

Clinical Report

Molecular Characterization of a Patient With Central Nervous System Dysmyelination and Cryptic Unbalanced Translocation Between Chromosomes 4q and 18q

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We report on a 12-year-old boy who presented with delayed development and CNS dysmyelination. Genetic studies showed a normal 46,XY karyotype by routine cytogenetic analysis, and 46,XY.ish del(18)(q23)-(D18Z1+, MBP-) by FISH using a locus-specific probe for the *MBP* gene (18q23). Though the patient appeared to have normal chromosome 18s by repeated high resolution banding analysis, his clinical features were suggestive of a deletion of 18q. These included hearing loss secondary to stenosis of the external auditory canals, abnormal facial features, and foot deformities. FISH studies with genomic probes from 18q22.3 to 18qter confirmed a cryptic deletion which encompassed the *MBP* gene. In an attempt to further characterize the deletion, whole genome screening was conducted using

array based comparative genomic hybridization (array CGH) analysis. The array CGH data not only confirmed a cryptic deletion in the 18q22.3 to 18qter region of approximately 7 Mb, it also showed a previously undetected 3.7 Mb gain of 4q material. FISH studies demonstrated that the gained 4q material was translocated distal to the 18qter deletion breakpoint. The 18q deletion contains, in addition to *MBP*, other known genes including *CYB5*, *ZNF236*, *GALR1*, and *NFATC1*, while the gained 4q material includes the genes *FACL1* and 2, *KLKB1*, *F11* and *MTNR1A*. The use of these combined methodologies has resulted in the first reported case in which array CGH has been used to characterize a congenital chromosomal abnormality, highlighting the need for innovative molecular cytogenetic techniques in the diagnosis of patients with idiopathic neurological abnormalities. © 2003 Wiley-Liss, Inc.

KEY WORDS: dysmyelination; cryptic unbalanced translocation; myelin basic protein (*MBP*) gene; fluorescence in situ hybridization (FISH); array CGH; 18q-syndrome; mental retardation

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INTRODUCTION

Central nervous system dysmyelination is a common, but nonspecific, radiological finding in pediatric patients. It is also the most common phenotypic feature

of children who are heterozygous for cytogenetically visible deletions of chromosome 18q that include the *Golli-MBP* (myelin basic protein) gene complex on chromosome 18 located to band q23 [Loevner et al., 1996; Gay et al., 1997]. Other common neurological findings in these patients include mental retardation, developmental delay and hypotonia [Wertelecki and Gerald, 1971; Miller et al., 1990; Kline et al., 1993]. Molecular analysis and genotype–phenotype correlations have focused on haploinsufficiency [Wilkie, 1994] of the *MBP* gene as a possible cause of dysmyelination and neurological abnormalities in individuals with 18q deletions [DuPont et al., 1995; Gay et al., 1997; Keppler-Noreuil et al., 1998]. Though it has been postulated that a decreased *MBP* gene dosage effect may be the cause of the neurological manifestations in these patients [Miller et al., 1990; Weiss et al., 1991; Ono et al., 1994; Loevner et al., 1996], specific genotype–phenotype correlations have been hindered by the unknown number of genes included in large cytogenetically visible deletions. These genotype–phenotype correlations have been further complicated by the complexity of the breakpoints in these patients [Brkanac et al., 1998] and the inability of site specific FISH studies to determine whether there is involvement of material from other chromosomes. The advent of new genome scanning technologies has introduced the means to determine whether unbalanced cryptic chromosomal abnormalities accentuate the phenotype in patients whose clinical presentation is inconsistent with their G-banded karyotype.

We report on a patient with dysmyelination, developmental delay and other distinctive and common features

seen in individuals with 18q deletions, who has a cryptic deletion of chromosome 18q22.3-qter and a gain of material from 4q35.1-qter. We have used a combination of FISH, and genome wide screening by array CGH to molecularly characterize the rearrangement.

CLINICAL REPORT

F.G. is a 10-year-old Hispanic male who was the product of an uncomplicated term pregnancy and spontaneous vaginal delivery to healthy unrelated parents in Bogota, Columbia. He has two older sisters who are in excellent health. The patient was noted at birth to have dysmorphic facial features, as well as foot deformities. He underwent cytogenetic testing, which revealed a normal 46,XY male karyotype. CT scan of the head and EEG were also normal. At the age of 8 months, the patient was diagnosed with hearing loss, secondary to bilateral stenosis of the external auditory canals and fitted with bilateral hearing aids. He was found to have a generalized hypotonia, and his motor, cognitive and language development have been severely delayed.

