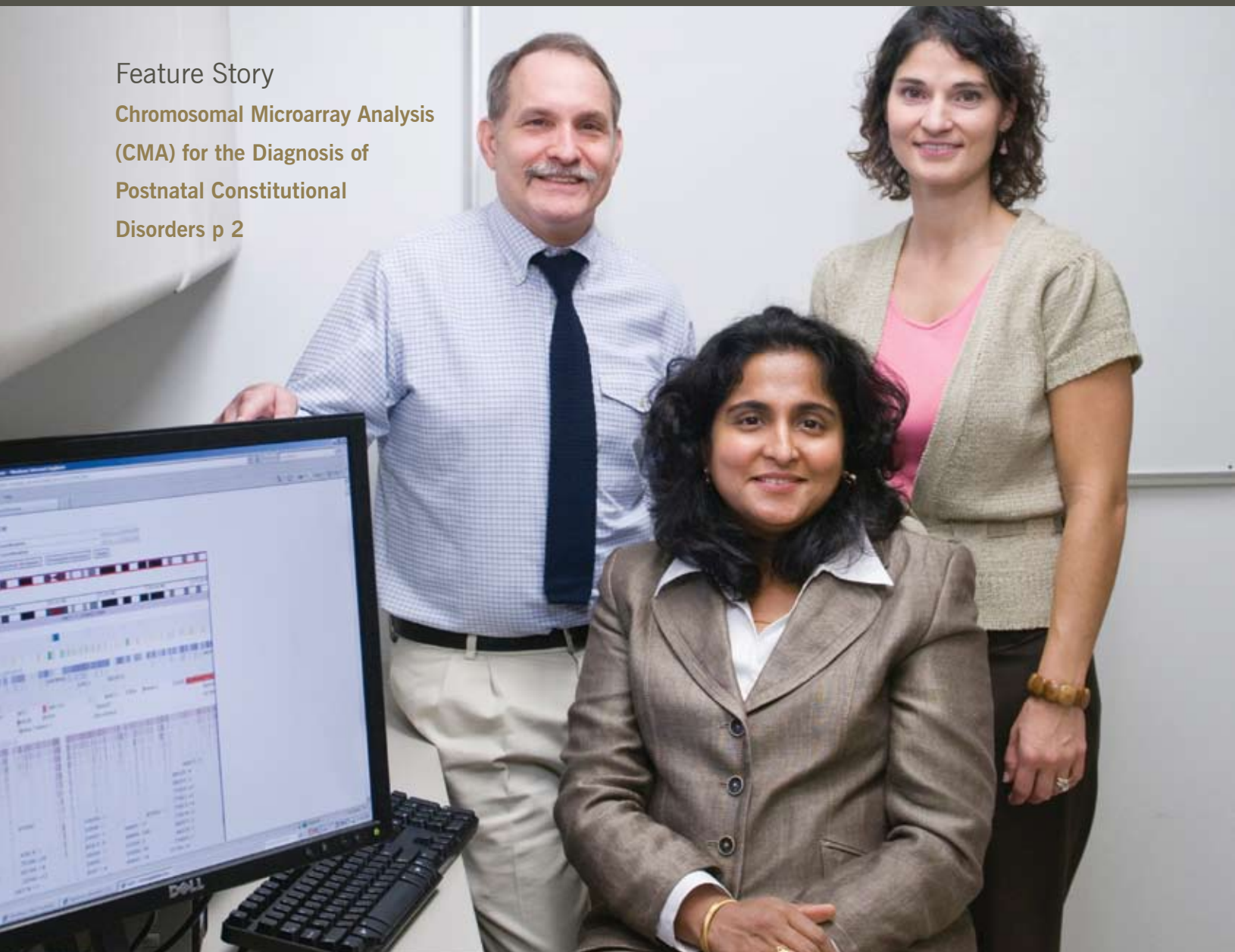


Pathology Research

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Feature Story

**Chromosomal Microarray Analysis
(CMA) for the Diagnosis of
Postnatal Constitutional
Disorders p 2**



Feature Story

Chromosomal Microarray Analysis (CMA) for the Diagnosis of Postnatal Constitutional Disorders

By Shashi Shetty, PhD

Evidence-based studies with the aid of technical developments in molecular cytogenetics are rapidly changing the way the human genome is being analyzed and interpreted. Until recently, cytogenetics evaluation has relied to a great extent on chromosome banding techniques for global genome analysis of chromosomal abnormalities at a resolution of five to 10 megabases (Mb). The advent of chromosomal microarray analysis (CMA), also known as array comparative genomic hybridization (aCGH), has changed the field of cytogenetics dramatically. Microarray CGH has been developed to identify large regions of DNA losses (deletions) or gains (amplifications). Such alterations in the DNA are often involved in both constitutional (germline) and acquired (somatic) conditions or disease. Microarray CGH is based on the use of differentially labeled test and reference genomic DNA samples that are simultaneously hybridized to DNA targets arrayed on a glass slide or other solid platform. Other types of microarrays are able to detect DNA sequences that bind to proteins or sequences that are chemically modified in the genome, such as by methylation or acetylation.

