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Development of new postnatal diagnostic methods for chromosome disorders

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SUMMARY

Chromosome imbalances are the leading cause of intellectual and developmental disabilities in the population. This paper reviews the current methods used to diagnose chromosome abnormalities in children including karyotyping, fluorescence in situ hybridization and microarray technologies. Advances in molecular cytogenetics, especially with the use of microarrays, have substantially increased the detection of chromosome abnormalities in children with disabilities and congenital anomalies above that achievable with conventional cytogenetic banding and light microscopy.

1. Introduction

Chromosome imbalance, whether through a numeric or structural change, is a leading cause of developmental and intellectual disabilities and congenital anomalies in humans. About 1% of all live births have a chromosome abnormality, and approximately half of these are unbalanced. Down syndrome is the most common disorder caused by a chromosome abnormality, affecting approximately one in every 800 newborns. Although Down syndrome is easily recognized both in the clinical phenotype and through the light microscope – by the presence of three, rather than two, copies of chromosome 21 – many other chromosome abnormalities can occur and are more difficult to diagnose accurately. This review will focus on the new technologies that are being used to uncover chromosome imbalance, at frequencies never before realized when banding and light microscopy were the only available methods.

2. Chromosome analysis

Chromosome analysis is made possible after the culture of cells, the arrest of the mitotic cell cycle in metaphase, and special staining and banding to delineate each individual chromosome pair. Normally, humans have 23 pairs of chromosomes, 22 autosomes and one pair of sex chromosomes. Chromosome analysis, or karyotyping, has been instrumental in identifying numerical and structural abnormalities of chromosomes. The most common, viable numerical abnormalities are trisomies of chromosomes 21 (Down syndrome), 13 (Patau syndrome), 18 (Edward syndrome), 47,XXX, 47,XXY (Klinefelter syndrome), 47,XYY and monosomy of the X chromosome (Turner syndrome). In general, whole chromosome monosomies of the autosomes do not exist, as they are thought to be lethal early in gestation. Trisomies of chromosomes other than the ones described earlier exist, but usually in a mosaic form. Mosaicism occurs when there is a normal cell population (46,XX or 46,XY) in an individual and another population of cells containing a chromosome abnormality, most typically a trisomy, with both populations originating from the same fertilized egg (zygote). In this situation, trisomies for chromosomes 8, 9, 14, 15, and 22 are most common, although mosaic trisomies of other autosomes are known to exist.

Traditional chromosome banding and analysis have served us well for almost 40 years. However, there are limitations to the imbalances that can be visualized through the light microscope. In general, monosomies and trisomies should always be detected, unless mosaicism is present and in such low levels that the occasional abnormal cell is 'missed' by the cytotechnologist during the microscopic examination of a limited number of cells (usually 20–30 cells). In addition, large structural alterations, such as translocations – the exchange of chromosomal segments between two or more chromosomes – can be visualized with light microscopy if the alteration is sufficiently large (greater than ~5–10 Mb) and the chromosome abnormality results in a detectable change in chromosome size or banding pattern.

In contrast to the situation described for numerical and large structural changes, there are many submicroscopic alterations such as 'microdeletions' and 'microduplications' that can occur in the genome and that are undetectable through the light microscope. In addition, some rearrangements of the ends of the chromosomes, termed telomere or subtelomeric regions, are too small to detect using traditional banding techniques and require molecular methodologies for their identification. Those methodologies that are reviewed in this article include fluorescence in situ hybridization (FISH) and genomic microarrays.