The patient's family moved to the United States when he was 10-years old, where he was seen for possible surgical correction of his foot deformities. He was at the 10th centile for height and weight, but his head circumference was less than the 2nd centile for OFC. He was brachycephalic with metopic fullness and bitemporal narrowing. His eye globes appeared large and he had a high nasal root and bridge with midfacial hypoplasia, micrognathia and very narrow ear canals (Fig. 1a). He had mild neck webbing, and his testes were

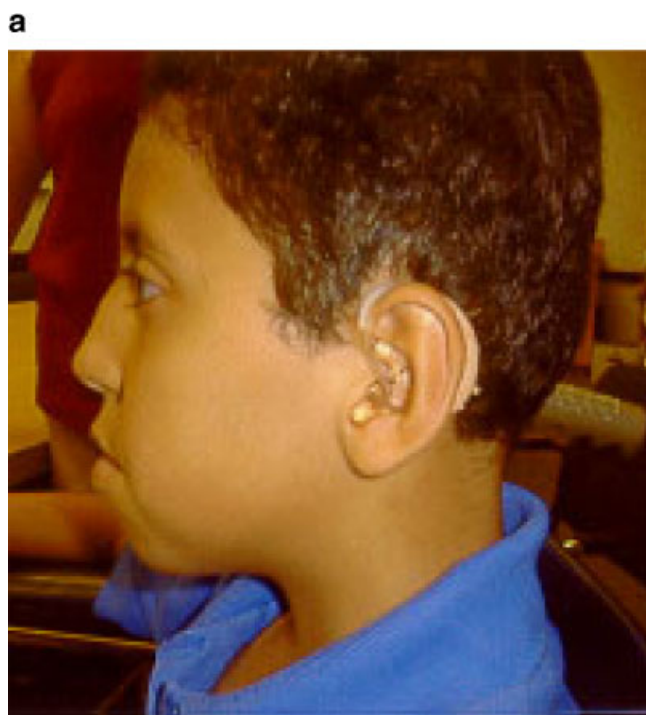


Fig. 1. Facial appearance (a) and lower extremities (b) of patient F.G.

small with mild penile chordae. He had underdeveloped lower leg musculature with apparent widening of the proximal tibiae (Fig. 1b). Due to the patient's significant developmental delays and physical deformities, a cytogenetics evaluation was conducted at that time which again showed a normal 46,XY male karyotype (Fig. 2). However, based on the patient's clinical presentation including stenotic ear canals and hypoplastic calf muscles, a chromosome 18q deletion was suspected. The patient's chromosome 18s were subsequently analyzed by FISH using a locus specific probe for the MBP gene. This revealed the karyotype to be 46,XY,ish del(18)(q23)(D18Z1+, MBP-).

FISH analysis of chromosome 18q23 using additional P1 and bacterial artificial chromosome (BAC) human genomic DNA derived probes confirmed the patient's apparent submicroscopic deletion. He subsequently underwent an extensive clinical workup as part of a protocol approved by the Institutional Review Board of the University of Texas Health Science Center for patients with 18q deletions which included radiological and endocrine testing. Magnetic resonance imaging of the brain showed no apparent abnormalities on T1 weighted images, but statistical analysis of T1 and T2 relaxation times were abnormal and similar to those recorded in other subjects with 18q deletions [Gay et al., 1997]. On T2 weighted images, the deep white matter of the cerebral hemispheres appeared diffusely hyperin-

tense relative to gray matter (Fig. 3) while the corpus callosum and brainstem appeared normal. Growth hormone (GH) stimulation with arginine and clonidine both showed peak values greater than 10, and therefore a normal response. Total T4 and TSH values were within normal limits. His workup also included a neuropsychological evaluation which confirmed mental retardation with significant delays in cognitive, language, and social skill development. Audiograms and auditory brainstem-evoked response (ABR) testing showed reduced ear canal volumes consistent with stenosis or atresia of the external auditory canals and bilateral hearing loss.

MATERIALS AND METHODS

Cytogenetic Analysis

A peripheral blood sample was obtained from the patient and whole blood lymphocytes were cultured with phytohemagglutinin (PHA) by using standard methods. Chromosome analysis was performed on trypsin-Giemsa banded chromosome preparations [Chen et al., 1997].

FISH Analysis

Chromosome painting, and telomere screening of 4q and 18q, was performed according to the manufacturer's protocol (Vysis, Down Groves, IL). FISH analyses of