With this technology, resolution of 100 to 500 kilobases (kb) or less is now possible. Instead of viewing the entire world from a bird's eye view to identify different countries (the whole genome, 5-10 Mb), with CMA we now have the technical capability to zoom into specific cities (100-500 kb) and, in some cases, even super-zoom to street level (30-50 kb, gene level). The zoom-in capability of CMA for high-resolution analysis is dependent on many factors. These include the performance of probes based on GC nucleotide

content, the analysis algorithm to interpret the aberrations, and other confounding factors inherent to the human genome like segmental duplications or normal variations found everywhere in the genome.

Current CMA technology does not completely replace conventional cytogenetics. It cannot detect balanced chromosome rearrangements (translocations, inversions and insertions), some forms of polyploidy (like 69,XXX and 92,XXYY) or low level mosaicism (< 20%). Further, despite its capability for increased resolution CMA does not allow for identification of small aberrations (< 30-50 kb), point mutations or other small changes within a gene.

Nonetheless, array technology is swiftly taking over the cytogenetics laboratory as it provides a quick method to scan the entire genome at a higher resolution for imbalances associated with copy number losses (deletions) and gains (amplifications), thereby improving diagnostic capabilities. In future, with improvements in probe designs and bioinformatics algorithms, the detection of mosaicism and types of abnormalities may expand.

Copy number change (CNC), defined as a DNA segment that is 1 kb or larger and present at variable copy number i.e., gain or loss in comparison with a reference genome, is the most prevalent structural variation in the human genome. To avoid confusion, the term CNC has been used in this article rather than CNV, as copy number variant (CNV) or "Variants" are generally considered alterations or uncommon forms of no clinical significance. In the past several years, the sequencing of the human genome, completion of the HapMap project and many other genetic initiatives have greatly extended our knowledge of CNCs in both healthy and disease cohorts. CNCs greater than 1 kb are found in normal individuals and have been identified in ~35% of the human genome (Database of Genomic Variants). CNCs influence gene expression, phenotypic variation and adaptation by disrupting genes and altering gene dosage. Such changes can cause disease, as in microdeletion or microduplication disorders, or confer

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On the Cover: Medical geneticist and clinical pathologist Marvin Natowicz, MD, PhD, genetic counselor Jacquelyn Riley, MS, CGC (r) and clinical cytogeneticist Shashirekha Shetty, PhD (seated) share an interest in the application chromosomal microarray for the diagnosis of autism and other developmental disorders.

risk to complex disease traits such as HIV-1 infection and glomerulonephritis and have been a focus of much interest in recent years.

Scope and purpose

Submicroscopic chromosomal rearrangements that lead to copy number changes have been shown to underlie distinctive and recognizable clinical phenotypes. The sensitivity to detect copy number change has improved with the advent of CGH, including BAC (bacterial artificial chromosome) - and oligonucleotide based platforms. Coupled with improved genome sequence data, these technologies are facilitating the identification of novel microdeletion and microduplication syndromes. Their characterization reveals the role of genome architecture in the etiology of many genetic disorders.

The challenges inherent in these newer technologies are not limited to the technical aspects but extend also to interpretation. Due to the genome's complex nature and potential variations, the interpretation of the CNCs can present challenges regarding the clinical relevance of the finding. The microarray data can present a spectrum of interpretation outcomes, ranging from clearly abnormal (established syndrome), to clinical significance unknown, to a benign finding (polymorphism or not known to be associated with a condition). CNCs of unknown significance can be due to lack of scientific evidence, benign variations partially overlapping the copy number change, gene-poor regions or genes with no associated function or disease.

Several genomic microarray platforms are appropriate for chromosomal microarray analysis. Copy number microarrays provide high-density whole genome analysis of copy number using intensity probes. Single nucleotide polymorphism (SNP) microarrays allow for high-density whole genome analysis for measuring genetic variations among individuals. The combination of the two allows for simultaneous whole genome analysis of both copy number (intensity probes) and allelic imbalances (SNP probes). Copy number alone or the combination of copy number plus SNP microarray platforms are most commonly used in clinical laboratories to detect copy number alterations or allelic imbalances. SNP-based arrays help with the detection of long continuous stretches of homozygosity (LCSH), which may be suggestive of uniparental disomy (UPD) associated

with imprinting or an increased risk of a recessive condition if it occurs across numerous independent regions.

Clinical significance

In the pediatric population many abnormal phenotypes are associated with chromosomal imbalances that can be identified using microarray analysis to detect copy number changes. Thus, whole-genome CMA has become the first-tier diagnostic test for the evaluation of children with unexplained developmental disabilities, intellectual disabilities, dysmorphic features, congenital anomalies, and autism. Based on numerous published studies, the yield of pathogenic or clinically significant CNCs by CMA is approximately 15-20% in a pediatric population, compared with a yield of 3-5% by standard cytogenetic analysis in the same population. Variants of uncertain clinical significance (VOUS), or clinical significance unknown, are found in a less than 10% and could play an important role in the clinical diagnosis. To a great extent, parental and family studies can be helpful in the clinical interpretation of these unknowns, as *de novo* occurrence of the CNC is most likely related to a pathogenic event.

