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Cell Free DNA Detection of Microdeletions: Microarray and FISH Follow-up with Unexpected and Complex Findings

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I. Introduction

Non-invasive prenatal genetic testing (NIPT) is a technology which detects fetal chromosomal aneuploidies by analyzing cell-free fetal DNA (cfDNA) in the blood of a pregnant woman. Since the introduction of testing of cfDNA in the maternal circulation into clinical practice in October of 2011, numerous series and advances have been reported. This technology has allowed clinicians to provide results for common aneuploidies (trisomy 21, 18, and 13) with a higher detection rate and lower false positive rate than traditional maternal serum screening tests. Numerous papers have been published that describe the efficacy of this test for the detection of autosomal aneuploidies. Most major professional societies in the area of obstetrics and genetics who previously recommended limiting testing with cfDNA for high risk population, now have also recommended making cfDNA screening available in all populations. As a result of deeper sequencing, several laboratories have recently introduced additional microdeletion testing as providing an additional benefit to this test. However, outcome data is limited and this test has not been endorsed for the detection of microdeletions, by any professional society to date.

The aim of this report is to review a large laboratory's experience with Chorionic Villus (CVS) and amniocentesis samples obtained from patients that screened positive for a microdeletion on a cfDNA. The performance characteristics of each microdeletion that screened positive and had a follow up testing done by this laboratory is reviewed. Comparisons between initial cfDNA findings and follow-up testing will be made for those cases that had follow up testing with either CVS or amniocentesis.

II. Material and Methods

SPECIMENS AND ASCERTAINMENT: Chorionic villous or amniotic fluid samples were obtained for standard cytogenetic analysis, FISH, or microarray studies at the discretion of the referring provider. Cytogenetic and FISH studies were done using standard analyses. For microarray studies, amniotic fluid was set up as a direct (uncultured) specimens if 16 weeks or greater gestation and at least 15 mL of fluid was available. (If the gestational age was 17 weeks or greater, only 8 mL of fluid was needed.) For CVS tissue, direct specimens were analyzed if more than 5 mg of CVS material was available. For all direct specimens, back-up cultures were established and utilized in case of direct analysis failure. Cultures could be established from as little as 5 mL of amniotic fluid or 2 mg of CVS material.

All the specimens were placed into one of several microdeletion groups as follows: 1p36, 4p (Wolf-Hirschhorn syndrome), 5p (Cri-du-Chat syndrome), 15q11-13 (Prader-Willi or Angeleman syndrome), or 22q11.2 microdeletions as designated by the testing laboratories.

Specimens were also grouped by the type of methodology utilized to detect microdeletions, when the information was available. These were broadly grouped into SNP versus non-SNP screen methodologies.

ARRAY METHODOLOGY: All studies were done utilizing the Affymetrix® Cytoscan® HD array [Affymetrix® and CytoScan® are Registered Trademarks of Affymetrix, Inc.]. This array contains approximately 2.695 million markers across the entire human genome. There are approximately 743,000 SNPs and 1,953,000 structural non-polymorphic probes (NPCNs). On the average there is approximately 0.88 kb between each marker. DNA was extracted utilizing standard methods and 250ng of total genomic DNA extracted was digested with NspI and then ligated to NspI adaptors, and amplified using Titanium Taq with a GeneAmp PCR System 9700. PCR products were purified using AMPure beads and quantified using NanoDrop 8000. Purified DNA was fragmented and biotin labeled and hybridized to the Affymetrix Cytoscan® HD GeneChip. Data was analyzed using Chromosome Analysis Suite. The analysis is based on the GRCh37/hg19 assembly.

The SNP array analysis is utilized to detect both copy number changes as well as copy neutral changes. This allows the detection of not only deletion and duplication, but also potential uniparental disomy and identity by descent. The presence of SNPs in the microarray also allows detection both of triploidy and complete moles with total homozygosity.