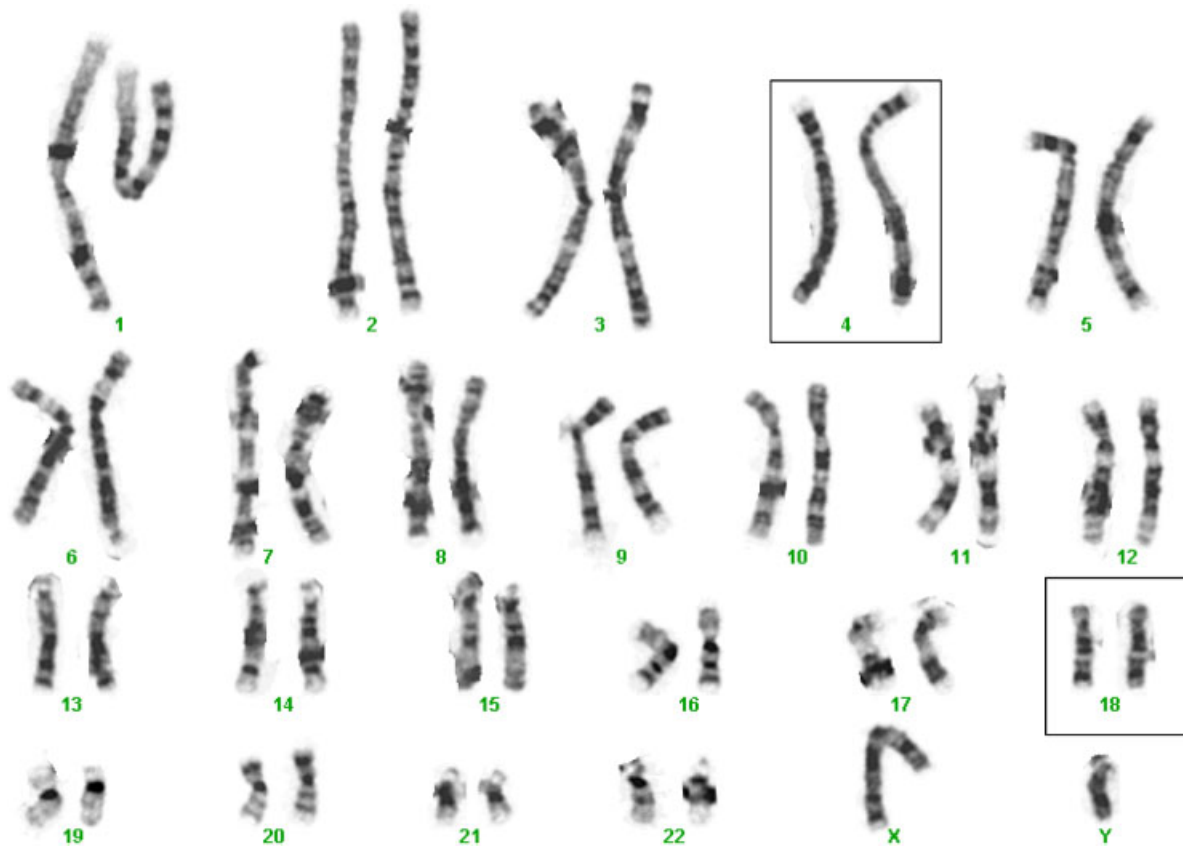


Fig. 2. G-banded 46XY karyotype of patient F.G. showing two apparently normal copies of chromosomes 4 and 18.



Fig. 3. T2 weighted MRI from patient F.G. showing diffuse hyperintensity of cerebral white matter.

both peripheral blood lymphocytes and transformed lymphoblastoid cell lines were performed using RP11-BAC derived probes identified by human genome browser UCSC to 18q22.3 and 18q23. Genomic clones encompassing the locus for myelin basic protein (MBP) and the telomeric microsatellite marker D18S553 were isolated from a human P1 library [Shephard et al., 1994] and had been shown to be useful for FISH analysis [Gay et al., 1997]. DNA was isolated from these genomic clones (Qiagen modified protocol obtained from Genome Systems) and labeled by bio-nick translation with biotin-14-dATP (BRL/Gibco, Carlsbad, CA). FISH slides were hybridized overnight using chromosome 18 α -satellite DNA (Vysis), and 40–100 ng of labeled genomic DNA. Slides were washed and fluorescently labeled with avidin-conjugated fluorescein isothiocyanate (FITC). Amplification was performed with biotinylated anti-avidin and avidin-FITC. Chromosomes were counterstained using DAPI (Vysis) and were viewed using a fluorescent microscope. Images were captured using Applied Imaging Probevision software.

Microarray Analysis

We used commercially available genomic DNA microarray slides (Human BAC Array 3 MB, Spectral GenomicsTM, Houston, TX) containing 1003 non-overlapping BAC and PAC clones spotted in duplicate. These microarrays provide an average of 3 Mb resolution for detection of chromosomal imbalances throughout the genome.

DNA sample preparation, labeling, and hybridization with human BAC array. Patient genomic DNA (test DNA) and reference genomic (a sample with no known chromosomal imbalances) were digested with *Eco*R1 for 16 hr at 37°C and re-purified by Zymo Research's Clean and ConcentratorTM (Orange, CA). The reference and test DNAs were labeled with Cy3 and Cy5 by Invitrogen's BioPrime random labeling kit. We obtained the majority of the probe between 100–500 bp