There are resources available for clinicians and researchers through public and restricted-access databases, including DECIPHER, Database of Genomic Variants (DGV), ISCA consortium and Genoglyphix, as well as the NCBI-established dbGaP (database of Genotypes and Phenotypes) and dbVar (database of genomic structural variation).

In accordance with the 2010 ACMG practice recommendations, we propose the following:

CMA testing for CNC is recommended as a first-line test in the initial postnatal evaluation of individuals with the following:

- Multiple anomalies not specific to a well-delineated genetic syndrome
- Apparently nonsyndromic DD/ID
- Autism spectrum disorders.

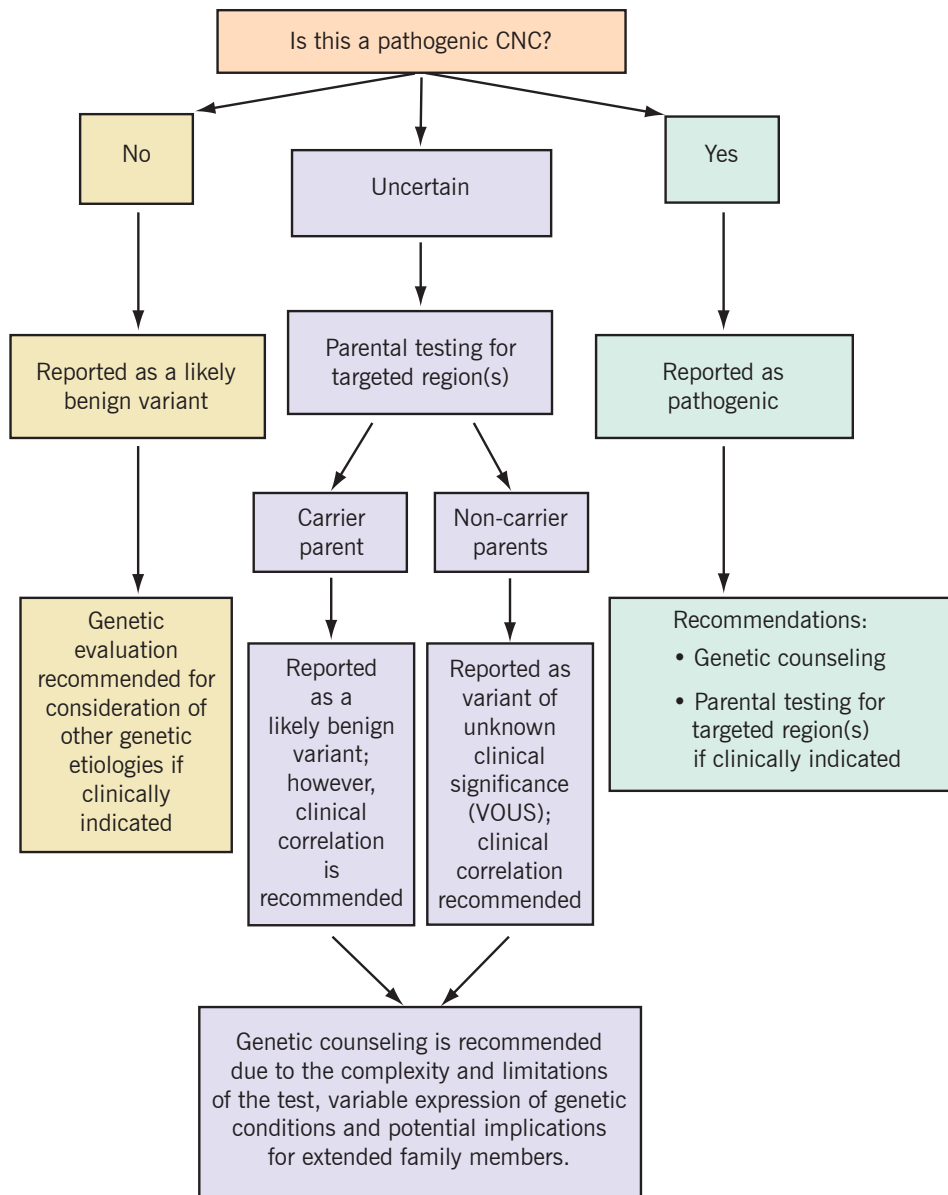
Further determination of the use of CMA testing for the evaluation of the child with growth retardation, speech delay, and other less well-studied indications is recommended, particularly by prospective studies and aftermarket analysis.

Appropriate follow-up is recommended in cases of chromosome imbalance identified by CMA, to include cytogenetic/FISH studies of the patient, parental evaluation, and clinical genetic evaluation and counseling.

