HHS Public Access

Author manuscript

Birth Defects Res A Clin Mol Teratol. Author manuscript; available in PMC 2015 July 29.

Published in final edited form as:

Birth Defects Res A Clin Mol Teratol. 2013 November ; 97(11): 726–729. doi:10.1002/bdra.23195.

Expanding Diagnostic Testing beyond Cytogenetics: Implications for Birth Defects Research and Surveillance

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For decades, classical cytogenetic techniques that yielded a karyotype were the mainstay for identifying and characterizing the causes of certain genetic syndromes and birth defects. The capacity to identify chromosome anomalies expanded in the late 1980s and the 1990s with the development and maturation of fluorescent in situ hybridization (FISH) techniques to interrogate submicroscopic regions of the chromosomes for deletions or duplications. Beyond karyotypes and FISH, new technologies—chromosomal microarrays and next generation DNA sequencing—have markedly increased the number of birth defects and genetic syndromes that now have a known cause. These new testing techniques that can unambiguously confirm a diagnosis—as occurred previously, for example, with FISH for 22q11.2 deletions—will increase the specificity and sensitivity for classifying birth defects and improve prevalence estimates. Although these testing techniques improve the resolution of analysis of smaller and more complex chromosome and DNA anomalies, their interpretation can be problematic, particularly when the test results are of unknown significance. Broader usage of new prenatal screening technologies, such as testing for chromosome and DNA anomalies in cell-free fetal DNA, will likely impact prevalence estimates of certain birth defects included in surveillance systems. These new advancements in genetic testing can create challenges for birth defects surveillance and research programs in learning how to abstract, interpret, classify, store, and incorporate new findings into surveillance systems, as well as categorizing the data in epidemiological studies. Birth defects research and surveillance programs must be mindful of these new challenges and thoughtful in addressing them.

TRADITIONAL CYTOGENETICS: HISTORICAL BACKGROUND

Advances in the field of cytogenetics over the years have yielded an increased understanding of the causes of numerous syndromes, diseases, and structural birth defects. These advances have arisen based on the desire to understand human chromosomes and the information that they contain. Although the interest in chromosomes waxed and waned during the late 1800s and early 1900s, chromosomes have been studied in ever-increasingly detailed and revealing

ways since the mid-1900s. The first recognizable drawings of human chromosomes were published in 1882 by Walther Flemming (Flemming, 1882), but it took until 1956 to realize that 46 was the correct number of chromosomes in human cells (Tjio and Levan, 1956). The subsequent development of banded karyotype analysis allowed for the identification of the aneuploidies responsible for Turner (monosomy X), Klinefelter (47,XXY), Down (trisomy 21), Edwards (trisomy 18), and Patau (trisomy 13) syndromes (Ford et al., 1959; Jacobs and Strongs, 1959; Lejeune et al., 1959; Edwards et al., 1960; Patau et al., 1960), as well as chromosomal translocations involved in cancer cytogenetics such as the Philadelphia chromosome (Nowell and Hungerford, 1960). Analysis of material from spontaneous abortions showed that over half of miscarriages are due to aneuploidies (Kajii et al., 1973; Boué et al., 1975; Carr and Gedeon, 1978). As cytogenetic analysis became commonplace, families of individuals affected by syndromes or birth defects could have cytogenetic testing whereby the results could refine the possibility of recurrence, thus informing future reproductive choices. Initially, all cytogenetic tests were done postnatally on cells derived from tissues such as blood, biopsies, bone marrow, and products of conception. Beginning around the late 1960s, the reproductive decision process could include an existing pregnancy because it became possible to diagnose chromosomal aberrations prenatally using the technique of amniocentesis and subsequent karyotype (Steele and Breg, 1966; Nadler, 1968).

GENETIC TESTING BEYOND TRADITIONAL CYTOGENETICS

Chromosome analysis expanded beyond banded karyotypes with the advent of fluorescent in situ hybridization (FISH) to diagnose submicroscopic chromosomal deletions and duplications (Langer et al., 1981; Langer-Safer et al., 1982), which together with polymerase chain reaction (PCR) (Saiki et al., 1985), became the mainstay technologies of modern day cytogenetics. However, recent genetic testing has begun to move beyond traditional cytogenetics. The American College of Medical Genetics and Genomics published guidelines in 2007 (Shaffer et al., 2007) regarding the clinical use of chromosomal microarrays (CMA) (an umbrella term encompassing both array-based comparative genomic hybridization [aCGH], which detects copy number variation, and single nucleotide polymorphism arrays), which has several advantages over FISH and traditional cytogenetic karyotyping. There is improved laboratory expediency with CMA as it allows for the simultaneous interrogation of hundreds of thousands of loci for potential duplication or deletion, which is not possible with FISH. aCGH has a resolution of 1 kilobase (Kb) while FISH can only detect changes approximately 100 Kb or larger and traditional karyotypes typically have a resolution of 5000 Kb or larger (Miller et al., 2010; Evangelidou et al., 2013). This improved resolution with aCGH facilitates the detection of many structural abnormalities that would have been missed using only a traditional karyotype; for example, in a recent study, aCGH detected losses and gains in approximately 20% of apparently balanced translocations previously analyzed by karyotype (Manning et al., 2010). Based on the advantages of aCGH over traditional karyotypes, Miller et al. (2010) recommended that CMA become the first-tier genetic test for patients with unexplained developmental disabilities, intellectual disabilities, autism spectrum disorder, or multiple congenital anomalies, while continuing to use karyotype and FISH first for individuals with suspected

