PRENATAL COUNSELING

"Molecular karyotyping" opens a new avenue of prenatal diagnosis

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IN THIS ARTICLE

Prenatal diagnosis became a reality almost 40 years ago, when advances in microscopy and cell culture made it possible to examine chromosomes in fetal cells drawn from amniotic fluid—the familiar karyotype analysis. Technical advances continue to sharpen the resolution of routine karyotype analysis on amniotic fluid or a specimen of chorionic villus, and to raise the level of detail obtained from such a study.

Yet examining chromosomes by light microscopy remains time- and labor-intensive. A cell culture typically requires 2 weeks before growth of cells is sufficient to undertake a karyotype analysis—after which the microscopic evaluation requires further time and significant skill to perform.

Change is coming to practice

Over the past 10 years, however, the human genome project has produced technologies that allow us to examine DNA at a level of resolution unattainable when chromosomes are evaluated under a light microscope. The exciting news is that these research technologies are being transferred to the clinical arena, where they will transform prenatal diagnosis and counseling in your practice.

One such technology that will have such a far-reaching effect, and that I focus on in this "Update," is known as molecular karyotyping.

What is "molecular karyotyping"? How is it performed?

- Refinements to hybridization technology yield new tools; a new term enters the lexicon of prenatal diagnosis

Vermeesch JR, Melotte C, Froyen G, et al. Molecular karyotyping: array CGH quality criteria for constitutional genetic diagnosis, J Histochem Cytochem. 2005;53:413–422.

Van den Veyver I, Beaudet A. Comparative genomic hybridization and prenatal diagnosis. Curr Opin Obstet Gynecol. 2006;18:185–191.

So-called molecular karyotyping utilizes the evolving technology of comparative genomic hybridization by microarray (or, simply, array CGH), which is a refinement of older CGH technology. Initial work with whole-genome hybridization involved applying fragmented and fluorescently labeled subject DNA to a normal metaphase chromosome spread. Deletions or duplications within the subject DNA

were then made evident by reduced, or increased, fluorescence at complementary sites along the metaphase chromosomes. The resolution afforded by this approach was comparable to that of light microscopy—namely, alterations of at least 5 to 10 megabases (Mb) could be detected.

Array technology emerged in the late 1990s and increased the resolution of genome hybridization by at least 10-fold. How does it work?

- An array comprises DNA fragments of known sequence that have been attached or printed on a platform, such as a glass slide.
- The array on the slide is analyzed by adding both subject DNA and normal, control DNA, with conditions optimized for hybridization between complementary strands. Subject and control DNA are differentially labeled with fluorescent dye—often, Cy3 (green) for subject and Cy5 (red) for control.
- As subject and control DNA compete for hybridization to the array DNA, imbalances in genomic content produce differential intensities of Cy3 and Cy5 signals as read by a laser scanner. Results are displayed relative to the control DNA. Deletions are revealed by a decrease in copy numbers; duplications, by an increase in copy numbers (**FIGURES 1 AND 2**).

Array CGH is still new but already being improved

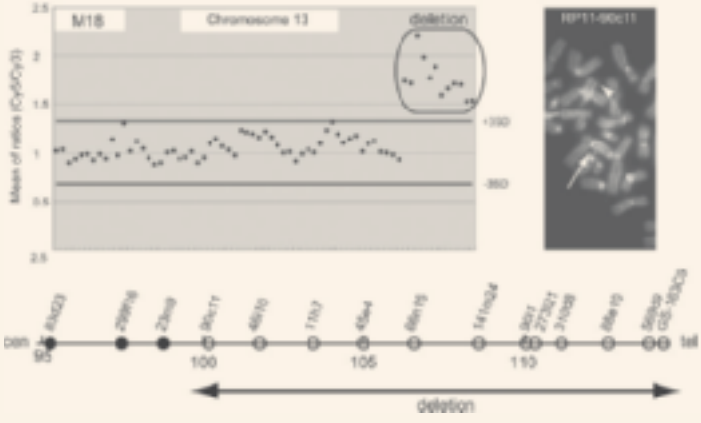
The 1st generation of array CGH slides covered the entire human genome with DNA fragments spaced approximately 1 Mb apart. Refinements have produced arrays of more than 30,000 overlapping DNA fragments. Such resolution allows detection of a gain or loss of segments as small as 100 to 200 kilobases (Kb). Compare this resolution with the best resolution of traditional microscopic cytogenetic analysis: approximately 5 Mb.