Cleveland Clinic Laboratories will offer CMA testing using the high resolution CGH/CNC 720K platform from Nimblegen (Roche)/Signature Genomics(Perkin Elmer). This platform has coverage for 95% of the CNCs represented by at least five probes (84% by at least 10 probes).

ALGORITHM FOR INTERPRETATION, PARENTAL FOLLOW-UP STUDIES AND GENETIC COUNSELING:

Copy Number Change (CNC) Detected by CMA/aCGH



Conclusions

Newer technologies come with challenges that are not limited to the technical aspects but also extend to interpretation. In the case of CMA, interpretation of the data has a spectrum of outcomes ranging from abnormal to variant of unknown clinical

significance (VOUS) to benign findings. Because of the complexities of the human genome and the sometimes non-specific results from CMA, genetic counseling for patients and families is a critical element in the appropriate clinical application of this technology.

Interesting cases to appreciate interpretation challenges and the role of genetic counseling

CASE 1:

Clinical indication: A newborn with congenital anomalies tested for copy number changes using CMA/aCGH.

Result: Abnormal (~410Kb gain), and verified by metaphase FISH using a RP11-clone.

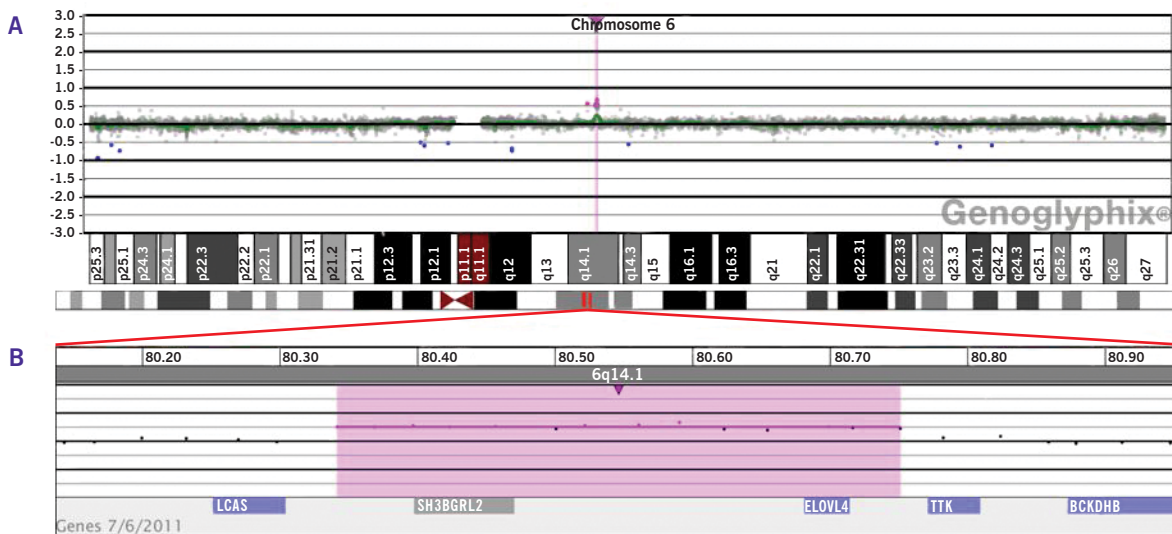


Figure 1. Microarray characterization of a 6q14.1 gain. (A) Microarray plot showing single-copy gain of 14 oligonucleotide probes from the long arm of chromosome 6 at 6q14.1, approximately 410 kb in size (chr6:80,341,528-80,751,497, hg18 coordinates). Probes are ordered on the x-axis according to physical mapping positions, with the most distal p-arm probes to the left and the most distal q-arm probes to the right. Values along the y-axis represent \log_2 ratios of patient:control signal intensities. (B) Zoomed-in view of microarray plot. Probe order and y-axis values are the same as in (A), with the most proximal 6q14.1 probes to the left and the most distal 6q14.1 probes to the right. Genes are shown as blue and grey boxes. Results are visualized using Genoglyphix (Signature Genomics, Spokane WA).

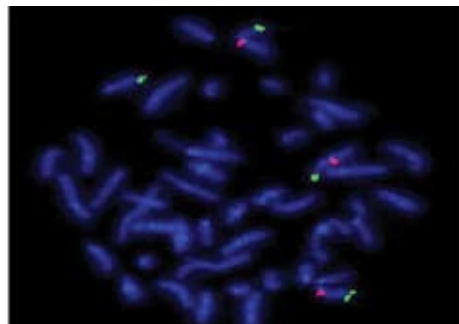


Figure 2. FISH visualization of an unbalanced 6q14.1 insertion into 13q in proband. FISH in the proband showing an insertion of 6q14.1 into the long arm of 13 at approximately 13q12. BAC clone RP11-177J12 from 6q14.1 is labeled in red, and BAC clones RP11-676K14 from 6p25.3 and RP11-63L17 from 13q34 are labeled in green as controls. The presence of a red signal on one chromosome 13 homologue indicates insertion of 6q14.1 into the der(13).