trisomies and for those with a history of recurrent miscarriage. A recent study concurred that CMA should be the first-tier test, but presented evidence that traditional cytogenetic analysis remains useful in some cases for detecting chromosomal mosaicism and characterizing structural chromosome rearrangements (Bi et al., 2013). Beginning in 2006, CMA has been performed prenatally with cells derived from amniocentesis or chorionic villus sampling (CVS) (reviewed in Evangelidou et al., 2013), although current recommendations are for it to be offered only as an optional screening tool with validation requiring karyotype and FISH analyses (American College of Obstetrics and Gynecology, 2009). In addition to CMA, another new technology changing the field of genetic diagnosis is next generation DNA sequencing (NGS), a massively parallel shotgun sequencing technique. NGS allows for the sequencing of whole genomes or whole exomes (the portion of the genome that codes for proteins) at a fraction of the cost with greatly increased coverage depth compared with first generation sequencing (Wetterstrand, 2013).

Perhaps the most significant recent development in genetic diagnosis, at least prenatally, is based on finding cell-free fetal DNA (cffDNA) in maternal blood in quantities substantial enough to be used to screen for aneuploidies. Although the discovery of cffDNA occurred in 1997 (Lo et al., 1997), it took the next ten years to develop the methods that would reliably differentiate between cffDNA and maternal cell-free DNA (Lo and Chiu, 2007, 2008), as fetal DNA represents just 10% of the cell-free DNA found in maternal plasma (Nygren et al., 2010). Numerous studies have used NGS to validate the use of cffDNA for prenatal detection of trisomy 13, 18, 21, and sex chromosome aneuploidies. The best results to date have shown a nearly 100% detection rate of trisomy 21 with a false-positive rate of less than 1%, and detection rates for trisomies 13 and 18 are now approaching those levels; however, it must be noted that these validation studies have been done in high risk pregnancies, not as population-based screenings (reviewed in Langlois et al., 2013). Because of this caveat and the varying detection rates, cffDNA analysis is currently being offered only as a screening test rather than as a diagnostic test, and results indicative of an aneuploidy require validation through karyotype analysis of cells collected by means of amniocentesis or CVS (Gregg et al., 2013). Further down the road is the prenatal use of NGS and cffDNA to obtain whole genome and exome fetal DNA sequences for prenatal diagnosis of a myriad of genetic conditions; however, this technology is still in the research phase and not yet clinically available (Kitzman et al., 2012).

INCORPORATING NEW GENETIC TESTING RESULTS INTO SURVEILLANCE ACTIVITIES: HOW MUCH INFORMATION IS TOO MUCH?

With each technological advancement in genetic testing, there have been challenges in learning how to interpret and incorporate the newly generated findings into clinical practice, as well as into surveillance activities and epidemiological studies. First and foremost, for those working in surveillance programs, it is not inherently obvious how to interpret the terminology used by laboratories to describe test results. Beyond standard karyotype nomenclature, which by itself has some degree of technical complexity, the terminology to describe FISH and CMA test results (ISCN Committee, 2013) might be even more perplexing to some who work in clinical medicine and population surveillance. Laboratory

reports are often long and packed with detail describing the results, as well as the technology that was used to perform the tests. It can be challenging to determine what components of the report need to be abstracted and included in a surveillance record; surveillance programs will need to make decisions regarding the extent of data that will be collected and train those who report or abstract test results according to surveillance protocols. Another related issue is how to classify actual test results. For years, surveillance systems captured only two types of cytogenetic tests: prenatal and postnatal chromosome analysis. With the current assortment of new tests and even newer ones on the horizon, it is not clear to many programs how to classify FISH, CMA, and now NGS test results because they do not exactly fit under the previous rubric. Surveillance programs will likely need to expand their diagnostic test categories to capture data that are generated by new advances in testing.

INCORPORATING NEW GENETIC TESTING RESULTS INTO SURVEILLANCE ACTIVITIES: WHAT IS TRULY SIGNIFICANT?