Into the clinical realm

Specialized "targeted" arrays can be applied to clinical work in several ways, including:

FIGURE 1

Sample display of array CGH and corresponding FISH analysis



At left: Hybridization ratios of normal sex-matched control DNA (Cy5) to sample DNA (Cy3) are plotted as a function of Cy5/Cy3 signal intensity. (Note that ratios of deleted clones are greater than +3SD.)

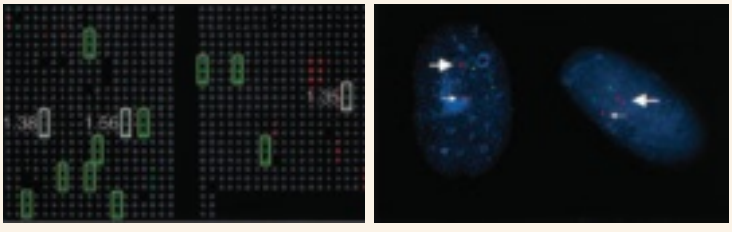
At right: Fluorescence in situ hybridization (FISH) analysis demonstrates intact (arrows) and deleted (arrowheads) signals.

Bottom: Clones are summarized schematically.

Modified from Yamagata et al. Am J Med Genet. 2006;140A:205–211.

FIGURE 2

Array CGH reveals a duplicated chromosome 15q



At left: Analysis by array CGH demonstrates trisomy 16 and duplication of the Prader-Willi/Angelman syndrome region on chromosome 15q in this patient. Each clone is spotted in triplicate on the array; clones with a gain in the specimen are represented in green; those with a loss, in red; and those with a normal copy number, in gray. Green boxes mark chromosome 16 clones that demonstrate trisomy. White boxes highlight clones from the Prader-Willi/Angelman syndrome region that are duplicated; corresponding ratios are shown next to each target. Other red and green signals correspond to clones from, respectively, the X and Y chromosomes.

At right: Interphase FISH analysis confirms the interstitial duplication of chromosome 15q that was identified by array CGH. The small arrow in each cell points to the normal signal for the *SNRPN* (Prader-Willi) gene; the large arrow indicates duplicated chromosome 15q, which shows two hybridization signals for *SNRPN*.

Modified from Schaffer. Am J Human Genet. 2004;74:1168.

- to detect known syndromes of chromosome duplication and deletion
- to assess whole chromosome gain or loss
- to provide information about telomere regions.

And consider what is anticipated: highly dense arrays that are capable of assessing single nucleotide alterations, making it possible to detect single-gene mutations.

Because array-CGH technology utilizes DNA and does not require cell culture, the time to results is significantly shorter. Furthermore, many aspects of the assessment are automated, providing both high resolution and rapid processing and reporting.

Array CGH uncovers genomic problems in the young

Causes of mental retardation, developmental deficits, congenital anomalies, and more are localized

Miyake N, Shimaokawa O, Harada N, et al. BAC array CGH genomic aberrations in idiopathic mental retardation. Am J Med Genet. 2006;140A:205–211.

Ming J, Geiger E, James A, et al. Rapid detection of submicroscopic chromosomal rearrangements in children with multiple congenital anomalies using high density oligonucleotide arrays. Hum Mutat. 2006;27:467–473.

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- **Submicroscopic abnormalities will be detected with increasing frequency**
- **The role of chromosomal abnormalities in 1st-trimester fetal loss will be better defined**

Early use of array CGH in the study of solid tumors was followed closely by its clinical application to children with mental retardation or developmental deficits, with or without birth defects. Historically, suspicion of a duplication or deletion syndrome despite a normal chromosome analysis in these children could prompt specific testing for that disorder. More often, however, it was impossible to delineate a specific syndrome, and disorder-by-disorder testing was not feasible. Today, estimates are that submicroscopic duplications and deletions on chromosomes, detected primarily by array CGH, occur in 1 of every 1, 000 births.

Initial work in the pediatric population by Vissers, in 2003, and Shaw-Smith, in 2004, showed that, with array CGH at a resolution of 1 Mb, 14% to 20% of children who were mentally retarded had duplications or deletions that could not be detected by routine karyotype analysis. Further detail on this approach, using an array with 1.4-Mb coverage, appears in the article by Miyake and co-workers. Among 30 children with idiopathic mental retardation and dysmorphic features, 17% (5 of 30) had submicroscopic deletions or duplications by array CGH. The imbalances ranged from 0.7 Mb to 1.0 Mb and spanned numerous and various chromosomes. The investigators emphasized the need to:

- validate the chromosomal locations of the array signals before clinical investigation
- include specimens from parents in the analysis to ensure that any identified imbalance represents a new event.

