Detection of Chromosomal Aberrations in Clinical Practice: From Karyotype to Genome Sequence

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Abstract

Since the inception of clinical cytogenetics in the late 1950s, the field has witnessed the evolution of multiple methodologies for the evaluation of chromosomal imbalances and rearrangements. From the replacement of solidly stained chromosomes by Giemsa banding (G-banding) to in situ hybridization and microarrays, each technique has sought to detect smaller and smaller chromosomal aberrations across the genome. Microarray analysis has revealed that copy-number variants—a class of mutation resulting from the loss (deletion) or gain (duplication) of genomic material that is >1 kb in size—are among the significant contributors to human disease and normal variation. Here, we evaluate the history and utility of various methodologies and their impact on the current practice of clinical cytogenetics.

INTRODUCTION

FISH: fluorescence in situ hybridization

CGH: comparative genomic hybridization

The detailed study of human chromosomes first became possible in 1952, when Hsu (15) discovered that hypotonic treatment of cells could spread metaphase chromosomes and allow each chromosome to be individually visualized. Shortly afterward, in 1956, Tjio & Levan (41) established the human chromosome number as 46, contrary to the previous number of 48 reported by Painter. The birth of clinical cytogenetics occurred in 1959, when Lejeune et al. (22) demonstrated that Down syndrome is caused by an extra copy of chromosome 21 and Jacobs & Strong (19) showed that an extra X chromosome is responsible for Klinefelter syndrome.

By the early 1970s, techniques had been developed that stained each human chromosome with a unique banding pattern, initially by using fluorescent stains and later by using trypsin-treated slides stained with Giemsa or other DNA stains. This chromosomal staining allowed investigators to identify deviations from the normal pattern (**Figure 1**). A nomenclature system was also established that identified each band uniquely at various levels of resolution and defined how to describe deviations from a normal karyotype. The ability to interrogate the whole genome via karyotype analysis soon led to the detection of deletions, duplications, and structural rearrangements across every chromosome, and the more common ones were linked to specific abnormal clinical phenotypes. At that time, however, no one could have imagined the impact that copy-number imbalances at a scale much smaller than that of whole chromosome or large segments of chromosomes would have on human disease as well as in normal genomic variation.

The late 1980s saw the advent of early molecular cytogenetic technologies such as fluorescence in situ hybridization (FISH) and comparative genomic hybridization (CGH), which use a combination of cytogenetic and DNA-based methods to detect and interrogate chromosomal abnormalities (reviewed in 37) (**Figure 1**). These methods had an advantage over Giemsa banding (G-banding) because of their ability to identify imbalances that are undetectable by microscope analysis of G-banded chromosomes, either because the imbalances are too small or because they are not discernible based on the banding patterns alone. FISH and early chromosomal CGH methods were both based on the use of a combination of DNA and metaphase chromosomes; however, the sources of the DNA and metaphase chromosomes differ. In FISH, metaphase chromosomes are prepared from patient cells, and the fluorescently labeled DNA probes that are hybridized to the chromosomes are derived from normal reference genomic DNA sequences. Conversely, in

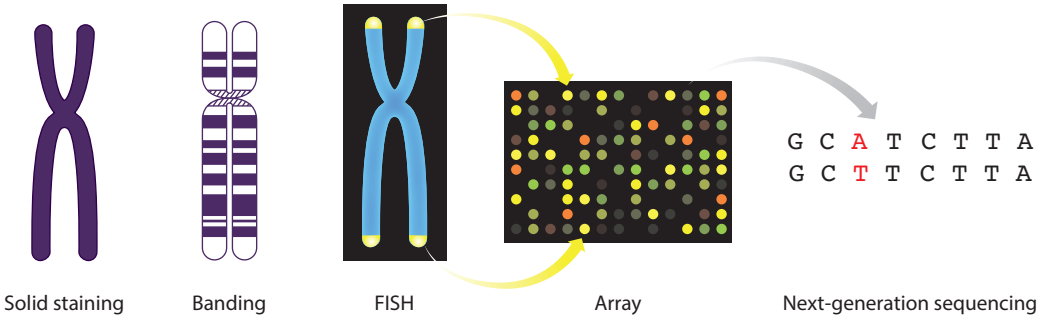

Figure 1

The evolution of technologies for detecting cytogenetic abnormalities. Early methods, such as solid staining and Giemsa banding (G-banding), were based on chromosome staining and identification through a light microscope. Later, molecular cytogenetic methods, such as fluorescence in situ hybridization (FISH) and copy-number arrays, were developed that incorporate DNA-based probes to detect cytogenetic abnormalities. Currently, next-generation sequencing methods are being developed to assess gains, losses, and rearrangements across the genome.

traditional CGH, the metaphase chromosomes are from normal control cells, and comparative hybridization of two differentially fluorescence-labeled DNA samples—one isolated from a test sample and the other from a normal reference sample—is carried out to compare the genomic content of the test with that of the control sample.

FISH provides a higher level of resolution for detecting targeted imbalances (corresponding to the size of the DNA probe used), whereas CGH is more like traditional G-banded chromosome analysis because it allows the analysis of the entire genomic complement. However, because early CGH techniques used normal metaphase chromosomes as the target, the size resolution was not any higher than that of traditional G-banding analysis. The major advantage of this technique is that it can use DNA stored from a patient or tissue rather than living cells. Therefore, this type of CGH analysis proved most useful in cancer cytogenetics, because DNA from tumor cells is required rather than metaphase chromosomes, which can be difficult to obtain from tumor cells in sufficient number and quality. Even though FISH can detect smaller copy-number differences than G-banding can, the technical limitation of using targeted probes does not allow for genome-wide analyses. Thus, although both of these techniques provided useful adjunct analyses to conventional cytogenetics, neither was a replacement for G-banding.