Technology has now progressed to the point where a single test has the potential to produce much more data than are needed to answer the question originally posed, with some results being of unknown clinical significance. For example, in CMA testing, the reports of results may include CNVs (copy number variations—duplications or deletions in chromosomal segments) that are known to be pathologic or benign, as well as CNVs of unknown significance (not known whether they are benign and represent normal variation in the population, or they are truly pathologic and related to the person's phenotype, i.e., birth defects and other features and medical problems). In some cases, the indication for performing the CMA test might have been for a phenotype that would have otherwise not been eligible for inclusion as a case in a birth defects surveillance system (e.g., seizures, low muscle tone); in such cases when the CMA test shows only CNVs of unknown significance, surveillance programs will need guidance whether or not to include these cases as true abnormalities. Even if a CNV is clearly known to be pathologic, the surveillance program might not be certain whether the presence of the CNV is related to the infant's phenotype or is just a coincidental finding. This abundance of uncertain data makes it challenging, not only in deciding whether to include certain cases in a surveillance system, but also how to classify cases with uncertain data into categories for epidemiological studies. In addition, when pathologic CNVs and particular phenotypes are clearly related, the fact that most surveillance programs will encounter very few cases with each particular CNV makes it problematic to evaluate these small numbers of cases in epidemiological and outcomes studies; only by combining resources and expertise from multiple surveillance programs might some of these classification and analysis issues be resolved.

Although there are several complexities for surveillance programs in dealing with results from CMA tests, these are potentially orders of magnitude larger when it comes to NGS results. Sequencing a person's exome or whole genome typically yields thousands of genetic sequence variants that need to be sorted through to determine if they are pathologic, benign, or of unknown significance, and among those potentially pathologic, which variants might or might not be responsible for the person's phenotype. With both CMA and NGS tests, CNVs or variants that are of unknown significance today could be determined in the future

to be either pathologic or benign; it will be a challenge for surveillance programs if they choose to update and reclassify cases based on new information or include cases that were initially excluded because of uncertain data. Finally, large amounts of data are generated by CMA or NGS tests, so there may be limitations on how much data could be recorded or stored long-term in a surveillance database. Learning how to incorporate data from these new tests within the confines of birth defect research and surveillance presents significant challenges to the field that will take time to resolve.

THE IMPACTS OF NEW GENETIC TESTING ON PREVALENCE

Could the newer diagnostic testing technologies impact the reported birth prevalence of certain birth defects that are ascertained by surveillance programs? In a word—possibly. As a result of CMA and NGS testing, there will definitely be a shift from cases of unknown etiology to those that have a known genetic cause. This was observed previously with the incorporation of FISH testing into clinical practice; for example, in population surveillance, cases with 22q11.2 deletion are now frequently identified (Tézenas Du Montcel et al., 1996; Devriendt et al., 1998; Goodship et al., 1998; Botto et al., 2003) when before the availability of FISH to diagnose this submicroscopic deletion, many such cases included in surveillance systems could have been of unknown etiology, clinically diagnosed as something else, or not ascertained at all (Emanuel et al., 1998; Katzman et al., 2005). Therefore, reported rates will likely increase for specific conditions that are diagnosed with expanded technologies. However, use of other technologies, such as cffDNA analysis might cause the reported birth prevalence of certain conditions to decrease. Several studies have reported that if surveillance programs fail to account for cases of trisomy 13, 18, or 21 that are stillborn or identified by prenatal diagnosis and electively terminated, there are significant underestimations of the prevalence (Stoll et al., 1998; Forrester and Merz, 1999, 2002a,b; Parker et al., 2003; Siffel et al., 2004; Crider et al., 2008). These under-ascertained cases typically occurred in women offered prenatal diagnosis because of their age, abnormal serum screening test results, or abnormal ultrasound findings. Because cffDNA analysis for prenatal cytogenetic abnormalities will likely be offered to the broader population, there is potential that this newer testing technology will further impact the estimated prevalence of aneuploidy disorders such as Down syndrome, as well as other conditions with cytogenetic abnormalities. Therefore, surveillance programs that do not account for cases identified by prenatal diagnosis may further underestimate population rates.

THE ROAD AHEAD: HOW TO AVOID FALLING OFF THE CLIFF

In summary, whether performed prenatally or postnatally, the myriad information available from CMA, NGS, and cffDNA is changing how genetic syndromes and birth defects are diagnosed and classified. The new genetic testing technologies are making significant contributions toward understanding the causes of birth defects and developmental disabilities. Birth defects surveillance programs need to keep abreast of these new diagnostic tests; develop the means to incorporate new genetic data for case ascertainment, classification, and etiological studies; and be cognizant of the potential effects on birth prevalence estimates. Finally, programs should also remain forward-thinking; if history is

any indicator, newer and even more exciting genetic testing opportunities are probably just around the corner.

Acknowledgments

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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