in size. The Cy3-labeled reference DNA and Cy5-labeled test DNA samples were combined with 50 μ g of human Cot-1 DNA and 30 μ g of sheared salmon sperm DNA. This mix was precipitated with ethanol, rinsed in 70% ethanol and air dried. The same procedure was repeated with the Cy5-labeled reference and Cy3-labeled test DNAs. The pellets were dissolved in 10 μ l of distilled water and mixed with 30 μ l of hybridization solution (50% formamide, 10% dextran sulphate in $2 \times$ SSC). The labeled DNAs were denatured at 72°C for 10 min followed by incubation at 37°C for 30 min to block repetitive sequences. Oppositely labeled DNA mixes (Cy3-labeled test and Cy5-labeled reference DNA, Cy3-labeled reference, and Cy5-labeled test DNA) were added onto duplicate microarray slides. Hybridization as per the Spectral Genomics protocol was overnight (approximately 16 hr) at 37°C. Slides were washed room temperature $2 \times$ SSC for 3–5 min, then washed at 50°C for 20 min with shaking in 50% formamide/ $2 \times$ SSC. The wash step was repeated with pre-warmed (50°C) 0.1%NP-40/ $2 \times$ SSC for 20 min and with $0.2 \times$ SSC for 10 min at 50°C. The microarrays were briefly rinsed with distilled water at room temperature for 3–5 sec and immediately centrifuged for 3 min at 500g for drying. Hybridized microarray slides were analyzed with GenePix 4000B scanner (Axon Ins., Inc., Union City, CA). Cy3 and Cy5 images were scanned independently through two separate channels. Two 16-bit TIFF images were created per array. The data obtained were analyzed using the Spectralware 1.0 software (Spectral Genomics).

The software recognizes the regions of fluorescent signal, determines signal intensity, and compiles the data into a spreadsheet that links the fluorescent signal of every clone on the array to the clone name, its duplicate position on the array and its position in the genome. SpectralWareTM was also used to normalize the C_{γ5}:C_{γ3} intensity ratios for each slide and each data point. Specifically, each slide is normalized such that the summed C_{γ5} signal equals the summed C_{γ3} signal. The normalized C_{γ5}:C_{γ3} intensity ratios were computed for each of the two slides and plotted together for each chromosome.

Arbitrarily, we assigned a ratio plot such that gains in DNA copy number at a particular locus are observed as the simultaneous deviation of the ratio plots from a modal value of 1.0, with the blue ratio plot showing a positive deviation (upward) while the red ratio plot shows a negative deviation at the same locus (downward). Conversely, DNA copy number losses show the opposite pattern. The linear order of the clones is reconstituted in the ratio plots consistent with an ideogram, such that the p terminus is to the left and the q terminus is to the right of the plot.

RESULTS

Clinical evaluation of this patient showed many of the most common features seen in individuals with 18q deletions foot deformities, bilateral hearing loss, abnormal myelination, and global developmental delay. However, repeated G-banding revealed a normal male

karyotype with no visible chromosomal abnormalities. To investigate the possibility of a submicroscopic deletion of chromosome 18q23, FISH was initially performed using a locus-specific probe (18q23-MBP gene, Genzyme Genetics, Santa Fe, NM), which showed a deletion on one chromosome in all 10 metaphase cells scored (data not shown.) FISH was then repeated using a P1 genomic clone for MBP which again showed a deletion of MBP in all metaphases analyzed (Fig. 4a). To further estimate the extent of the deletion, FISH with BAC-derived probes for additional loci on 18q22.3 and 18q23 were used to analyze the patient's chromosomes. A list of the probes used and results obtained from them are included in Table I. From this analysis, we estimate that the patient has a 7 Mb terminal deletion spanning 18q22.3-qter which includes the *Golli-MBP* gene and at least five other previously described genes.

This patient was clinically affected with many of the most common and severe manifestations seen in patients with larger 18q deletions, and those with complicated rearrangements [Brkanac et al., 1998]. Seeking to explain the apparent discrepancy between this relatively small deletion and the patient's phenotypic presentation, we considered the possibility of an undetected cryptic unbalanced translocation. Whole gen-

ome analysis on our patient's DNA was performed using array CGH with, on average, 3 Mb resolution (Spectral Genomics, Houston, TX). This confirmed the 7 Mb terminal deletion of chromosome 18 (Fig. 5a) and also showed a gain of 4q35.1-q35.2 (Fig. 5b). FISH analysis with BAC probes derived from the array CGH revealed the gain of 4q material to be on chromosome 18q (Fig. 4b). Additional FISH analysis using two color labeling of MBP and the 4q BAC probes confirmed that the gained 4q material is on the derivative chromosome 18 (data not shown). The extent of the 4q gained material was estimated to be at least 4 Mb, and was calculated by subtracting the start number (185,160,576) of the most proximal BAC present in three copies (RP11-90E7), from the stop number (190,160,576) of the terminal BAC present in three copies (RP11-91J3). Start and stop numbers are from the UCSC genome browser April 2002 Freeze. FISH with a commercial probe for the 4q telomere (Vysis) also demonstrated gain of a 4q material on the derivative 18 (data not shown). The commercially available probe for 18q telomere was missing on the derivative chromosome, which was consistent with the array CGH data showing a terminal deletion of 18q, thus making the final karyotype 46,XY,ish der(18)t(4;18)(q35.1;q22.3).