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3. Fluorescence in situ hybridization

In FISH, DNA is labeled with a fluorescent reporter molecule. The labeled probe is then hybridized onto metaphase chromosomes or interphase nuclei so that the fluorescent signal on the target chromosomes or nuclei can be visualized by fluorescent microscopy.[1] The DNA probes that are used can contain repetitive or unique sequences. The most common repetitive sequence probes that are used in the clinical laboratory are those to discrete centromeres (the primary constriction within a chromosome). Centromere probes are often used to detect aneuploidy (trisomies and monosomies), and to identify the chromosomal origin of marker chromosomes, which are small, extra structurally abnormal chromosomes whose sizes are too small to determine their origins. These repetitive probes hybridize to their targets quite efficiently, providing a rapid enumeration of the number of chromosomes targeted in interphase nuclei.[2] Unique-sequence or locus-specific probes are most commonly used to identify deletions on the metaphase chromosome of specific and unique regions of the genome associated with known genetic syndromes. For example, a probe from the chromosomal region 22q11.2 is used to identify a common deletion that causes the DiGeorge syndrome.[3] Similarly, diagnosis of Williams syndrome and Duchenne and Becker muscular dystrophy can be made by demonstrating deletions of the elastin gene at 7q11.23 and deletions of the dystrophin gene at Xp21, respectively.[4–6] Duplications can also be detected, although most commonly in the interphase nucleus rather than on metaphase chromosomes due to limitations in the resolution of the duplicated FISH signals on the tightly packed metaphase chromosomes. One of the first FISH assays to be developed for duplication visualization was for the detection of duplications of the *PMP22* gene on 17p12 in Charcot–Marie–Tooth disease.[7] Locus-specific FISH probes have been combined to provide a rapid screen for microdeletion syndromes and the common trisomies.[8,9]

The subtelomeric regions of the chromosomes tend to be involved frequently in rearrangements and deletions. To help identify these often subtle or cryptic changes, sets of FISH probes were developed that targeted the unique ends of all the chromosomes.[10,11] Although these probes have been instrumental in uncovering pathogenic alterations of these regions,[12–15] apparently benign imbalances have also been identified, which need to be distinguished from disease-causing deletions and duplications.[16]

Other probe types were developed, such as those that coated or 'painted' an entire chromosome.[17] These painting probes were also combined in sets to produce 24-color karyotypes.[18] These probes are helpful in identifying the additional material on unbalanced translocations and in delineating complex rearrangements often seen in certain malignancies.[19]

Although probe sets can be combined to interrogate specific regions of the genome,[8,11] they do not allow for a comprehensive evaluation of the whole genome. Thus, chromosome analysis provides an overview of the genome at a relatively low resolution, whereas FISH provides a high-resolution analysis of only targeted locations. To overcome the limitations of both karyotyping and FISH, researchers and diagnosticians have turned their attention to microarrays.

4. Genomic microarrays

Microarrays are constructed from various-sized targets ranging from bacterial artificial chromosomes (BACs), 80–200 kb in size, to synthetic oligonucleotides, 25–85 bp in length. The targets, representing various segments of the genome, can number in the thousands and up to more than a million targets on some commercially available arrays. Of the arrays available, two general types exist: comparative genomic hybridization (CGH)-based arrays and single nucleotide polymorphism (SNP)-based arrays. In CGH arrays, the patient sample and a control sample are each labeled with a distinct fluorescent dye, hybridized in the same concentration to the same microarray, and the fluorescent intensity of each dye is captured by computer imaging. The amount of fluorescence, or dosage of the dyes for a particular locus, is compared between the patient and control, and the ratio of the two dyes is plotted on a graph. When the patient has a genomic gain or loss, as compared to the control, the difference in the fluorescent intensity of the dyes at this genomic location can be visualized on the graph (Fig. 1). CGH arrays can be both BAC- and oligonucleotide-based. Depending on the genomic coverage contained within the targets on the array, in general, oligonucleotide arrays have the potential for higher-resolution analysis, with the ability to detect smaller alterations than can be identified by BAC arrays.