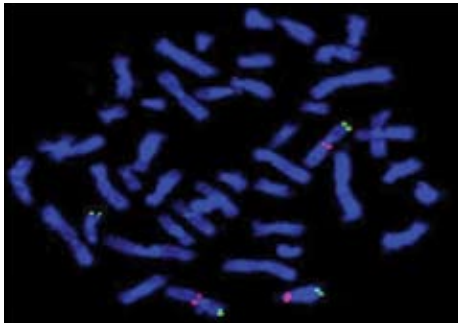


Figure 3. FISH visualization of an unbalanced 6q14.1 insertion into 13q in father. FISH in the father showing an insertion of 6q14.1 into the long arm of 13 at approximately 13q12. BAC clone RP11-177J12 from 6q14.1 is labeled in red, and BAC clones RP11-676K14 from 6p25.3 and RP11-63L17 from 13q34 are labeled in green as controls. The presence of a red signal on one chromosome 13 homologue indicates insertion of 6q14.1 into the der(13).

Interpretation: Is this a pathogenic copy number change?

Parental follow-up study strongly recommended to determine the clinical significance and the origin or mechanism.

Parental testing performed using targeted FISH with the same clone used for the proband.

Three possible outcomes from parental testing:

1. If the parent is not a carrier, the CNC is interpreted as *de novo* or new mutation most probably a pathogenic event (paternity is assumed to be as stated).
2. If the parent is a balanced carrier, the CNC is interpreted as most likely a benign event.
3. If the parent is an unbalanced carrier, the CNC could represent a benign or pathogenic finding. In this case it is important to clinically assess the carrier parent to determine the significance of this finding.

Parental FISH test results: Abnormal, one parent carries the same unbalanced abnormality.

The determination of whether a CNC contributes to the abnormal phenotype depends on many factors, including the genomic region, especially OMIM genes, previous evidence of imbalances of the region, size of the imbalance (as related to gene content), type of imbalance (deletion or duplication), inheritance pattern, and frequency in unaffected populations.

Final interpretation: Called “Uncertain or Variant of Unknown Significance” as a phenotypically normal parent carries it. In this particular case there is one OMIM gene; however, the phenotype of the proband could not be explained.

Why is genetic counseling important?

This case exemplifies the complexities of CMA that can challenge even experts in the field. The nuances of such testing and its implications can be extremely difficult to convey to families, especially when they are confronting significant medical problems or health crises. Although the CMA result was reported as “Abnormal,” it could not be confirmed as causative of this child’s congenital anomalies. Two known genes are within the duplicated area, including one OMIM gene. However, the disease association has been made based on previously identified deletions and point mutations, not duplications; furthermore, the child’s phenotype does not correlate to the known gene function and disease association.

Testing the parents in hopes of clarification instead further confounded the results. An apparently normal parent with no personal history of congenital anomalies was found to carry the same duplication. Generally, such a finding would support the duplication being incidental instead of causative. A review of the family history, however, revealed one previous neonatal death and a spontaneous abortion for the parents of this child. More extensive family history review of the carrier parent may be indicated to probe for additional cases of miscarriage, stillbirth and birth defects. Siblings of the carrier parent may also carry the duplication.

Although no definite cause of the clinical findings was found, future reproductive risk for this family cannot be ruled out. The duplication may in fact be causative, or there may be a separate genetic basis to explain the child's phenotype and the family history of pregnancy and neonatal loss.

CASE 2:

Clinical indication:

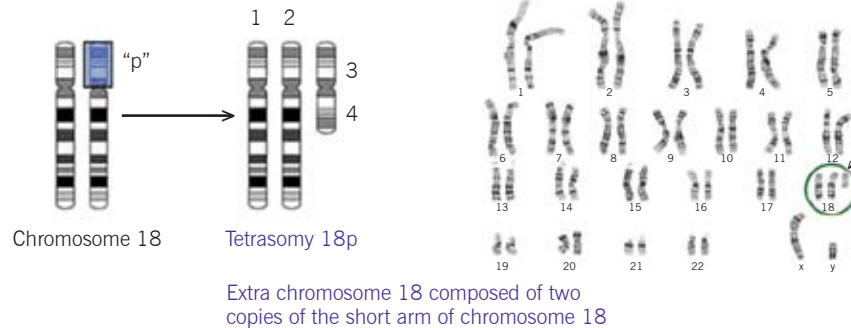
Developmental delay

Result:

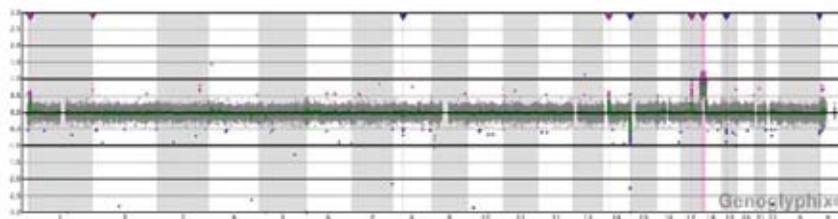
Abnormal (gain), verified by chromosome analysis