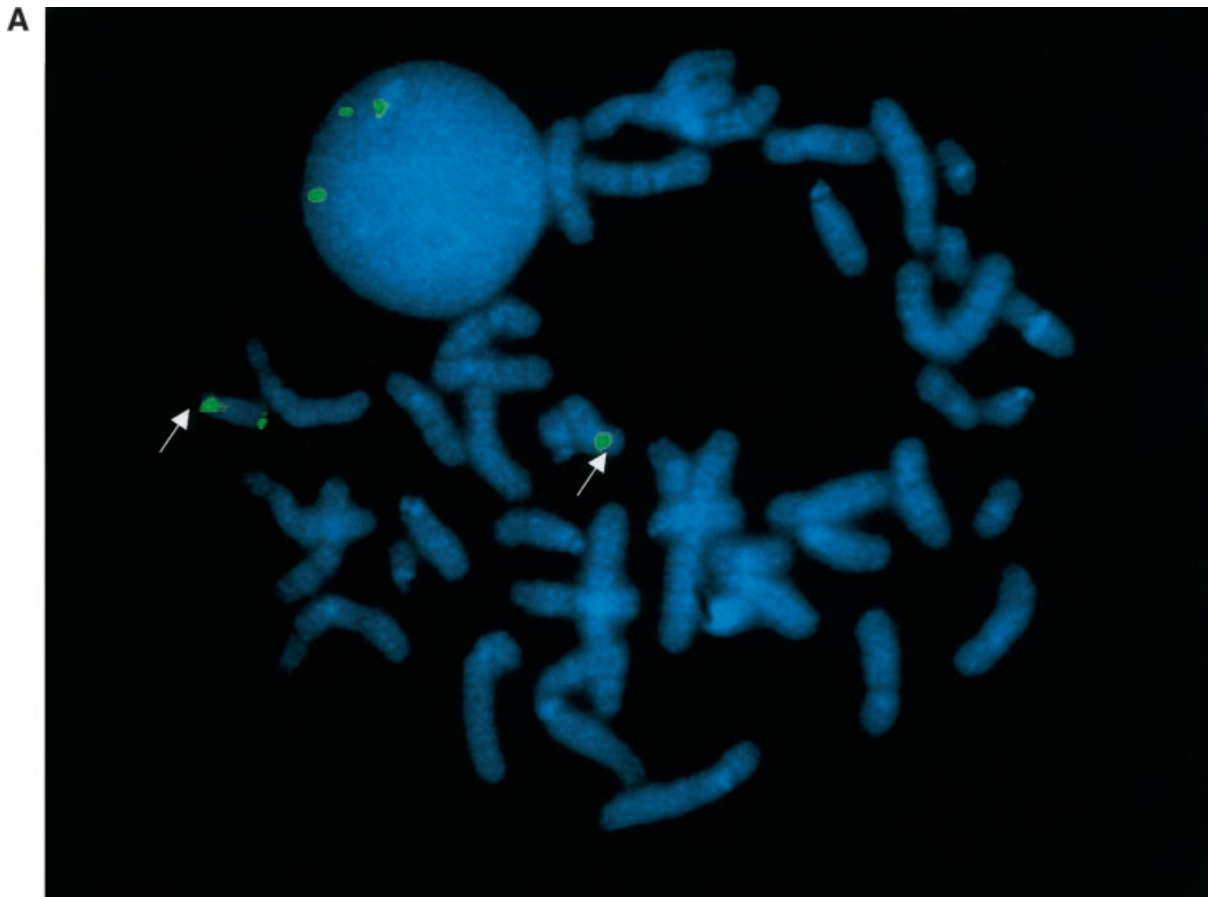


Fig. 4A. FISH using a P1 probe for the MBP gene shows heterozygous deletion of one copy of MBP in all 10 metaphases analyzed. The deletion is also apparent in the interphase nucleus. Arrows indicate the chromosome 18 specific alpha-satellites.