Unlike CGH arrays, SNP arrays do not directly compare a patient and a control specimen. SNP arrays compare the dosage of the patient at any given locus to a database of control individuals. As with CGH arrays, gains and losses of the genome are readily detectable using this method. SNP arrays have the added advantage of being able to detect DNA base alterations, or genotyping, for any given SNP. The combination of multiple SNPs can show regions that have loss of heterozygosity, either resulting from uniparental isodisomy, such as seen in rare cases of Angleman syndrome,[20] or in offspring from closely related parents (consanguinity). Although CGH arrays can detect some cases of triploidy, based on the dosage over the sex chromosomes, SNP arrays readily detect triploidy. Both CGH and SNP arrays will detect mosaicism greater than about 20–30% abnormal cells.[21,22]

The first genomic microarrays used to detect chromosome abnormalities in children with developmental disabilities demonstrated the advantages over traditional karyotyping,[23] including the ability to detect alterations smaller than those that can be visualized through the light microscope and the comprehensive nature of a whole-genome assay. The resolution of the array is determined by the size of the target and the genomic coverage or density of the targets. The clinical utility of the array is determined by the specific genomic coverage and the potential pathogenicity of the particular loci targeted. For example, microarrays cannot discriminate between potentially pathogenic loci and segmental duplications, which can be found throughout the genome of normal individuals. Thus, if a repetitive region, such as a segmental duplication, is represented on the array, gains and losses of this region can be detected and potentially misinterpreted. Therefore, arrays for clinical use should be designed specifically for particular applications by individuals knowledgeable in the intended use and interpreted by experts in the field.

5. Interpretation of copy number alterations

The ability of microarrays to interrogate thousands of loci simultaneously has changed the practice of medical genetics.[24] Gains and losses of the genome, also termed copy number alterations (CNAs) or copy number variants (CNVs), have been identified in individuals with developmental and intellectual disabilities or physical congenital anomalies at unprecedented detection rates. Compared with karyotyping, which will detect a chromosome abnormality in about 3–4% of children with global developmental delay,[25] one study detected a pathogenic CNA by a targeted BAC array in an additional 6.4% of children.[26] A recent review of 33 published studies using microarrays in children with developmental disabilities showed that most reported studies had detection rates of chromosomal gains and losses of 15–20%.[24] Thus,