Numerous "copy number polymorphisms" have been uncovered—do they always matter?

Work with array CGH among the pediatric population was expanded by Ming and colleagues, who obtained greater resolution and coverage of the genome by utilizing a 2nd-generation array of oligonucleotides with >100,000 single-nucleotide polymorphisms. With this array, intermarker distance is estimated at 25 Kb—a resolution at which very small genomic imbalances can be identified. Of 10 children evaluated using this greater-density array, 2 (20%) had a previously unidentified genomic imbalance—both deletions.

Ming also put forward concerns that more non–disease-causing "copy number

polymorphisms" (CNPs) will be uncovered as higher-density arrays increase the resolution of array CGH. These polymorphisms are encountered in healthy persons and are considered clinically insignificant. Consequently, when a copy number imbalance is detected by array, several actions are warranted: comparison with normal controls, evaluation of published CNP databases, and—most important—array CGH analysis of both parents' DNA.

Such an approach adds to the labor-intensity of array CGH, but is necessary to ensure that imbalances that are clinically relevant and causative are distinguished from normal variants. With more than 250 discrete CNPs reported in normal controls, the use of denser arrays will uncover more CNPs than arrays targeted to significant fetal and pediatric disorders. Applying array CGH to clinical practice will entail (1) ongoing assessment of the technology and the results it provides and (2) perhaps, targeting of arrays to particular populations—the goal being to balance the yield of useful information against the increase in reported CNPs.

Where is the potential of array CGH in prenatal diagnosis?

Le Caignec C, Boceno M, Saugier-Veber P, et al. Detection of genomic imbalances by array based comparative genomic hybridization in fetuses with multiple malformations. J Med Genet. 2005;42:121–128.

Rickman L, Fiegler H, Shaw-Smith C, et al. Prenatal detection of unbalanced chromosomal rearrangements by array CGH. J Med Genet. 2006;43:353–361.

Sahoo T, Cheung S, Ward P, et al. Prenatal diagnosis of chromosomal abnormalities using array-based comparative genomic hybridization. Genet Med. 2006;8:719–727.

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Five years from now, look for array CGH to provide:

- Faster detection of aneuploidy
- Routine assessment of more disorders from fetal cells

Prenatal diagnosis can be enhanced by array CGH

If ongoing research on array CGH can accomplish any of the following goals, it is likely that the technology will be propelled into clinical use as part of prenatal counseling within the next 5 years:

- detection of aneuploidies more quickly (and, perhaps, more economically)
- expansion of the number of disorders routinely assessed from fetal cells
- genomic assessment of nonviable tissues (such as from fetal loss).

Le Caignec and colleagues' work on applying array CGH to DNA specimens from fetuses that had multiple malformations—but in whom cytogenetic study was normal—have provided a foundation for subsequent prenatal studies. Using an array that targeted subtelomeres and specific DNA loci that are important in cytogenetic deletion–duplication syndromes, Le Caignec found that 5 of 49 (10.2%) fetuses studied had clinically significant genomic imbalances. These included:

- subtelomeric deletions
- interstitial deletions
- submicroscopic duplications
- multiplex genomic imbalance.

The fetuses studied by Le Caignec had at least three malformations—variously in the cardiovascular, urogenital, skeletal, digestive, and central nervous systems. But when the list of identified anomalies was assessed, most of those fetuses, if examined by high-resolution ultrasonography, would have had anomalies identified in only 2 systems; the 3rd involved system would have been detectable only on fetopsy.

Rickman and colleagues used a custom array that focused on prenatal and pediatric abnormalities to examine the sensitivity and specificity of array CGH for detecting common aneuploidies in amniotic fluid specimens. All but 1 of the 30 subjects' unbalanced chromosome rearrangements could be detected by array CGH—in some cases, from a specimen of amniotic fluid as small as 1 cc.

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In Rickman's hands, as well as in the hands of others, triploidy could not be detected, however—a problem that has been addressed in newer array platforms. In an additional 30 cases, no false positives were noted.