B

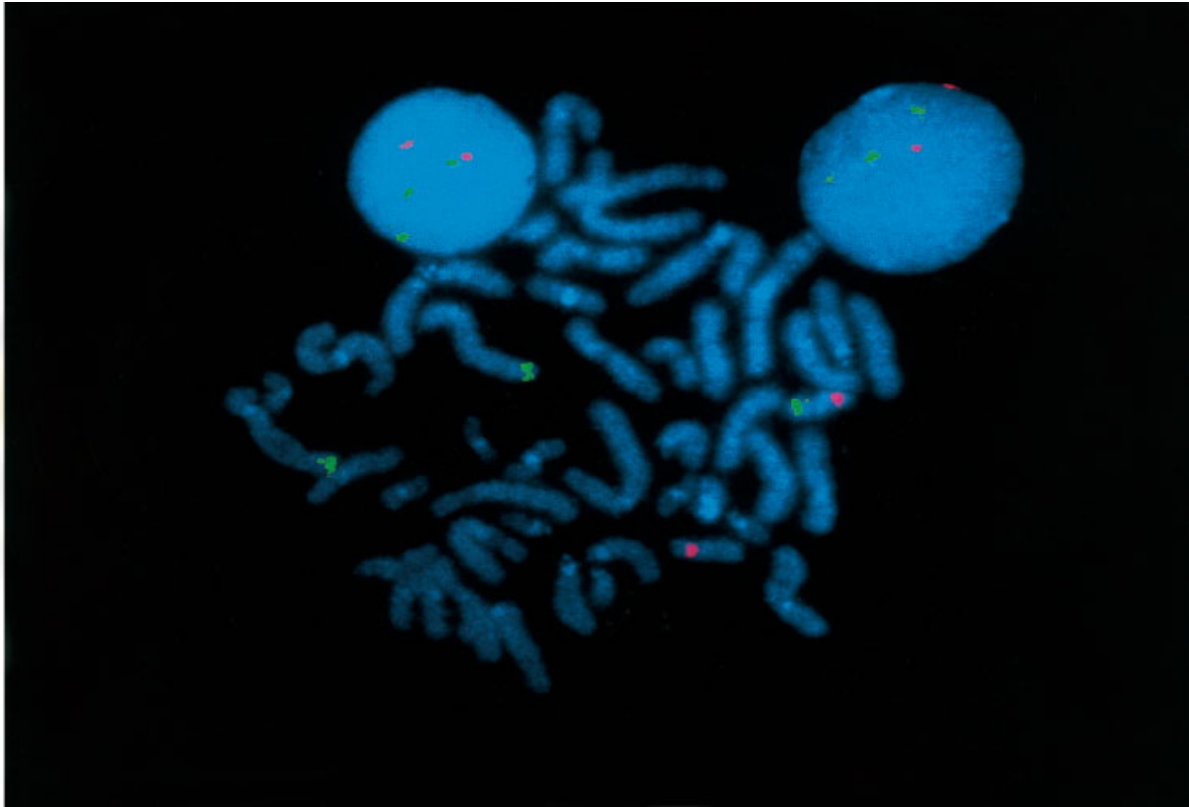


Fig. 4B. FISH analysis using the probe RP11-90E7 shows three copies in all 10 metaphases examined. Gained 4q material is localized to 18q23. The chromosome 18 alpha satellite is labeled red.

DISCUSSION

Cytogenetically normal patients with mental retardation, abnormal myelination, and other clinical features suggestive of a chromosomal anomaly present a diagnostic challenge for physicians. Several molecular cytogenetic techniques such as telomere screening [Ghaffari et al., 1998; Knight and Flint, 2000], have been tested in these individuals because it has been shown that the gene rich ends of chromosomes [Saccone et al., 1992] are areas in which many anomalies occur. However, these methods cannot detect more proximal chromosomal rearrangements that do not include the telomere, and unless a patient's clinical picture is suggestive of a

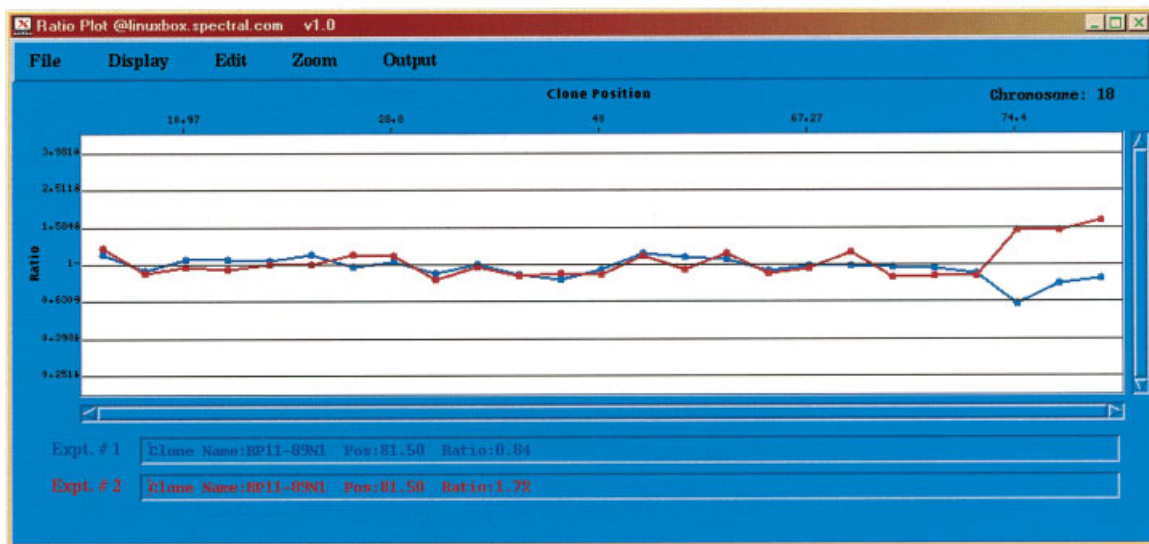
particular syndrome, it is difficult to know where in the genome to screen for cryptic chromosomal imbalances. We were fortunate that our patient's phenotype was highly suggestive of a chromosomal defect, specifically an 18q deletion, and that the first attempt to molecularly characterize his condition by FISH analysis led to a partial diagnosis.