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III. Results

OVERALL POSITIVE PREDICTIVE VALUE AND FREQUENCY OF ABNORMALITIES: Of the 335 microdeletion that were identified by cfDNA testing, subsequent diagnostic testing revealed that only 29 had a confirmed microdeletion (**Table 1**) yielding an overall positive predictive value (PPV) of 8.7%.

Ninety-seven of the samples were studied with CVS samples and 9 confirmed the presence of a microdeletion (PPV=9.3%) while 238 were with amniotic fluid samples, demonstrating 20 microdeletions (PPV=8.4%).

The number of cases ascertained for each microdeletion type varied from 9 cases (4p) to 180 (22q). Positive predictive values were determined for each type individually (**Table 1**).

	CHROME/FISH (# DELETED)	ARRAY (# DELETED)	TOTAL (# DELETED)	PPV
1P	1(0)	22(2)	23(2)	8.7%
4P	0	9(4)	9(4)	44.4%
5P	11(1)	33(4)	44(5)	11.4%
15Q	8(1)	71(4)	79(5)	6.3%
22Q	56(3)	124(10)	180(13)	7.2%
TOTAL	76(5)	259(24)	335(29)	
	PPV=6.6%	PPV=9.3%	PPV=8.7%	

FREQUENCY OF ADDITIONAL ABNORMALITIES WHEN A MICRODELETION WAS CONFIRMED: Microarray analysis confirmed 24 of the 29 detected cases of microdeletions while the other 5 were confirmed by chromosome analysis/FISH analysis. Additionally, 10 (41.6%) of the 24 cases confirmed by microarray studies displayed additional or unusual findings likely yielding a phenotype that would not be consistent with a standard microdeletion (**Table 1**). Three patients had a duplication identified (derivative chromosomes) in addition to the microdeletion. Two patients had additional unrelated abnormalities (one deletion and one duplication). One patient had a contiguous duplication/deletion (**Figure 1**). Four patients had unusual microdeletions leading to unexpected phenotypes (one smaller deletion without the syndrome critical region and three larger deletions with more deleterious phenotypes, **Figure 2**).

Figure 1: This figure demonstrates that the array analysis confirmed the deletion (an 11.49 Mb deletion in 1p) that was detected by the cfDNA studies, but it also demonstrates the presence of an additional abnormality, a 5.31 Mb contiguous to the deletion.

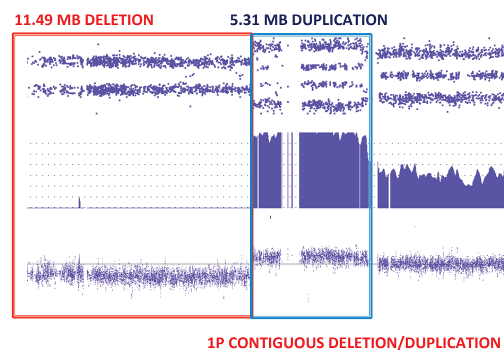
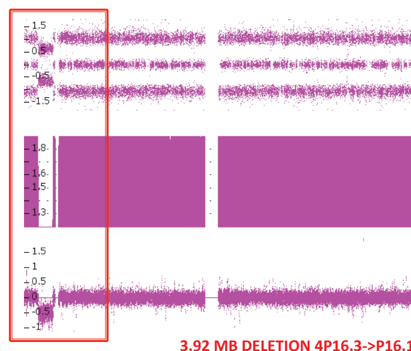


Figure 2: This figure demonstrates that the array analysis confirmed the deletion (a 3.92 Mb deletion in 4p16.3->p16.1) that was detected by the cfDNA studies; however, this deletion does not contain the critical region for the Wolf-Hirschhorn syndrome. It was initially believed to be a variant of unknown significance.