Array CGH Data: Tetrasomy 18p

Karyotype: 47,XX,+i(18)(pter→p10::p10→pter)



Whole Genome



CASE 3:

Clinical indication:

Rule out Down Syndrome

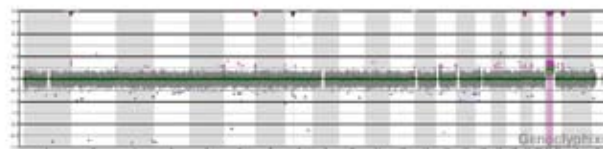
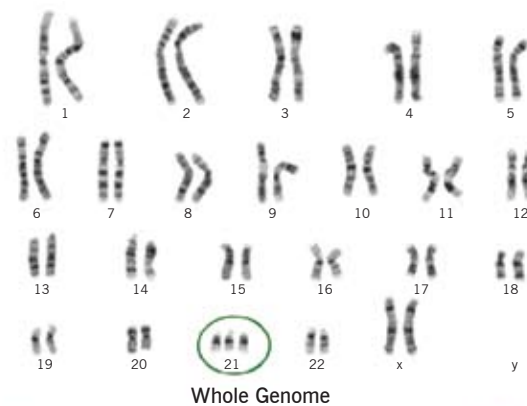
Result:

Abnormal (gain), verified by chromosome analysis and found to be a pure trisomy 21.

Microarray will detect genomic loss or gain, but it will not provide insight into the mechanism causing the abnormality. In these two cases (Case 2 and Case 3), it is important to know the type of structural abnormality for genetic counseling purposes as the recurrence risks for a couple may be very different. In a *de novo* case of tetrasomy 18p, the predicted recurrence risk is low. However, if the isochromosome is due to a chromosome abnormality in one parent, the risk could be significant. In the case of trisomy 21, the recurrence risk would be about 1% or the applicable maternal age risk. If the chromosome 21 gain were due to a translocation (something CMA cannot distinguish), the risk to future pregnancies could be much higher.

Array CGH Data: Trisomy 21 (Down Syndrome)

Karyotype: 47,XX,+21



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FISH for Non-Hodgkin Lymphoma	Vitamin D 25 Hydroxy
	Stool Culture for <i>Yersinia Enterocolitica</i>

Collagen Binding Assay for Diagnosis of von Willebrand's Disease

By Joyce Heesun Rogers, MD, PhD

Von Willebrand disease (vWD) is the most common inherited bleeding disorder with a prevalence of approximately 1 percent in the general population. It can also occur as an acquired bleeding disorder. Patients with vWD typically experience mild mucocutaneous bleeding, including lifelong epistaxis, easy bruising, menorrhagia or surgical bleeding. VWD is classified into several subtypes based on the degree of deficiency or dysfunction of von Willebrand factor (vWF).

vWF is a large, complex, high molecular weight (HMW) multimeric glycoprotein that mediates initial adhesion of platelets to collagen in injured sub-endothelium and to the platelet surface receptor glycoprotein (GP) Ib and serves as the carrier protein for coagulation factor VIII (FVIII) in plasma. It also plays a major role in primary hemostasis and coagulation. vWF is synthesized by endothelial cells and megakaryocytes. Its complexity and multimeric structure make vWF susceptible to a range of quantitative structural and functional defects.

A vWF antigen level of less than 30% (or 30 IU/dL) is designated as a definitive diagnosis of vWD by 2008 National Institutes of Health, National Heart, Lung, and Blood Institute guidelines. Several factors are known to affect plasma vWF levels. Individuals with blood type O will have approximately 25% lower vWF levels compared with non-O individuals. Stress, inflammation, pregnancy, estrogen therapy, trauma, chronic renal failure, infancy and advanced age can increase vWF levels and potentially mask the diagnosis of vWD.

VWD is classified into two major categories, quantitative and qualitative defects. Quantitative vWF defects include type 1 (partial deficiency of vWF) and type 3 (complete absence of vWF) in plasma and/or platelets. Qualitative vWF defects include type 2, which is further classified into four subtypes by different pathophysiologic mechanisms. At the molecular level, the vWF gene is located on chromosome 12, at 12p13.3, containing 52 exons and spanning approximately 180kb. Compared to a type 1 mutation that can occur at any location in the vWF gene, type 2A, 2B and 2M vWF mutations are primarily located in exon 28, which encodes for the A1 and A2 domains of vWF where platelet GPIb, collagen or heparin can bind.

The acquired form of vWD is caused by defects of vWF concentration, structure or function and can be associated

with certain malignancies, including myeloproliferative neoplasm, lymphoproliferative disorder or plasma cell dyscrasia, autoimmune disease or aortic stenosis (AS). It also can develop in patients with ventricular assist devices (VAD). The high shear stress associated with AS or VAD can increase the proteolysis of vWF by ADAMTS13 enough to deplete HMW forms of multimers and cause bleeding that resembles type 2A vWD.