The full potential for detecting submicroscopic imbalances across the genome was revealed only when Pinkel et al. (32) used a microarray containing genomic clones in a CGH assay instead of a slide containing normal metaphase chromosomes. Further modifications of the array template by incorporating DNA sequences, such as oligonucleotide or single-nucleotide polymorphism (SNP) probes, helped to increase the resolution for detecting imbalances by using thousands of probes spaced across every chromosome. For the first time, chromosomal microarray analysis (CMA) had the capacity to surpass traditional G-banded karyotyping in the number of small but clinically significant imbalances that could be detected. As with any new methodology, CMA still has some shortcomings compared with its predecessors: It cannot detect balanced rearrangements or some mosaics, and it cannot define the chromosomal position of inserted or deleted segments, thus being unable to establish the underlying mechanism for a particular gain or loss of genomic material. As discussed below, it is necessary to weigh the relative strengths of each technique to determine the best approach for cytogenetic testing.

This new technology brought with it new terminology. Small (1 kb to ~3–5 Mb) chromosomal deletions and duplications are referred to as copy-number variants (CNVs) and are usually detectable by CMA. Research and clinical practice quickly demonstrated the important contribution of CNVs to human disease and normal variation, carving out an important role in genomic medicine. In this review, we highlight the evolution of cytogenetic methodologies and compare and contrast their utility in clinical cytogenetic practice for diagnosing constitutional abnormalities.

SNP: single-nucleotide polymorphism

CMA: chromosomal microarray analysis

CNV: copy-number variant

CYTOGENETIC ANALYSIS BY KARYOTYPE: THE SENSITIVITY OF G-BANDED KARYOTYPING FOR CONSTITUTIONAL ABNORMALITIES

Since 1959, chromosome preparations from human tissues have been used to detect changes in chromosome number and structure. All major autosomal and sex chromosome aneuploidy syndromes and several deletion syndromes, such as cri-du-chat and Wolf-Hirschhorn syndromes, were described before the development of banding techniques. Structural rearrangements—including Robertsonian translocations, balanced and unbalanced reciprocal translocations, ring chromosomes, and pericentromeric inversions—had also been observed. Newborn surveys and studies of embryonic and fetal material from spontaneous abortions established the major impact of chromosomal aberrations on human morbidity and mortality.

The advent of quinacrine banding (Q-banding) and G-banding enabled the identification of many more rearrangements. Jacobs et al. (18) estimated that moderate banding levels increased the frequency of unbalanced translocation detection by approximately 20% and the frequency of balanced rearrangement detection by approximately 75%. They also reported that in prenatal testing using banding, the total frequency of balanced rearrangements was 3.4/1,000, whereas unbalanced arrangements were much less common, with a frequency of 0.4/1,000. Hook et al. (13) estimated very similar rates in newborns: 2/1,000 for balanced rearrangements and 0.5/1,000 for unbalanced rearrangements. Further studies by Hook et al. (14) used New York State Chromosome Registry data to estimate that across all prenatal diagnoses in New York State, the rates of balanced and unbalanced abnormalities were 3.6/1,000 and 1.7/1,000, respectively. The higher rate of unbalanced abnormalities in prenatal specimens in this study may reflect the inclusion of marker chromosomes and selection against unbalanced progeny after the time of a chorionic villus sampling or amniocentesis.

G-banding at a resolution greater than approximately 550 bands has not been practical for routine clinical use, although in special cases a somewhat higher resolution can be achieved through culture synchronization and elongating additives such as aminopterin (27). The introduction of FISH and microarray techniques for the routine detection of common deletions and duplications not visible by G-banding and for the refinement of breakpoints has led to a phase-out of high-resolution banding in most laboratories.

Because a haploid human chromosome complement comprises approximately 3 billion base pairs of DNA, an "average" chromosome band includes approximately 6 Mb of DNA. Detection of a copy-number change by routine G-banded karyotype analysis depends not only on its size but also on its chromosomal position and the particular banding pattern of that chromosomal region. In G-banded karyotypes, deletions are easier to detect than duplications, and the same is true for FISH. Experience with CMA has shown that 5–10 Mb is approximately the limit of detection for a deletion or duplication in a routine G-banded karyotype. Thus, higher-resolution molecular cytogenetic technologies increased the ability to detect pathogenic copy-number changes and the yield of diagnostic testing.

FISH ANALYSIS: AN ADJUNCT FOR BOTH G-BANDING AND CHROMOSOMAL MICROARRAY ANALYSIS

Since the late 1980s, FISH techniques have been used to greatly expand the sensitivity of karyotype analysis to detect small, submicroscopic abnormalities such as microdeletions/duplications and telomeric imbalances or rearrangements. For example, telomere FISH analysis, which uses probes for the most distal unique DNA at every telomere region, has a diagnostic yield of approximately 3–6% in individuals with a normal G-banded karyotype analysis (8, 33) and became a standard clinical test in conjunction with G-banded karyotype analysis in individuals with unexplained cognitive disorders and/or congenital malformations.

FISH also has the added advantage that it can be used on interphase cells to provide quick results for aneuploidy, unbalanced translocations, single-cell analysis (preimplantation diagnosis), and paraffin sections. The use of interphase FISH has been particularly fruitful for cancer cytogenetics, where interphase testing may be crucial and mosaicism is common. FISH analysis of uncultured interphase cells is also a standard procedure for prenatal testing with amniotic fluid or chorionic villus samples and alleviates much anxiety for patients and physicians. No other test can be done with the speed of FISH (most results can be reported out in a matter of hours), making it the first-tier diagnostic test for suspected aneuploidy and some specified smaller deletions or duplications that characterize well-described microdeletion/duplication syndromes.

The size limit for FISH detection can be as low as 10 kb, but without genomic amplification, 50 kb (the average size of a fosmid) is approximately the lower limit for obtaining results robust enough for routine testing. Bacterial artificial chromosome (BAC) clones, which are usually in the neighborhood of 100 kb in size, make excellent FISH probes and have become the preferred clone for use in FISH assays. FISH is the method of choice for validating CMA results because metaphase FISH gives information about not only copy number but also chromosomal positioning, which can determine the mechanism for how an imbalance occurred. Although molecular methods such as quantitative polymerase chain reaction and multiplex ligation-dependent probe amplification are adequate for validating copy number, these methods do not give any information regarding location in the genome. Therefore, they cannot determine the mechanism of an imbalance, which is essential for accurate recurrence risk determination and family counseling.