Our molecular cytogenetic analysis revealed a deletion of distal 18q encompassing approximately 7 Mb which was not apparent with repeated G-banding by two separate cytogenetic laboratories. We now know that this was due to the gain of material from chromosome 4q, and it is becoming increasingly clear that G-banded cytogenetics is often inadequate for identifying small chromosomal rearrangements that cause clinically significant consequences [Biesecker, 2002]. This is especially true in areas where chromosomal banding patterns are not distinctive, and especially at the G band negative ends of chromosomes [Lamb et al., 1989]. Screening of our patient's entire genome by array CGH was an important adjunct to our polymorphic marker analysis and FISH characterization of his chromosomes. The array CGH not only confirmed our data but also revealed a cryptic unbalanced translocation, involving 4q. However, FISH was necessary for localizing the gain of 4q material to the derivative 18q, and screening for the presence of the 4q and 18q telomeres. Therefore, genome wide screening with array CGH, at the currently available levels of resolution and coverage, provides valuable information that still must be interpreted

TABLE I. BAC and P1 Derived Probes Used for FISH Analysis

Probe name	Cytogenetic band	Result
RP11-800G9	18q22.3	Two Copies
RP11-125M20	18q22.3	Two Copies
RP11-25L3	18q22.3	Deleted
MBP	18q23	Deleted
Golli	18q23	Deleted
RP11-875C21	18q23	Deleted
RP11-467N12	18q23	Deleted
D18S553 (P1-46E4)	18q23	Two Copies
18q tel (Vysis)	18q23	Deleted
RP11-80P4	4q34.3	Two Copies
RP11-90E7	4q35.1	Three Copies
RP11-91J3	4q35.2	Three Copies
4q tel (Vysis)	4q35.2	Three Copies

a



b

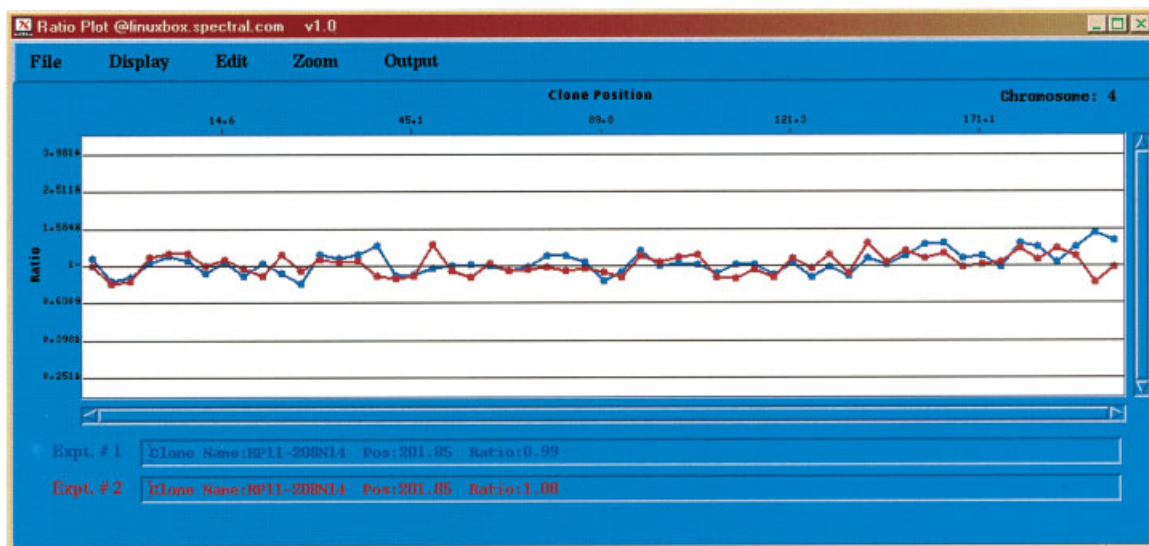


Fig. 5. Ratio plots from microarray data for chromosomes 18 (a) and 4 (b) from patient F.G. Each ratio plot comprises of normalized data from two independent arrays such that the normalized data from the array in which the test sample was labeled with Cy3 is shown in red while the normalized data from which the test sample was labeled with Cy5 is shown in blue. Individual spots along the ratio plot represent the normalized ratio of individual clones linearly ordered such that the left most clone is consistent with the p-arm terminus while the right most clone is consistent with the q-arm terminus. Since the normalized Cy5: Cy3 ratio was computed for both

arrays, a loss of a particular clone is manifested as the simultaneous deviation of the ratio plots from a modal value of 1.0, with the red ratio plot showing a positive deviation (upward) while the blue ratio plot shows a negative deviation at the same locus (downward). Conversely, DNA copy number gains show the opposite pattern. The gain at the 4q terminus is illustrated by the simultaneous deviation of both ratio plots with the blue ratio above the red, while the deletion at 18q22.3 is illustrated by the simultaneous deviation of both ratio plots with the red ratio above the blue.

by FISH in order to clarify the chromosomal localization of gains and losses of genetic material.