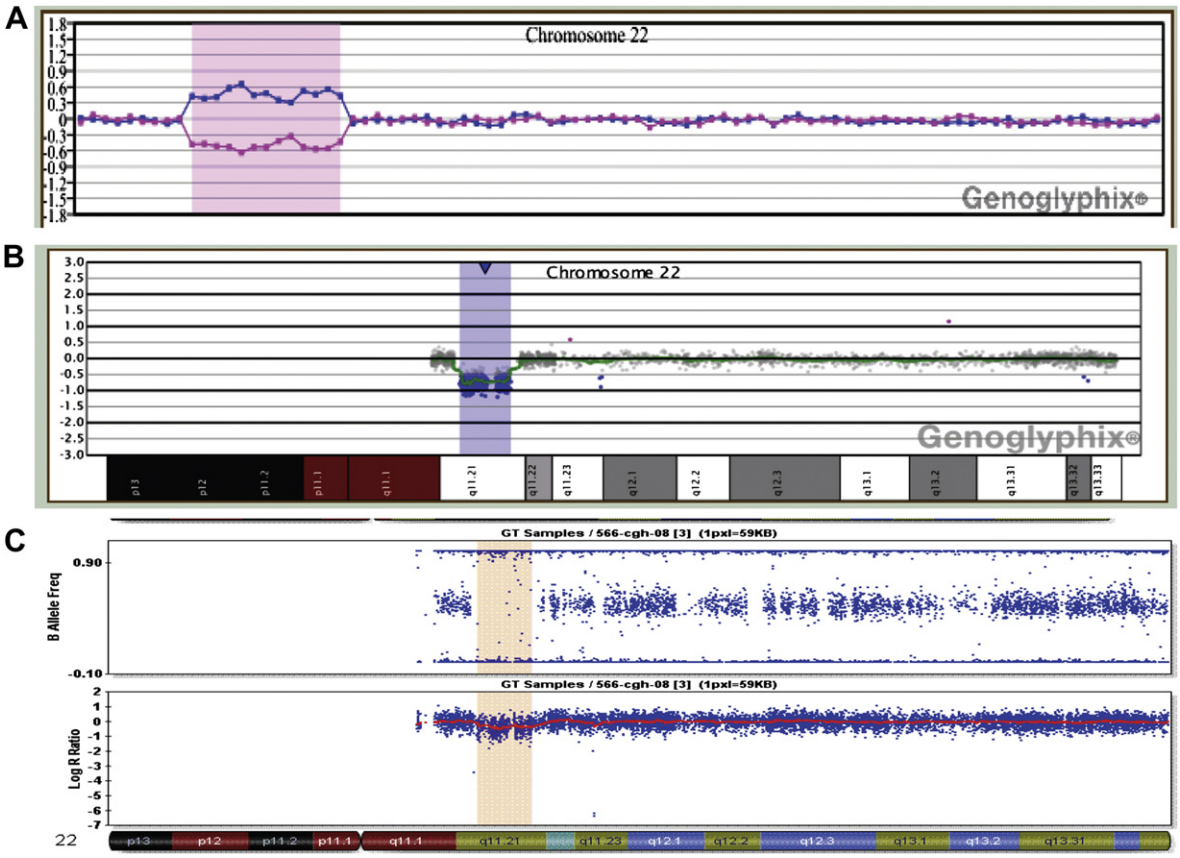

Figure 1. Examples of microarray results for three cases of DiGeorge syndrome and deletion 22q11.2. In each, probes are ordered on the *x*-axis according to physical mapping positions, with the short arm oriented to the left and the long arm to the right. Because chromosome 22 is an acrocentric chromosome, the short arm is not represented on the arrays. (A) Bacterial artificial chromosome (BAC) array. Two experiments were performed, one shown as a pink line and one shown as a blue line. Regions of chromosome 22 in a normal dose of two copies plot at a $\log_2$ ratio of zero, and the lines come together. Region of deletion shows a deviation of the two lines, as the shading indicates. (B) Oligonucleotide comparative genomic hybridization (CGH) array. All of the data points at zero indicate normal dosage of two copies. The blue data points and shading indicate a loss of chromosome 22 over the DiGeorge syndrome region. (C) Single nucleotide polymorphism (SNP) array. Upper panel shows the B-allele frequencies. Deletions show either A or B alleles, but no AB alleles, as indicated by the gap in the middle of the plot. Lower panel shows the dosage of the SNPs on chromosome 22. The red line indicates a dip in the dosage over the DiGeorge syndrome deletion region.

microarray analysis has been shown to identify more clinically relevant genomic imbalances than can be detected with conventional karyotyping.

Although some CNAs can be clearly classified as pathogenic, others can be classified as benign, conferring susceptibility to some abnormal phenotype, or of unclear clinical relevance. CNVs across the genome have been demonstrated in normal individuals and in some cases likely represent normal population variation.[27–29] These benign variants are identified in both patient and control populations in roughly equal frequencies. However, there are other regions of the genome that appear to confer susceptibility to certain phenotypes.[29,30] These susceptibility loci can be carried by an apparently unaffected parent, are enriched in patient populations and have a relatively low frequency in control populations. Recent examples include deletions and duplications of 1q21, deletions and duplications of 16p11.2, and deletions of 16p13.3.[29–37] In addition, CNVs of unclear clinical significance are often found in patient populations tested by microarrays.[38,39] These alterations are considered unclear because of their very low frequency in patient populations, inheritance by a clinically normal parent, and absence in control populations. The interpretation of these CNVs as causative to the patient's phenotype is challenging, and the study of parents is often not helpful because a rare, novel change, inherited from a normal parent, may represent a susceptibility locus, and this possibility cannot be excluded without further population studies. Even the finding of a small de novo change in a patient's DNA may not indicate a causal relationship between the alteration and the phenotype; however, it is generally accepted that de novo changes are more likely to be causative than inherited CNVs. Even with these challenges in interpretation, most CNAs have clear clinical implications, and those that are determined to be pathogenic do provide answers to families seeking the reason for their child's medical problems. Identifying the genetic etiology of disease allows for accurate genetic counseling, reproductive management, and anticipation of potentially serious medical problems in the child.[40]