BAC: bacterial artificial chromosome

CHROMOSOMAL MICROARRAY ANALYSIS FOR COPY-NUMBER DETECTION

CMA first entered the clinical diagnostic arena in 2004, with the introduction of arrays that contained BAC clones corresponding to known clinically relevant regions, such as microdeletion/duplication syndrome regions and telomere and centromere regions. In essence, this assay was a compilation of multiple assays targeting various chromosomal regions that had been previously shown to cause a genetic disorder when deleted or duplicated. With rapid advances in microarray technologies, the replacement of BAC clones with oligonucleotide sequence or SNP probes allowed the initial targeted-region arrays to be expanded to interrogate the whole genome, mirroring a G-banded karyotype analysis at a much higher resolution (**Figure 2**).

The addition of more probes (up to 3 million) across the genome led to an increased diagnostic yield. A review by Miller et al. (26) showed that the use of a whole-genome array resulted in a 9–15% increased yield over karyotyping alone for detecting cytogenomic imbalances in patients with cognitive disorders and/or congenital malformations. This increased diagnostic yield includes imbalances previously detected by telomere FISH, eliminating the need for this test as a part of routine testing. The data in **Table 1**, from probands where both CMA and karyotyping were done, show that CMA increased diagnostic yield by 6% among postnatal specimens and 2% among prenatal specimens compared with karyotyping (5, 42, 44). Additional data from **Table 1** are discussed below.

The use of SNP probes, rather than just spaced oligonucleotide probes, also adds additional capabilities to CMA testing. SNP probes can detect a specific genotype (allele) at the location of the probe. By using the allele track of array analysis software, SNP probes can detect regions with a loss of heterozygosity, which may indicate genomic regions that either have uniparental disomy or are identical by descent (38). Follow-up testing is needed in either case because the detection of absence of heterozygosity alone is not a diagnostic test. Uniparental disomy requires chromosome-specific testing, and individual gene sequence analysis is needed for any region with absence of heterozygosity suspected to cause an autosomal recessive disorder. SNP probes also allow the detection of polyploidy for prenatal CMA, which is an advantage over the use of oligonucleotide probes alone for fetal specimens.

Genome-wide CMA has redefined cytogenetic testing, including the terminology used to describe very small cytogenetic imbalances. Once the technology allowed the detection of very small imbalances, the term copy-number variant was adopted to describe a class of mutation resulting from the loss (deletion) or gain (duplication) of genomic material that is >1 kb in size (17). Furthermore, in addition to a band designation, cytogenomic imbalances detected by CMA are described based on the genomic coordinates and genome build of the probes used to delineate