Molecular characterization of this patient's chromosomal rearrangement shows that it includes deletion of five genes, and a gain of four genes, for which the function of the protein product has been fully or partially characterized. Genes included in the deleted and gained chromosomal regions are summarized in Figure 6. The loss of one copy of *MBP* and the patient's dysmyelination

phenotype provide further evidence to support the hypothesis that haploinsufficiency of *MBP* is responsible for dysmyelination in individuals with 18q23 deletions [DuPont et al., 1995; Gay et al., 1997]. The increased gene dosage of the *FACL1* and *FACL2* genes, (which code for the fatty acid metabolism enzyme-palmitoyl CoA ligase) is of interest in light of the patient's mental retardation, which is more pronounced than what is normally seen in those with small 18q

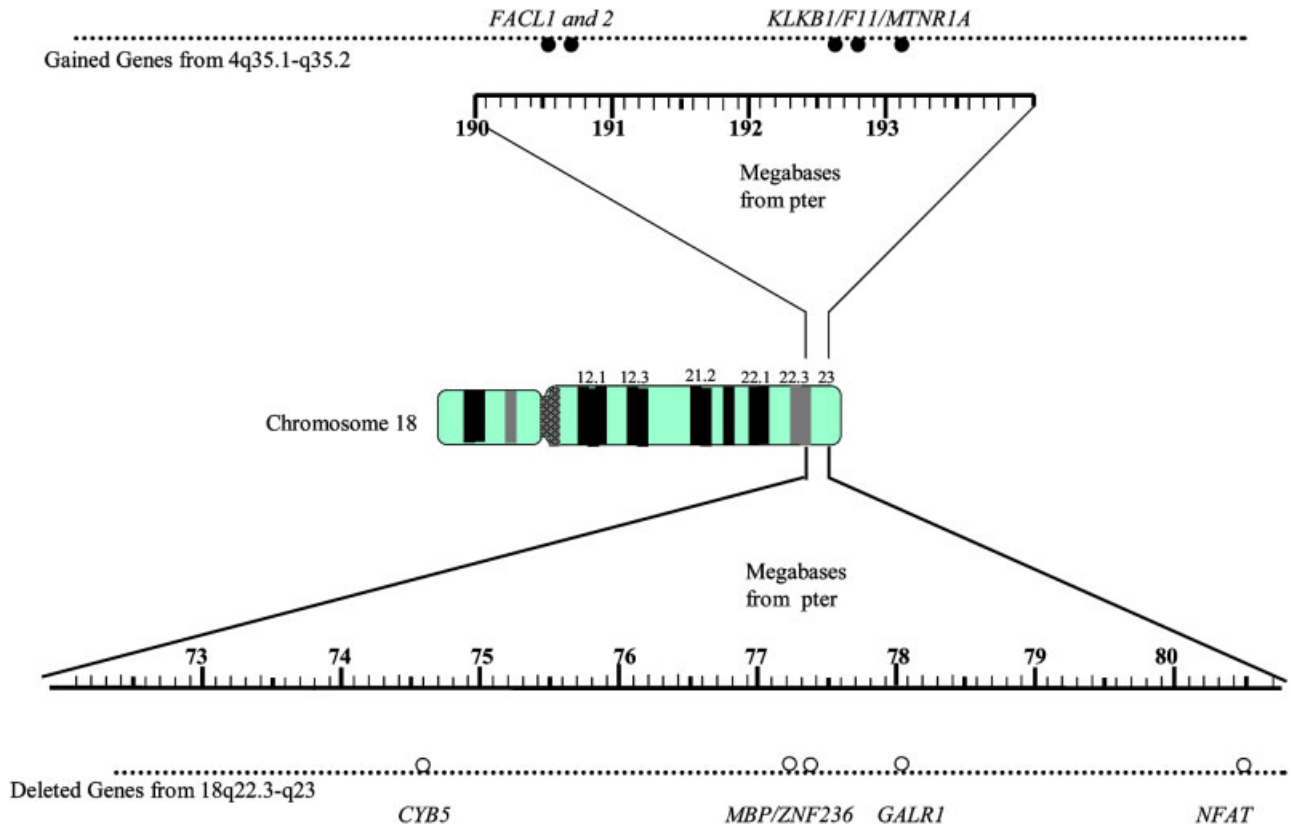


Fig. 6. Genes with known functions gained and lost in cryptic translocation between 4q and 18q in patient F.G.

deletions. It was recently shown that point mutations in a similar gene, *FACL4*, are involved in non-specific X-linked mental retardation [Meloni et al., 2002].

New methods of molecular analysis, specifically FISH in combination with array CGH, make it possible to scan the entire genome for cryptic chromosomal imbalances in patients with a normal G-banded karyotype and clinical features that suggest a chromosomal abnormality. Genotype–phenotype correlations of candidate genes to the clinical features seen in these patients can answer many fundamental questions about the phenotypic effects of gene dosage. Reevaluation of previously described 18q deletions, using these new methods of molecular analysis, could provide valuable information about what genes on 18q must be lost in order for patients to show phenotypic features, and possibly demonstrate submicroscopic involvement of other chromosomal material. These cryptic and unexpected gene dosage effects may have contributed to the clinical presentations of these patients, and would explain the tremendous phenotypic variability observed in individuals with deletions of 18q.

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