The functional activity of vWF traditionally has been assessed using the ristocetin cofactor activity (vWF:RiCof) assay. The vWF:RiCof assay measures vWF-mediated platelet agglutination in the presence of ristocetin, which cause platelets to bind at the GPIb binding site in domain A1 after conformational change of vWF. However, this assay has limited usefulness due to high inter-assay variability, lower sensitivity and poor reproducibility.

In recent years, the vWF:collagen-binding activity (vWF:CBA) assay has been recognized in the literature as being superior to vWF:RiCof activity as a functional screening diagnostic test for vWD. The vWF:CBA is a functional assay that relies on the property of vWF adhesion to collagen at the injury site and is helpful for identifying vWD with loss of HMW multimer forms. The discrepancy between vWF:CBA and vWF antigen (vWF:Ag) levels is a sensitive screen for subtypes of vWD and has relatively less inter-assay and inter-laboratory variability than the vWF:RiCof assay.

The vWF:CBA assay's greatest advantage is its ability to selectively detect primarily HMW forms of vWF, which are known to be the most functional and most adhesive. The vWF:CBA assay is a useful adjunctive to the vWF:RiCof assay for the diagnosis of vWD. It is clinically useful to differentiate type 2A and 2B vWD, both of which lack the HMW vWF forms, from type 1. It also can differentiate very low levels of vWF in severe type 1 from complete absence of vWF in type 3.

Concordant decrease of vWF:CBA and vWF:Ag plus low vWF:Ag levels suggests type 1 vWD. Absence of both vWF:Ag and vWF:CBA suggests type 3 vWD. The vWF:CBA assay has been reported as an effective marker of functional HMW vWF for therapeutic efficacy of treatment with DDAVP® (desmopressin) and FVIII concentrate. The vWF:CBA is being investigated in various diseases, including thrombotic thrombocytopenic purpura showing high levels of vWF:CBA; essential thrombo-

cytosis and end-stage renal disease showing low levels of vWF:CBA correlating to the loss of HMW forms; and neurofibromatosis-1 showing reduction of vWF:CBA and reduced collagen-based platelet responsiveness.

The vWF:CBA assay is a useful adjunctive to the vWF:RiCof assay; however, it cannot entirely replace the vWF:RiCof assay for the diagnosis of vWD. For example, some patients with type 2M may have a mutation in the platelet GPIb/IX binding site in the A1 domain, which can not be detected by the vWF:RiCof assay. However, others with type 2M may have a mutation in the platelet-collagen binding site, which can not be detected by the vWF:CBA assay.

The vWF:CBA assay is an enzyme immunoassay (REAADS® Collagen Binding Assay ELISA kit, Corgenix, Inc. Broomfield, Colo.) that quantifies the binding of vWF to a collagen-coated microwell plate. The primary site of fibrillar collagen binding is in the A3 domain of vWF. Like the vWF:RiCof assay, the vWF:CBA assay is dependent on vWF multimeric size, with the largest multimers binding more avidly than the smaller forms. After binding peroxidase conjugated anti-vWF antibodies to vWF multimers, the resulting color intensity, determined photometrically, is proportional to the HMW forms of vWF present in the plasma (the higher the intensity, the more HMW forms of vWF are present).

In situ evaluation of the precision and accuracy of the vWF:CBA assay shows a low coefficient of variation (6.3-11.1%) with the lower limit of detection 0.2% (linearity 1-530%). However, the assay's performance and sensitivity to vWD detection or discrimination among vWD subtypes is highly dependent on the source of collagen, as well as on whether type 1 collagen or a mixture of type 1/3 collagen is used. A few patients have been identified who have specific collagen-binding defects that are independent of multimer size, and these defects have been associated with a mutation of vWF in the A3 domain. The prevalence of such defects is unknown.

vWF:CBA results are reported as percent of the reference value for vWF:CBA (reference range: 41-161%). The ratio of vWF:CBA to vWF:Ag is calculated to provide a ratio of vWF activity to protein amount (reference range: greater than 0.6).

Subtype Interpretation

- Type 1 vWD patients have concordantly decreased vWF: CBA, vWF:RiCof, vWF:Ag and FVIII levels.
- Type 3 vWD patients have markedly decreased or nearly absent vWF:CBA, vWF:RiCof, vWF:Ag and FVIII levels.