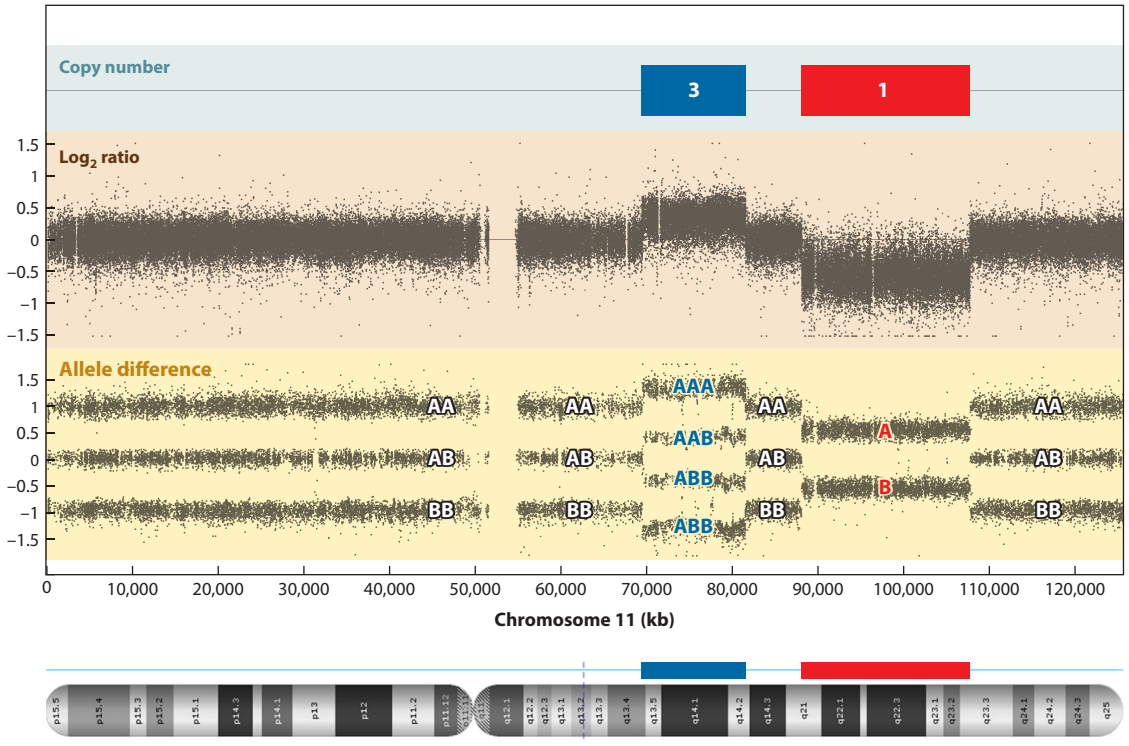

Figure 2

Single-nucleotide polymorphism (SNP) microarray data showing the algorithm-computed copy-number gains and losses (*light blue*, *top*), the $\log_2$ ratio (*light brown*, *middle*), and the allele difference plots (*light yellow*, *bottom*). Dark blue boxes indicate gains, and red boxes indicate losses (copy number shown within each box). The corresponding chromosomal breakpoints of gains and losses are visible in the chromosome ideogram (*horizontal below the main image*). A $\log_2$ ratio of zero indicates a copy number of 2. A positive shift in the $\log_2$ ratio (upward) indicates a gain, and a negative shift (downward) indicates a loss. The allele difference plots show the various SNP genotypes for each SNP locus. In the presence of two chromosomal regions (copy number 2), there are only three possible SNP combinations—AA, AB, and BB—which can be visualized on the allele difference graph as three distinct tracks. In the presence of three chromosomal regions (copy number 3), there are four possible SNP combinations—AAA, AAB, ABB, and BBB—which can be visualized as four distinct tracks on the allele difference graph. In the presence of a single chromosomal region (copy number 1), the genotype can be either A or B, and thus only two distinct tracks are visible on the allele difference graph. Normal diploid females therefore show the characteristic three tracks for all their chromosomes, whereas normal diploid males display the characteristic three tracks for all their autosomes and two tracks for their X and Y chromosomes.

the imbalance. For example, a deletion of ~1 Mb in band 16p11.2 detected using genome build GRCh37/hg19 is designated as "arr 16p11.2(29,500,000–30,500,000) × 1 (GRCh37/hg19)."

New classes of rearrangements have also been defined based on their underlying mechanism of formation. CNVs are described as recurrent if they use common breakpoints and are mediated by a specific underlying mechanism, such as nonallelic homologous recombination via segmental duplications (25). CNVs can also be nonrecurrent, with variable breakpoints occurring throughout the genome. A large study of almost 16,000 clinical cases showed that 76% of rearrangements are nonrecurrent; the remaining 24% are recurrent and mediated by segmental duplications (20). Common patterns of CNVs, such as a small deletion preceding a duplication, are also being identified, and detailed sequence analysis at the breakpoints of rearrangements such as these is identifying new breakage and repair mechanisms, such as microhomology-mediated repair, that mediate chromosome rearrangements based on the underlying genomic architecture (10).

Table 1 Diagnostic comparisons of the specimens tested by both chromosomal microarray analysis (CMA) and karyotyping

	Postnatal						Prenatal					
	Bi et al. (5)			Warburton et al. (44)			Warburton et al. (44)			Wapner et al. (42)[a]		
	N	% of abnormal results	% of cases	*N*	% of abnormal results	% of cases	*N*	% of abnormal results	% of cases	*N*	% of abnormal results	% of cases
Total specimens	3,710			480			428[b]			4,265[c]		
Normal result in both tests	3,206			362			367			3,730		
Same abnormal result in both tests	238			21			34[b]			302		
Abnormal result in CMA only	179	35.5	4.8	36	50.7	7.5	8	14.3	1.9	96	17.9	2.3
Abnormal result in karyotyping only	35	6.9	0.9	6	8.5	1.3	6	10.7	1.4	41[c]	7.7	1.0
Abnormal result requiring both tests for full interpretation	52	10.3	1.4	8	11.3	1.7	8	14.3	1.9	96[d]	17.9	2.3
All abnormal results requiring karyotyping for detection or interpretation (total of above two rows)	87	17.3	2.3	14	19.7	2.9	14	25.0	3.3	137	25.6	3.2
Total abnormal results	504		13.6	71		14.8	56[b]		13.1	535		12.5

[a]Omits cases with mosaic karyotypes.

[b]The standard prenatal protocol tested for aneuploidy by fluorescence in situ hybridization (FISH) and did not do CMA on positive cases. We added back 29 aneuploid cases where CMA was not done but would have revealed the abnormal karyotype.

[c]Triploid cases have been removed because these abnormalities can be detected by using data from a single-nucleotide polymorphism (SNP) array, which was not used in this study.

[d]Estimated from comments in text.

INTERPRETING THE CLINICAL SIGNIFICANCE OF COPY-NUMBER VARIANTS

ClinGen: the Clinical Genome Resource

In the mid-to-late 2000s, the research and clinical communities quickly adopted CMA to examine copy-number imbalances across the genome. This rapid uptake allowed investigators to determine the contributions of CNVs to multiple clinical disorders, which ultimately led to a change in the guidelines for diagnostic cytogenetic testing (see Current Guidelines Concerning the Use of Microarrays and Karyotyping, below). CMA has most commonly been used in postnatal testing to investigate individuals with intellectual disability, developmental delay, and autism spectrum disorder. Other large clinical subgroups examined include individuals with epilepsy, schizophrenia, and congenital malformations, such as heart defects. CMA has shown that approximately 5–25% of individuals in these patient populations have a pathogenic CNV that apparently caused their disorder, with a higher percentage observed in individuals with a more severe clinical presentation (4).

More recently, studies to evaluate the use of CMA for prenatal testing have emerged. In the largest single study to date, Wapner et al. (42) analyzed more than 4,000 amniotic fluid or chorionic villus samples using both G-banding and CMA, and found that CMA identified all aneuploidies and unbalanced rearrangements identified by karyotyping. In addition, in samples with normal karyotypes, CMA revealed clinically relevant CNVs in 6% of patients with an ultrasound anomaly and in 1.7% of patients referred for advanced maternal age or positive screening results. More recently, a systematic review of more than 12,000 prenatal cases with normal karyotypes reported in the literature showed that 2.4% of the samples, which were referred for various indications, had a clinically significant CNV (9); in those with ultrasound anomalies, the rate was 6.5%, similar to that in the Wapner et al. (42) study.

The utility of CMA has also been evaluated in stillbirths (34). G-banding analysis and CMA of more than 500 stillbirth samples showed that cytogenetic test results could be obtained more frequently with CMA than with karyotyping. In addition, CMA had a higher diagnostic yield (8.3%) compared with karyotyping (5.8%) for detecting aneuploidies and clinically relevant CNVs.

CMA has also revealed common variants that occur frequently in normal populations. CNVs have been identified across approximately 70% of the human genome in apparently normal individuals (23). Although these CNVs have not been associated with overt clinical disorders, investigations about their possible relevance for the risk of certain common human conditions are ongoing.

