely benign CNVs near these regions we identify here, as well as additional likely benign variants also found near these regions, may be even more common than the pathogenic variants tested for on the cfDNA assay (for example, ~1 in 3000 for pathogenic 22q11.2 microdeletion versus >1/1000 for other variants in the region based on the DGV database). If these alternate CNVs routinely lead to positive testing, this mechanism would

strongly limit even the theoretical positive predictive value of cfDNA for microduplication/microdeletions.

Our finding is similar to that seen by Yatsenko *et al*.[6] in their recent report of a single patient with a high risk of DiGeorge syndrome identified using cfDNA. Similar to Case 1 here, their patient had a deletion downstream of the DiGeorge critical region that is present in <0.1% of the phenotypically normal population. A more recent report of a variety of non-trisomic chromosomal anomalies reported by cfDNA has also described limited concordance with cytogenomic microarray[7]. Here we report additional variants both in the 22q11 and 15q11 regions that suggest a general mechanism for false positive cfDNA testing. A recent study found that false positive results for trisomy 18 on cfDNA testing may be caused by maternal copy number duplications on this chromosome[8]. While we were unable to obtain maternal follow-up samples for Cases 1 and 2, our findings in Case 3 suggest these false positive screens for microdeletion syndromes may be more likely to occur in the case of inherited maternal copy number variants. If maternal copy number variants are frequently causative of cfDNA false positives in microdeletion testing, this result would further emphasize the importance of parental testing to alleviate anxiety and inform testing decisions in future pregnancies. Our experience also further reaffirms the necessity of confirming all positive findings from cfDNA by cytogenetic analysis, using cytogenomic microarray for microduplication/microdeletion calls in particular. Our results also further emphasize the necessity of additional clinical and analytical validation of these testing methodologies before routine use in clinical practice.

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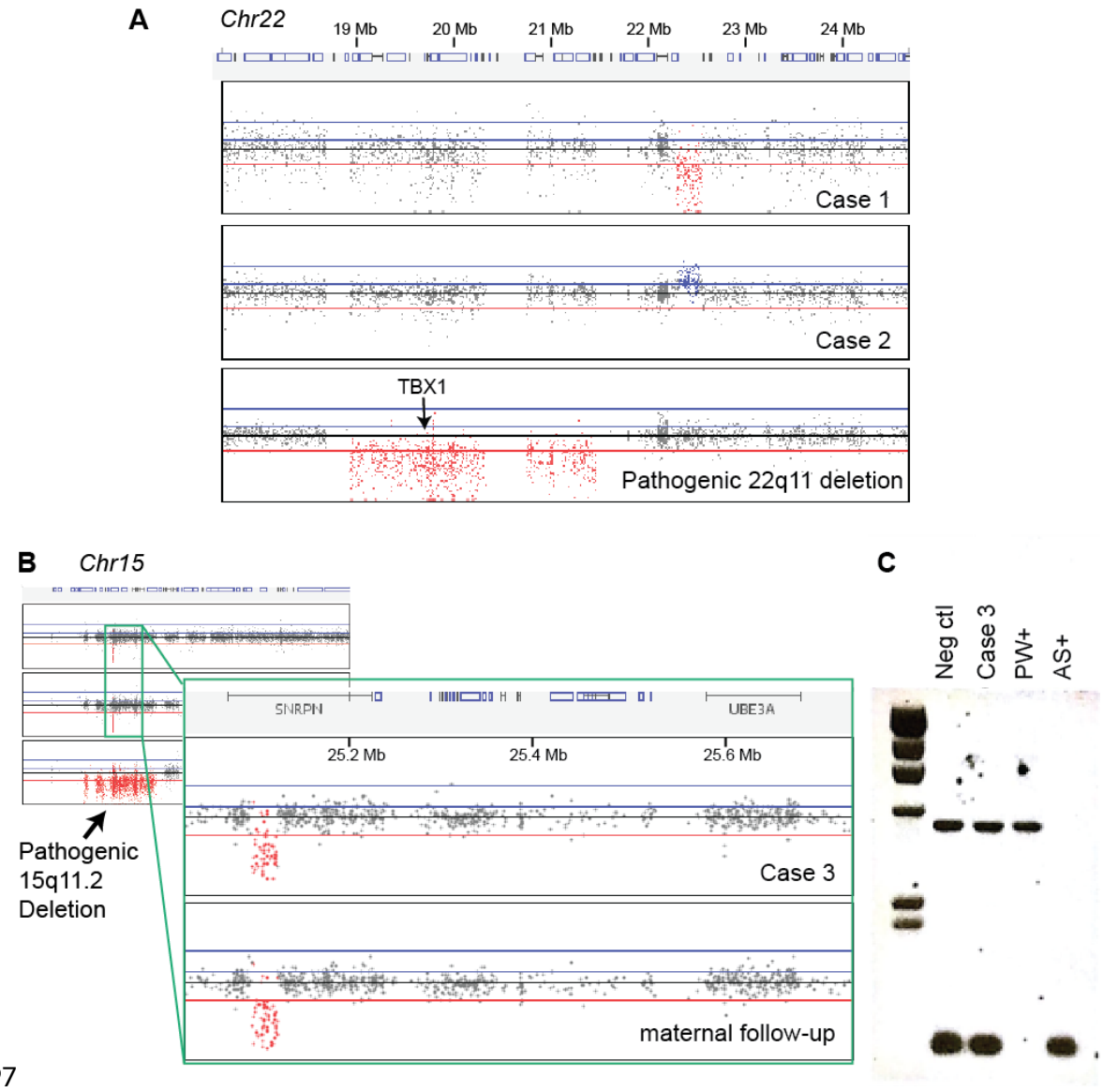

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198**Figure 1. A.** Positive cfDNA testing resulting from microdeletion (top panel) and
199microduplication (middle panel) downstream of the typical region deleted in DiGeorge syndrome
200(bottom panel: unrelated case including the critical gene TBX1). **B.** Maternally inherited small
2010.026 Mb Microdeletion in intron 1 of the *SNRPN* gene (zoom in, right), which is much smaller
202than a typical pathogenic deletion within the 15q11.2 region (zoom out, left; unrelated case), not
203associated with any imprinting defects, and seen at low frequency in the normal population. **C.**
204Southern blot for Prader-Willi and Angelman syndrome reveals normal methylation of both
205alleles. PW+ and AS+ = positive controls.