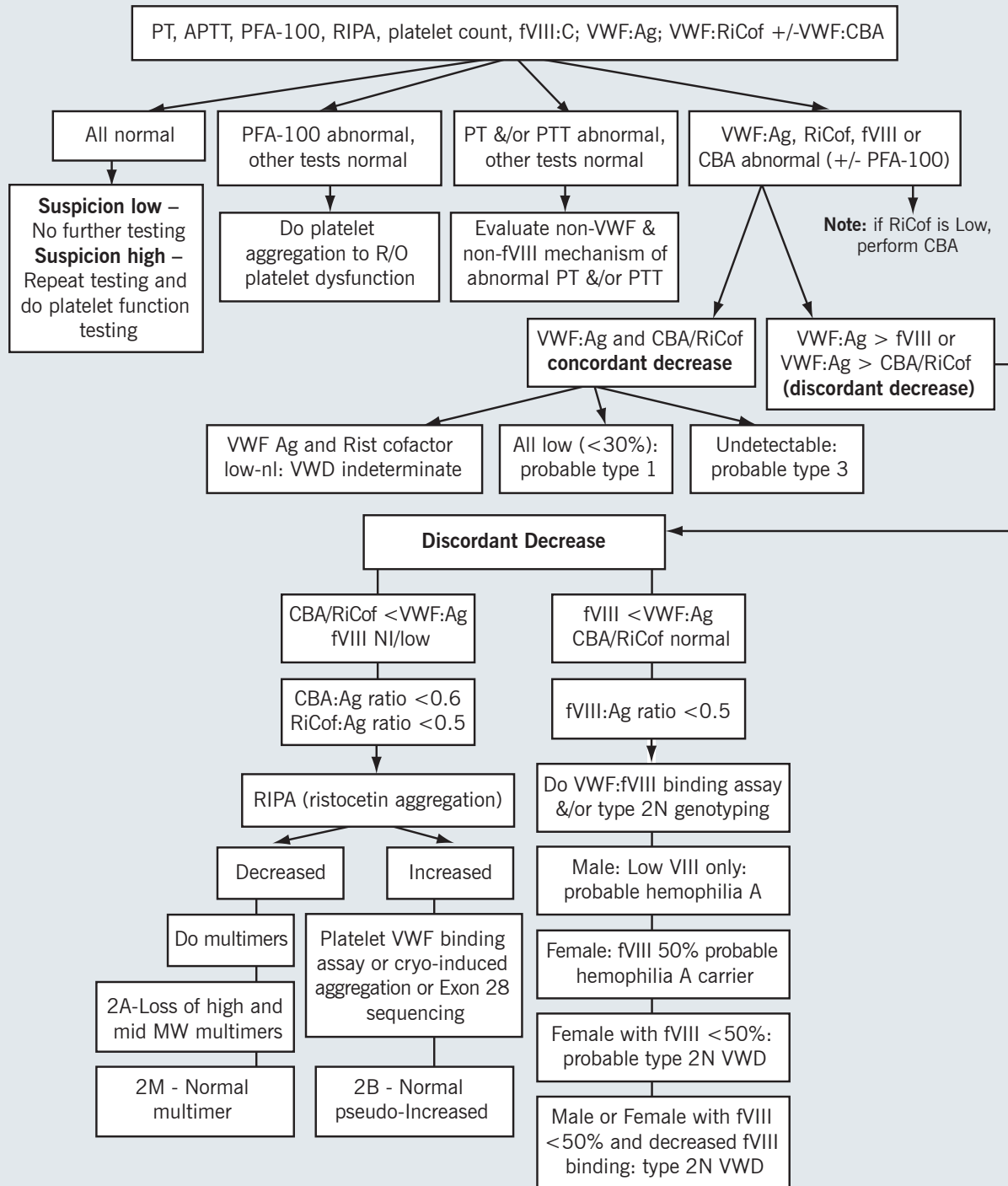
- Type 2A vWD and type 2B vWD patients have discordantly decreased vWF:CBA and/or vWF:RiCof compared to vWF: Ag levels with markedly decreased vWF:CBA and/or vWF: RiCof, normal or decreased vWF:Ag level, and loss of high and/or intermediate MW multimers. The ristocetin-induced platelet aggregation may be helpful to distinguish type 2B with an increased dose response at the lower concentration of ristocetin.
- Type 2M vWD patients have discordantly decreased vWF: CBA and/or vWF:RiCof with normal or decreased vWF:Ag without loss of HMW multimers.
- Type 2N vWD patients have normal vWF:CBA, vWF:RiCof, and vWF:Ag with discordantly decreased FVIII coagulant activity.

For patients in whom the initial clinical evaluation suggests a bleeding disorder, accurate laboratory diagnosis and classification of vWD using both quantitative (antigenic) and qualitative (functional) assays based on the vWD diagnostic algorithm (see figure) are crucial. These subtypes represent a range of hemorrhagic risk as defined by the biologic level of vWF. Clinical management for the disease subtypes differs, thus underscoring the need for precise identification of the disease subtype at diagnosis.

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DIAGNOSTIC ALGORITHM FOR VON WILLEBRAND PANEL



Abbreviations: **Ag** - antigen; **CBA** - collagen binding activity; **fVIII:C** - factor VIII: coagulant; **MW** - molecular weight; **NL** - normal; **PFA-100** - platelet function screen; **PT** - prothrombin time; **PTT** - partial thromboplastin time; **RiCof** - ristocetin cofactor; **RIPA** - ristocetin-induced platelet aggregation; **VWF** - von Willebrand factor

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