Data sharing of large-scale genomic data sets from clinical and research laboratories is needed to aid in the interpretation of rare genomic variants. To fulfill this need, online databases have been created as publicly available resources to catalog CNVs associated with disease or observed in normal populations. **Table 2** lists some of these databases and indicates whether they contain CNVs from cases, controls, or both. Many of these databases also contain phenotype and sequence-level variant information in addition to structural-level CNV data. The Online Mendelian Inheritance in Man (OMIM) database is another resource that contains information about specific genes as they relate to human disease. The Clinical Genome Resource (ClinGen), a newly funded National Institutes of Health project, is an emerging resource being developed to help better integrate and standardize the use of genomic variants in routine medical care. The goal of ClinGen is to investigate which genomic variants play a role in disease through an evidence-based review process and to make those data available through the National Center for Biotechnology Information's ClinVar database.

The advent of CNV discovery has raised new diagnostic and counseling dilemmas. However, this phenomenon is not different from previous experiences; with each new technology

Table 2 Copy-number variant (CNV) databases of disease (case) and control populations

Database	Case CNV data	Control CNV data
ClinVar	✓	
dbVar	✓	✓
DECIPHER	✓	
DGV		✓

Abbreviations: DECIPHER, Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources; DGV, Database of Genomic Variation.

comes improved resolution and capacity for detecting genetic variants, and often the ability to detect genetic variants surpasses our ability to accurately interpret them. Often for CNVs, there are simply no data about dosage effects for particular genes, particularly for duplications. Therefore, a particular CNV's potential for pathogenicity is assessed based on several factors. First, databases and literature reviews can help determine whether the CNV overlaps a known disease-causing region or a common normal polymorphism. If a CNV has not been previously reported, then certain guidelines can help in evaluating pathogenicity. For example, in general, CNVs that cause overt disease are larger in size, have a higher density of genes, and are de novo. Compared with CNVs that cause disease, nonpathogenic CNVs tend to be smaller in size, to contain fewer genes, and to be located in highly variable regions of the genome, such as in pericentromeric regions. CNVs frequently observed in normal populations also tend to be inherited. Genes within a CNV region can also be evaluated to determine whether they are dosage sensitive, i.e., haploinsufficient or triplosensitive, which can aid in interpreting the relevance of a deletion or duplication, respectively. Online tools such as the ClinGen Dosage Sensitivity Map [previously known as the International Standards for Cytogenomic Arrays (ISCA) Dosage Map; **http://www.ncbi.nlm.nih.gov/projects/dbvar/clingen**] and the Haploinsufficiency Score (HI Index), a prediction tool available as part of the gene information in the Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources (DECIPHER; **http://decipher.sanger.ac.uk**), are freely available to help with these analyses (16, 35).

To standardize CNV interpretation and reporting across laboratories, the American College of Medical Genetics and Genomics (ACMG) has defined five categories to define the clinical significance of a CNV: (*a*) Pathogenic, (*b*) Uncertain—Likely Pathogenic, (*c*) Uncertain, (*d*) Uncertain—Likely Benign, and (*e*) Benign (21). The Pathogenic category is used for CNVs known to cause disease, whereas the Benign category is used for common, well-characterized normal polymorphisms. The three Uncertain categories are used when no clear conclusion can be drawn from existing data, with Likely Pathogenic and Likely Benign then used when the evidence suggests a CNV is likely to be pathogenic or benign, respectively, based on data used in evaluating the CNV, as described above.

CURRENT GUIDELINES CONCERNING THE USE OF MICROARRAYS AND KARYOTYPING

In 2010, both the ACMG and ISCA (now called ClinGen) recommended that CMA be used as the first-tier test for cytogenetic analysis for clinical testing purposes (24, 26). CMA used for clinical testing should at a minimum be able to detect any imbalance 400 kb or larger across the genome (21, 26, 38). This lower required limit for genomic resolution is much greater than the

ISCA: International Standards for Cytogenomic Arrays

ACMG: American College of Medical Genetics and Genomics

resolution of a G-banded karyotype (estimated to be 5–10 Mb), and many laboratories even test for imbalances much smaller than 400 kb—including down to the size of a single exon.

Given that CMA can detect many more deletions and duplications than karyotype analysis can, and that most abnormalities are detected by both techniques, the question has been raised of whether it is still necessary to perform karyotyping during routine testing. ISCA/ClinGen was the first group to publish a consensus statement recommending CMA as a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies, stating that "G-banded karyotype analysis should be reserved for patients with obvious chromosomal syndromes (e.g., Down syndrome), a family history of chromosomal rearrangement, or a history of multiple miscarriages" (26, p. 749). Although recognizing that CMA will miss truly balanced rearrangements, the authors argued that these are seldom clinically significant. However, they also recognized that in some cases a karyotype analysis will be needed to verify or clarify the origin of changes detected by CMA.

Similarly, the ACMG's newest laboratory guidelines for CMA testing also recommend that CMA testing be the first-tier test for diagnostic testing (24, 38). These guidelines do not explicitly state that karyotype analysis is not required and point out that performing only CMA would miss most carriers of chromosome rearrangements, may at times be slower than karyotype analysis, and is not appropriate for some kinds of diagnoses, e.g., complex sex chromosome abnormalities, mosaicism, and trisomies that may occur via rearrangements rather than whole-chromosome nondisjunction.

For prenatal CMA testing, the American College of Obstetricians and Gynecologists and the Society for Maternal-Fetal Medicine have released new recommendations for the use of CMA in the prenatal setting (2). In cases where the fetus has a structural abnormality identified by ultrasound and an invasive prenatal test is being performed, they recommend CMA in place of fetal G-banded karyotyping. In a structurally normal fetus, if an invasive test is being performed, either CMA or karyotyping is endorsed. The recommendations also support the use of CMA as a first-tier test over karyotyping in cases of intrauterine demise or stillbirth.