Similar results were obtained by Sahoo and co-workers: In 98 prenatal specimens (obtained by CVS or amniocentesis), there was complete concordance between the results of karyotype analysis and array CGH studies. In most cases, specimens were obtained because of advanced maternal age; only 19% represented concern over a sonographic abnormality. This study population included 4 cases of trisomy 21 and 1 case of an unbalanced translocation.

Notably, among the 98 specimens, 30 were thought to be characterized by gain or loss of copy number of 1 or more clones. Because these copy number repeats are recognized as normal variants (based on analyses of normal populations), they were considered copy number polymorphisms (CNPs) and without clinical significance to the fetus.

In addition, 12 cases contained a copy number imbalance that had not been recognized among normal controls. In 9 of those cases, the same loss or gain was demonstrated in 1 parent. In 1 other case, the parents elected not to be studied and, in the 2 others, the array finding was not confirmed on further testing (although low-level mosaicism could not be excluded). Sahoo's team emphasizes both the targeted specificity of their custom array for well-characterized disorders, the reference to normal population databases being constructed for CNPs, the use of at least 3 clones for each disease locus, and the necessity for parental specimens to appropriately counsel the family about the presence of CNPs.

The work of Rickman and Sahoo reveals the potential for applying array CGH to a small volume of amniotic fluid or a specimen from direct CVS—a process that begins with whole-genome amplification. As this approach is refined to decrease the sample size and shorten the time to results even more, we can expect to see array CGH applied to areas where analysis has been constrained by the fact of small specimen size—such as preimplantation genetic screening.

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So-called copy number polymorphisms are common in molecular karyotyping but often of no clinical significance

Analysis of fetal loss will mean better counseling about recurrence

Fritz B, Hallerman C, Olert J, et al. Cytogenetic analyses of culture failures by comparative genomic hybridization (CGH)—re-evaluation of chromosome aberration rates in early spontaneous abortions. Eur J Hum Genet. 2001;9:539–547.

Schaeffer A, Chung J, Heretis K, et al. Comparative genomic hybridization-array analysis enhances the detection of aneuploidy and submicroscopic imbalances in spontaneous miscarriages. Am J Hum Genet. 2004;74:1168–1174.

Approximately 50% of 1st-trimester pregnancy losses are considered to be the results of chromosomal abnormalities. Often, however, it isn't productive to analyze the products of conception because fetal cells fail to grow in culture or are overgrown by maternal cells. And, although chromosomal abnormalities play less of a role in 2nd-trimester fetal loss or in stillbirth, the rate of nondiagnostic results from classical cytogenetic study in such cases is high.

Sampling of the placenta or amniocentesis at the time fetal loss/stillbirth is recognized can lower the no-growth rate, but these methods have not been incorporated into practice universally. With array CGH, however, results can be obtained from uncultured cells, and that capability offers the opportunity to assess a demised fetus for common aneuploidies.

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Array CGH will also provide an assessment of genomic imbalances that aren't otherwise detectable at the resolution of metaphase chromosomes. Identification of a genomic imbalance—during a 1st- or 2nd-trimester loss—would facilitate an appropriate workup and lead to more accurate counseling about the risk of recurrence.

Assessment of nondividing cells reveals an unexpectedly high rate of chromosomal abnormality

Fritz and co-workers used array CGH to assess 60 cases of 1st-trimester spontaneous loss in which culture did not yield a karyotype result. Utilizing the older methodology of genomic CGH (ie, resolution is comparable to that of karyotype analysis), 72% of fetuses were found to have an underlying chromosome abnormality.

The work of this team supports what is increasingly reported:

- Genomic imbalance may play a larger role in 1st-trimester loss than the rate of 50% that is often cited.
- The rate of no-growth in particular trisomies (eg, chromosome 7) and triploidies may be higher than is now believed.

These data warrant expanding array CGH to the evaluation of loss in 2nd and 3rd trimesters.

Schaffer and colleagues assessed a population of 41 products of conception using conventional cytogenetic analysis and array CGH. The conventional karyotype study and the array CGH were concordant in 37 of 41 cases—with 100% concordance for normal karyotypes, 10 cases of trisomy, 2 cases of sex chromosome aneuploidy, and 2 cases of deletion. More important, 4 cases (9.8%) that had been interpreted as normal on a conventional karyotype study were found by array CGH to have submicroscopic genomic imbalances, including trisomic mosaicism, interstitial deletion, and subtelomeric deletion. ■

The author reports no financial relationships relevant to this article.

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An imbalance in the genome of the fetus may play a larger role than we've thought in 1st-trimester loss