6. New syndrome identification by microarray analysis

Historically, new genetic syndromes have been described after identification of a collection of cases with consistent, specific and overlapping phenotypic features. In many cases, a chromosomal etiology was established after the clinical syndrome was delineated. Examples include deletions of 15q11.2 and Prader–Willi syndrome, deletions of 7q11.23 and Williams syndrome, and deletions of 8q24.1 in Langer–Giedion syndrome. The relative rarity of patients with certain syndromes made the identification through a 'phenotype-first' approach a difficult and lengthy process.

More recently, microarrays have allowed for the identification and grouping of patients with identical, similar or overlapping alterations. This 'genotype-first' approach[41] allows for the

delineation of new syndromes based on the genomic alteration, rather than the clinical presentation. Such an approach leads to the grouping of patients, sometimes with disparate phenotypic presentations including deletion 3q29, deletion 9q22.3, deletion 15q13.3, distal duplications of 22q11.2, and deletion 17q23.[42–46] Several other new syndromes have been identified through overlapping alterations found after microarray analysis, and these patients were found to have common clinical features. These include deletion 15q24, deletion 16p11.2p12, deletion 1q41q42, and deletion 2q33.1.[41,47–49]

In addition to the identification of new microdeletion syndromes, reciprocal microduplications of some of the common microdeletions have been identified after microarray analysis including duplication 22q11.2, duplication 7q11.23, and duplication 15q12.[50–53] Many of these microduplication syndromes exhibit clinical features much milder and often more varied than the reciprocal microdeletions which would preclude clinicians from grouping them together as a syndrome.

7. Summary

Advances in molecular cytogenetics have aided in the identification of chromosome abnormalities in children with developmental and intellectual disabilities and physical congenital anomalies. FISH has been an instrumental screening tool to confirm the clinical suspicion of a particular syndrome due to a chromosomal microdeletion or to screen the ends of all human chromosomes to uncover terminal deletions and unbalanced translocations. However, the advent of microarrays in clinical diagnostics effected a phenomenal change in genetic medicine. The use of microarrays has identified about a fivefold increase in chromosome abnormalities, above that achieved with traditional cytogenetics. These advances have led to the discovery of new syndromes; the clinical characterization of these new disorders has led to additional diagnoses in children that might otherwise been unwarranted. The most striking examples of the benefits of microarray testing are those cases that would not have been diagnosed if it were not for microarrays. For example, Shah et al.[54] described the identification of the common deletion found in Williams syndrome in a preterm infant with a phenotype suggestive of Alagille syndrome. Cases such as this also illustrate the wide variability that even the common microdeletion syndromes may display. The variable phenotypes were underappreciated prior to the use of microarrays. Without the clinical suspicion of Williams syndrome, the physician would not have ordered the FISH test for Williams syndrome and a diagnosis would not have been made. The genome-wide view that microarrays provide is the only current mechanism for identifying unsuspected anomalies. In addition, homozygous deletions of loci have been identified with microarrays, providing novel mechanisms for diseases.[55] Finally, the resolution of microarrays provides the ability to identify single gene deletions in the child suspected of having a chromosome anomaly.[49,56] Currently, microarrays should be used as the first approach to the child suspected of having a chromosome abnormality to provide the highest chance of making a diagnosis and sparing the patient unnecessary diagnostic testing.

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Conflict of interest statement

Lisa G. Shaffer and Bassem A. Bejjani are employees of Signature Genomic Laboratories, a subsidiary of PerkinElmer.

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