COMPARISON OF CHROMOSOMAL MICROARRAY ANALYSIS AND KARYOTYPING ON THE SAME PATIENTS

The best way to evaluate how the use of CMA without karyotyping may affect clinical diagnosis is to examine an unselected series of cases where both tests were performed on the same specimen. **Table 1** shows a compilation of data from two surveys using postnatal specimens (5, 44) and two surveys using prenatal specimens (42, 44). Cases were classified as (*a*) normal result in both tests, (*b*) same abnormal result in both tests, (*c*) abnormal result in CMA only, (*d*) abnormal result in karyotyping only, or (*e*) abnormal result requiring both tests for full interpretation.

The table presents data on the frequency of each category among the total number of cases and the total number of abnormal cases. Although the Warburton et al. (44) sample is much smaller, the results are similar and support those of the two larger studies, by Bi et al. (5) for postnatal samples and Wapner et al. (42) for prenatal samples. These data clearly demonstrate the increase in detected abnormalities when CMA is used, which is attributable to pathogenic gains and losses of 100 kb to 5 Mb that are undetectable by karyotyping. In postnatal studies (with an abnormal proband), an average of 5% of specimens, or 37% of abnormalities, had such a lesion. In prenatal studies, the frequency of abnormalities detected only by CMA was lower, as would be expected because this population is largely unselected for an abnormal clinical phenotype: An average of 2% of all specimens and 18% of all abnormalities had an imbalance detected only by CMA. A study using another prenatal sample cohort, reported by Sahoo et al. (36), resulted in an even higher rate

for detection by CMA only: 3.1% of all prenatal specimens and 27% of all abnormalities. These pre- and postnatal data are a strong basis for clinical guidelines supporting CMA as the first-tier test in clinical testing.

WGS: whole-genome sequencing

Although many other studies have reported an increased rate of abnormality detection using CMA, few report the other relevant category, i.e., cases where an abnormality is detected only with karyotyping (including cases where CMA was abnormal, but the results were not sufficient to determine a final diagnosis). **Table 1** shows that for postnatal cases, this was true for ~1% of all cases and ~7% of all abnormalities detected in these cases (the "abnormal result in karyotyping only" category). In prenatal specimens, the frequencies were very similar: ~1% of all specimens and ~8% of all abnormalities. Again, in the sample presented by Sahoo et al. (36), the frequency was higher: 2.6% of all cases and 22% of abnormalities. The higher frequency in this study may reflect the inclusion in this category of cases needing both tests. Overall, the majority of abnormalities missed by CMA in these studies are apparently balanced rearrangements (translocations, inversions, or insertions), and the remainder can be explained mostly by mosaicism that was undetected or misinterpreted by analysis of the CMA results.

DETECTION OF BALANCED REARRANGEMENTS

The majority of abnormal karyotypes not detected by CMA are apparently balanced rearrangements. Several CMA studies have reported finding CNVs at the breakpoints of de novo apparently balanced rearrangements detected by karyotyping (3, 11). Baptista et al. (3) showed that this type of imbalance occurred in 4 of 14 probands with clinical abnormalities and in 0 of 31 probands without abnormalities. This finding is compatible with the documented increased rate of phenotypic abnormalities in carriers of de novo balanced translocations and inversions (31, 43). However, it is remarkable that no CNVs were detected at or near the putative breakpoints in any of the 74 balanced translocations detected in the cases in **Table 1** (a subset of the "abnormal result in karyotyping only" category). This finding is in spite of the fact that all of the postnatal cases had developmental abnormalities in the Bi et al. (5) study and most of them did in the Warburton et al. (44) sample. How many of the balanced rearrangements in the Bi et al. (5) and Wapner et al. (42) studies were de novo is not known, although the latter study stated that "most" were inherited. In the Warburton et al. (44) study, all were inherited. Thus, although it is possible that apparently balanced translocations may be unmasked by the identification of CNVs at the breakpoints, it does not appear that any sizable proportion will be.

However, even the high resolution now attained with CMA does not rule out copy-number changes of a few hundred base pairs or breakage within a gene as a cause of an abnormal phenotype associated with a balanced rearrangement. New technologies, including the targeted next-generation sequencing method described by Talkowski et al. (39), can reveal small and complex changes often associated with chromosome rearrangements and not detected by CMA. This method has also revealed pathological changes in noncoding DNA. The turnaround time for this method is decreasing such that it has the potential to provide a result in the time period needed for prenatal diagnosis (40). A new system of nomenclature has been proposed to describe these rearrangements characterized by sequencing (29). Although this method has the power to greatly expand our knowledge of a given rearrangement, it still relies on the initial discovery of the abnormality by karyotyping.

By contrast, untargeted whole-genome sequencing (WGS) is a rapidly emerging, high-resolution methodology that can function as a stand-alone assay for revealing balanced chromosome rearrangements and interrogating apparently balanced rearrangements that are associated with sequence changes at the breakpoints. WGS has the potential to, and probably will, eventually

replace most other techniques, but it is currently impractical for routine clinical testing. What proportion of translocations will remain truly balanced (with no changes in DNA sequence when fully analyzed) and what proportion of the observed changes are truly pathogenic are not known. Nevertheless, the majority of balanced translocations are carried by normal individuals and are not pathogenic except in the context of unbalanced segregants that lead to miscarriage or offspring with abnormalities. Thus, the importance of their detection lies mostly in information that may impact the reproductive outcomes of the carrier and other relatives.

MOSAICISM

The other major karyotypic abnormality that may not be detectable with CMA is mosaicism. All studies in **Table 1** reported mosaicism by karyotyping and FISH that was not detected by CMA, and sometimes the reverse. In two prenatal cases, karyotype analysis revealed a low level of 46,XY cells in a 45,X fetus that was not detected by CMA. This mosaicism was confirmed in another tissue at birth and was clinically significant in raising the question of whether a gonadectomy should be performed. Although CMA can detect a cell line as rare as ~10% for a whole trisomy, the sensitivity for smaller lesions is not known, and the extent of the mosaicism is not quantified by the test. The sensitivity of karyotyping to detect mosaicism depends entirely on the number of cells examined (12); the number of cells needed can be very large, especially if FISH is used. Examining 100 cells will exclude 3% mosaicism or higher with 95% confidence, whereas the usual 20 cells examined for karyotyping will exclude only 14% mosaicism or higher. Of course, using very large numbers of cells leads to a problem when the sensitivity to detect mosaicism and the inherent error of the technique coincide.