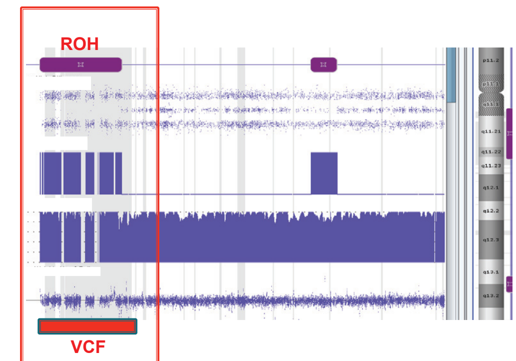
FREQUENCY OF DETECTING AN ABNORMALITY IF A MICRODELETION IS NOT CONFIRMED: There were 235 patients in which microdeletions inferred by cfDNA studies that were not confirmed by microarray. A total of 25 cases (10.6%) displayed other results from the microarray analysis which could have phenotypic consequences (**Table 1**). Seventeen patients (7.2%) had an increased number of homozygotic stretches consistent with consanguinity. Three patients had a single chromosome run of homozygosity suggestive of uniparental disomy, one had a mosaic pathogenic deletion, two had a different microdeletion syndrome, one had a deletion involving an autism susceptibility gene, and one had a derivative X chromosome.

There were also other alterations that were detected that would not likely have phenotypic consequences. These include 4 patients with variants of unknown significance in which the changes are not thought to be associated with phenotypic consequences and 5 patients with recessive gene deletions (NPHP1 (Joubert syndrome) – 1 patient; OTOA (Hearing loss) – 1 patient; CTNS (cystinosis) – 3 patients).

FREQUENCY OF DETECTING RUNS OF HOMOZYGOSITY IF A MICRODELETION IS NOT CONFIRMED: Because of the findings of an increased number of patients with homozygotic stretches associated with consanguinity, an effort was undertaken to look for an association between microdeletion homozygosity and false positive detection of a microdeletion. Both 15q and 22q deletions were examined in detail, as their regions were delineated by specific low-copy repeats (whereas the sizes for 1p, 4p, and 5p deletions are more variable). For 22q deletion that were not confirmed by the microarray analysis, 15.8% of patients demonstrated homozygosity in the 22q11.21 region (**Figure 3**), whereas only 2.8% did in our general clinical pediatric population (unpublished data not shown; p<0.0001). For 15q deletion that were not confirmed by the microarray analysis, 3 (4.5%) of the patients demonstrated homozygosity in the 15q11-q13 region, whereas only 0.6% did in our general clinical pediatric population (unpublished data not shown; p<0.01).

POSITIVE PREDICTIVE VALUE BASED ON TESTING METHODOLOGIES: The cfDNA testing was done by seven different laboratories, in which the technology could broadly be separated into laboratories either using NGS with a SNP based approach versus an NGS approach not using a SNP technology. The testing company was identified in 66.6% of the patients and the SNP based approach had a PPV=3.8% (7 positive microdeletion of 184 identified), while the non-SNP approach had a PPV=32.3% (10 positive abnormalities of 31 identified). This difference is likely to be caused by false positive results due to the presence of copy-neutral homozygotic runs as discussed above.

Figure 3: This figure illustrates that the array analysis confirms that the presence of a homozygous region that covers the VCF genomic locus. The deletion was not confirmed but a region of homozygosity (ROH) was found.



IV. Conclusions

This large follow-up study of 335 microdeletions positive on cfDNA studies has yielded an overall low positive predictive value. As reported, over 90% of the detected microdeletions were not confirmed using diagnostic testing. This is true whether the confirmation was by cytogenetics/FISH or microarray or whether the deletion was detected early enough for a CVS or later when an amniocentesis was used.

This study clearly shows that when a microdeletion was screen positive by cfDNA, not only is confirmation necessary, but the confirmation with a microarray is highly recommended. In addition to the needed microdeletion confirmation, the array provided additional useful information in 10 of 24 confirmed cases (41.6%) that would help to more precisely provide prognosis, then just knowing that an alteration was present. It also illustrates the importance of counseling following a screen positive result.

Lastly, our data suggest that another reason for a large number of false positives may be due to the underlying genomic structure. The percent of copy-neutral homozygosity in the 22q11.21 in patients with 22q deletion calls that were not confirmed by the microarray analysis was almost 14 times greater than our general clinical pediatric population seen in our laboratory (unpublished data) consistent with an underlying reason for some of the false-negative results.