Wapner et al. (42) suggested that the best estimate of mosaicism would come from combining FISH and CMA, but this would take place after primary detection by one method or another. The point remains that, with mosaicism, the chromosomal constitution is dependent on the cell population being studied, which may be different for G-banding, FISH, and CMA. Overall, it is probably best to acknowledge that some cases will be picked up or missed by each technology.

CASES REQUIRING BOTH CHROMOSOMAL MICROARRAY ANALYSIS AND KARYOTYPING

The other relevant category is cases classified as "abnormal result requiring both tests for full interpretation" in **Table 1**. These are cases where an abnormality was detected by both techniques and both sets of results were necessary for a complete analysis, which would include obtaining a precise definition of the size of a duplication or deletion found by karyotyping, clarifying the chromosomal configuration of a dosage change (e.g., two unrelated changes resulting in a translocation or a gain caused by a supernumerary marker chromosome or tandem duplication), or achieving a correct interpretation when mosaicism, especially for sex chromosomes, is present. Verification of the CMA results for anything except simple deletions requires using FISH, if possible, to confirm the lesion and define the nature of the gain and/or loss. This classification is somewhat subjective and so may vary among interpreters. **Table 1** shows that an estimated 18% of abnormalities detected by CMA in postnatal testing needed karyotyping for a complete interpretation, whereas for prenatal testing this value was 13%. **Figure 3** shows other examples from the Warburton et al. (44) study of how a CMA diagnosis without karyotyping would have led to an error with serious health consequences for the patient or family. Of note is that CMA does not distinguish trisomies resulting from Robertsonian translocations. Therefore, the time-honored rule still applies that

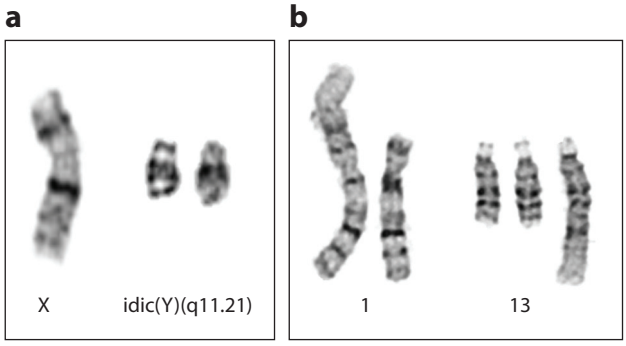

Figure 3

Examples from the Warburton et al. (44) study showing how karyotyping is needed to correctly interpret some chromosomal microarray analysis (CMA) results. (*a*) Sex chromosome results from a prenatal diagnosis. Karyotype analysis showed mosaicism, with approximately 75% of cells having a 45,X karyotype and 25% having a 47,X,idic(Y)(q11.2) × 2 karyotype. The CMA algorithm and visual inspection interpreted this as a male with del(Yq), because the relative dosage across both cell lines is 1X:1Yp [(0.75 × 0) + (0.25 × 4)] :0 Yq. The two interpretations have major differences in their predictions for outcome: The CMA result suggests a phenotypic male with infertility problems, whereas the karyotype result suggests a Turner syndrome phenotype with the presence of a Y chromosome, giving an increased risk for gonadoblastoma. Sex chromosome abnormalities are particularly prone to CMA misinterpretation because of both frequent mosaicism and the complex dosage relationships of sex chromosome loci. (*b*) A partial karyotype with an unbalanced translocation between 1p and 13q, found in a prenatal case interpreted by CMA as trisomy 13. Subsequent karyotype analysis of parental samples showed that the mother was a carrier of a balanced translocation: t(1;13)(p22;q33.2). The trisomy 13 was due to a 3:1 segregation of this maternal translocation. This pregnancy was a young couple's first child; they have received genetic counseling that their risk of another affected child is much higher than their age would indicate and that early prenatal diagnosis is possible for them. CMA results alone will not distinguish this situation from a straightforward trisomy with a low rate of recurrence.

children with autosomal trisomies should have a karyotype analysis even if the diagnosis is not in question, in order to determine the mechanism and inform recurrence risks.

CMA is, however, the easiest way to define imbalances very precisely, regardless of whether they are visible by karyotyping, so that predictions of outcome can be made on an individual basis by examining the implicated genes. CMA does not always detect marker chromosomes, but when it does, it also immediately detects the nature of the duplicated material, with no need for additional FISH studies to determine whether the marker chromosome contains euchromatin. Most marker chromosomes that are not detected by CMA are likely not pathogenic, because they contain only highly repetitive sequences that are not represented by the unique DNA probes present on most clinical arrays.

The "all abnormal results requiring karyotyping for detection or interpretation" category in **Table 1** refers to both cases in the "abnormal result in karyotyping only" category and cases in the "abnormal result requiring both tests for full interpretation" category, i.e., those in which karyotyping or FISH was needed in addition to CMA to correctly interpret the results. Such cases made up 2–3% of both postnatal and prenatal cases and ~22% of all abnormal cases. This number reflects the true proportion of cases from these studies where CMA without karyotyping would not provide a correct interpretation. These numbers are not trivial, and they approach the range where the disclosure of new abnormalities would suggest routinely adding karyotyping to a first-tier CMA test for certain CMA findings.

PROTOCOLS COMBINING CHROMOSOMAL MICROARRAY ANALYSIS AND KARYOTYPING

A remaining question is whether the number and type of diagnoses that would be missed without a karyotype analysis are so significant in terms of patient care that such analyses are necessary. The answer hinges partly, but not completely, on whether the detection of balanced abnormalities is clinically worthwhile.

At this point, it is hard to imagine a situation where balanced translocations are no longer routinely detected. This change would mean that carriers would be diagnosed only after an unbalanced offspring or miscarriage occurs or after other tests for infertility have been done, and that de novo translocations that could be pathogenic are no longer discovered in routine testing. For years, in addition to the importance of discovering balanced translocations for counseling (see below), such rearrangements have also provided fruitful material for gene discovery, and they are the basis for several ongoing projects (11). However, for postnatal testing in a child with an abnormal phenotype, one can argue that detection of a balanced translocation is unlikely to advance the goal of discovering the cause of the problems. For prenatal testing, one can also argue that balanced translocations are not a consideration that would lead to early diagnosis of a disorder, the goal of prenatal diagnosis.

Most importantly, however, the discovery of a balanced translocation is significant for family planning in the families involved. It may prevent the birth of severely disabled children in multiple branches of the family and provide information on the reason for pregnancy losses. The best strategy would be to perform karyotyping only in those identified as being at the highest risk for carrying a translocation. The ISCA/ClinGen guidelines referred to this idea when they suggested karyotyping only in couples with multiple miscarriages or a known history of a translocation in the family (26). This begs the question of how we will know about familial translocations if they are not routinely identified during chromosomal studies. Also, in most cases, a history of miscarriage in the parents is either not known or not elicited by the laboratory when a prenatal study or study of an affected child is done. There is currently no way to screen for situations with the highest likelihood of balanced rearrangements or other situations where a karyotype analysis is needed. In addition, when CMA is used as a first-tier test, karyotyping is still necessary in the 10–15% of cases where an abnormality identified by CMA requires karyotyping to complete the description. A complete description of the chromosomal status requires sequential testing using both methods.

Such a protocol could begin with CMA and add karyotype analysis when the CMA result is normal or karyotyping is needed for interpretation; alternatively, one could perform karyotype analysis first and then do CMA only when the karyotype is normal or CMA would enhance the karyotype findings. Based on the numbers from the Bi et al. (5) study in **Table 1**, the first scenario would require 3,710 CMAs followed by 3,293 karyotype analyses (7,003 total tests) to detect and define 504 abnormalities in postnatal testing, whereas the second scenario would require 3,710 karyotype analyses followed by 3,437 CMAs (7,147 total tests). Cost effectiveness depends on the relative costs of the two tests as well as which finds the most abnormalities. At present, the costs of the tests are quite similar, but CMA testing has the highest yield of abnormalities, with karyotyping adding additional information in ~2–3% of cases (**Table 1**). Thus, the use of CMA as the first-tier test is now the preferred method for clinical practice.

As laboratorians and other clinical providers learn from experience, a consensus will likely be reached that both CMA and karyotyping are useful tests in different situations. For example, some laboratories first use FISH to screen prenatal samples for aneuploidy, and when the results are positive, they then perform karyotyping. One other potential option to reduce the cost and labor of a secondary karyotype analysis is to relax the present standards for a completed test (e.g., fewer

cells analyzed, fewer cultures set up, fewer or no hard-copy images). This option can be justified following a normal CMA result.

WES: whole-exome sequencing

NEW TECHNOLOGIES CONTINUE TO EMERGE FOR THE DETECTION OF CHROMOSOME ABERRATIONS

With the advent of next-generation sequencing technologies capable of rapidly generating WGS and whole-exome sequencing (WES) data came the possibility of accurately deriving copy-number information from these data (1, 45). Many groups are evaluating the accuracy of WGS and WES for detecting deletions and duplications of varying sizes and are developing new algorithms based on read depth of coverage to enhance CNV calling capabilities. Assessment of these sequence-based methodologies compared with CMA-based techniques for CNV calling will provide another opportunity to evaluate which approach provides the most accurate and highest-resolution data for clinical testing.

In the prenatal testing arena, noninvasive prenatal screening, also known as noninvasive prenatal testing, has rapidly joined the landscape of chromosome aneuploidy screening (6). This methodology is based on the detection of cell-free fetal DNA in maternal plasma using next-generation sequencing or other methods for fetal DNA enrichment. It allows the fetus to be tested for the most common aneuploidies by using a DNA sample derived from the mother's blood, thus eliminating the need in most women for an invasive procedure, such as amniocentesis or chorionic villus sampling (7, 28, 30). With improved methodologies currently under development, this screening test could potentially be expanded to scan for smaller CNVs across the genome. Only technological limitations will ultimately determine whether noninvasive testing could eventually replace invasive cytogenetic testing for prenatal detection of genome-wide CNVs.

CONCLUSIONS

The data and discussions presented here support the use of CMA as the first-tier diagnostic test for detection of chromosome aberrations because it provides the highest diagnostic yield for detecting clinically relevant chromosome aberrations. An abbreviated karyotype analysis should be considered after normal CMA results because some abnormalities, such as balanced chromosome rearrangements, are not detectable by CMA. An abnormal CMA test may also need to be augmented at times with additional G-banding and/or FISH analyses to fully characterize a specific finding and provide accurate recurrence risk estimates. Furthermore, because CMA cannot detect balanced rearrangements, G-banding should still be used first for some indications, such as couples with infertility or recurrent miscarriages where detection of a balanced rearrangement is particularly important for genetic counseling purposes.

New methodologies such as WGS and noninvasive prenatal screening will continue to evolve for copy-number detection and eventually will likely replace the use of CMA for detecting copy-number abnormalities. Because WGS can also detect structural chromosome rearrangements, it is now easy to imagine the use of a single methodology for routine clinical genetic testing to evaluate copy-number changes, structural rearrangements, and sequence-level variants. It clearly is an exciting time for genomic medicine, where the integration of genomic variation into everyday health care will allow truly personalized medicine.

DISCLOSURE STATEMENT

C.L.M. is on the Board of Directors of the American College of Medical Genetics and Genomics and is an employee of Geisinger Health System.

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