Chapter 3

Cytogenetic Evaluation by High Resolution Banding and Molecular Genetic Screening of *FMR1* Mutation
3.1 Introduction

Generally, the phenotype of an individual is determined by the quintuple; which in turn is regulated by the sequential arrangement of genes into chromosomes. The chromosomes undergo an order of dynamic changes during the cell cycle and eventually maintain their uniform structure and functions. Occasionally, some changes may become detrimental and lead to abnormalities in chromosome structure, and as result, lead to phenotypic anomalies. Since the advent of clinical cytogenetic analysis, it has been recognized that chromosomal deletions and duplications, such as autosomal trisomies, can produce recognizable syndromes. Chromosomal abnormalities are responsible for up to 28% of all ID cases [1].

However, depending on the variation in the study design among published reports, a wide range (2% to 50%) of frequencies of chromosome abnormalities causing ID was observed irrespective of the category of ID (mild to profound) or gender. Thus, cytogenetic studies are considered a valuable diagnostic technique in the evaluation of children with ID/DD [2]. G-banded karyotyping has been the standard first-tier test to detect genomic imbalance in children with ID/GDD worldwide for the past 35 years. Cytogenetically visible abnormalities include gross alterations involving whole or partial regions of chromosomes of more than 5 Mb in size, which can be detected with conventional GTG banding. Subtle chromosome alterations such as interstitial and terminal microdeletions are not visible at the resolution of conventional cytogenetic analysis [3].

In contrast, high resolution banding (HRB) is a modification of the routine PHA stimulated blood culture procedure designed to yield, at harvest, a large number of mitotic plates in late prophase or prometaphase stage [4]. At this stage of mitosis, the chromosomes are longer, less condensed and appear at 550 or greater level of band resolution, showing sub-bands not seen in routine karyotype analyses. This modification allows extra information and more detailed analysis of the karyotype. It is of particular use in the evaluation of children with ID associated with microdeletions or rearrangements of...
specific chromosomes. It is also useful for fine breakpoint determination of previously diagnosed rearrangements and their potential parental carriers.

Next to chromosomal abnormalities, Fragile X syndrome (FXS) is the most frequent and best-studied X-linked syndrome often reported to be associated with ID [5]. Most often, the disorder results from mutations caused by the abnormal expansion of CGG repeats located in the 5′ promotor region of the \textit{FMR1} gene [6]. Upon expansion of the repeat region in fragile X patients, the promotor region of \textit{FMR1} becomes hypermethylated resulting in the transcriptional silencing of the gene. Consequently, \textit{FMR1} mRNA and protein are absent in fragile X patients [3]. Despite the fact that the analysis of CGG expansion is primarily performed using Southern blot analysis, which is able to detect alleles spanning the range from normal to large full mutation alleles, the method lacks the resolution to accurately size alleles [7]. Alternatively, using polymerase chain reaction (PCR) amplification of the region spanning the CGG repeat, provides much greater resolution, although it suffers from the difficulty of amplifying CGG repeats greater than 100 to 150 repeats owing to the high GC content of the sequence being amplified [8–11]. Thus, it was suggested that a combination of PCR and Southern blot methods reliably detects all alleles, and the recent incorporation of capillary-based methods dramatically increases throughput [12]. However, such approaches are neither time- nor cost-effective for screening a large population.

Although improved PCR methods using the osmolyte, betaine, are capable of detecting alleles as large as 300 CGG repeats [7], they are unable to distinguish between females who are homozygous for normal \textit{FMR1} alleles (single normal band following PCR; 40% of all females) and females with one normal allele and a second full-mutation allele. Whereas, a modified betaine-based approach with a second PCR with a “chimeric” primer suggested by Tassone et al [13], could overcome the problem. That is, the “chimeric” primer targets the triplet repeat region and generates different sized amplicons due to multiple annealing sites on the template. Hence, the approach allows
rapid determination of the allele status of males and females.

In the genetic evaluation of the child with idiopathic ID, chromosome analysis and fragile X mutation screening are the primary and essential steps in establishing an etiological diagnosis. Hence, in this study, the first-level screening of subjects included probing for microscopically visible chromosomal abnormalities and FMR1 mutation.

3.2 Methodology

The subjects (n=130) were recruited as described in Section 2.2.2. The age group of study subjects varied between 7 months and 14 years. Psychological and clinical evaluation of dysmorphism (microcephaly, hypertelorism, clinodactyly) and prenatal and postnatal growth defects (intrauterine growth retardation, seizures, cardiac defects, speech delay) were conducted, wherever possible. Blood samples were collected in heparin and ethylene diamine tetraacetic acid (EDTA) vacutainers for genetic testing. Lymphocyte preparation and banding of slides were performed as described in Section 2.2.4 through Section 2.2.5. The modified method of Yunis et al [4] was adopted for HRB analysis of the chromosomes. The heparinized blood samples from the study subjects were PHA-stimulated and cultured for 72 hours in duplicates. Cultures were blocked at S phase with the addition of $10^{-5}$ M methotrexate at 48 hours after culture set-up. The block was released by incorporation of $10^{-3}$ M thymidine to the cultures. Subsequently, ethidium bromide ($2.54 \times 10^{-3}$ M) and colchicine (10 µg/mL) were added and the lymphocytes harvested. Multiple slides were prepared and GTG-banded. A minimum of 25 metaphase spreads were analyzed for each sample and the representative images documented.

Blood collected in EDTA vacutainers was used to isolate DNA and, subsequently, FMR1 screening. The DNA was isolated using the Qiagen QIAamp blood DNA mini kit (Section 2.2.6). Screening for FXS was performed using two steps of PCR as described in Section 2.2.7. The primers were verified by sequencing a control sample in which a
normal number of CGG repeats was counted. The first PCR was performed for screening of all samples for the identification of expanded alleles. PCR reaction was set up with the reaction mix and program settings described in Table 2.2 and Table 2.3, respectively. The PCR products were electrophoresed in a 2% agarose gel and visualized using a UV transilluminator and documented. To distinguish between normal homozygous females and full mutation females, a second PCR was performed with the reaction mix and program described in Table 2.2 and Table 2.3, respectively. Similar to PCR1, the PCR products were electrophoresed in a 2% agarose gel and visualized using a UV transilluminator and documented.

3.3 Results

Demographic details of study subjects

The demographic details of the study subjects are described in Table 3.1. Of the 130 subjects, a majority (82%) were below the age of 10 years with a clinical diagnosis of developmental delay. Most of subjects were male (n=97), and only about a fourth were female (n=33). The subjects were categorized by psychological evaluation: 11% of the individuals were moderately disabled and 12% were severely disabled. Dysmorphism (microcephaly, hypertelorism, clinodactyly) and growth defects (seizures, cardiac defects, speech delay) were observed in 60% and 45% of study subjects, respectively, while prenatal abnormalities (IUGR) and abnormal pedigree were observed in 9% and 10%, respectively.

Chromosome analysis by HRB

Chromosome analysis was conducted in all the samples by high resolution banding. Normal males showed a chromosome complement of 46,XY (Figure 2.2) and normal females showed a chromosome complement of 46,XX (Figure 2.1). Of the 130 samples analyzed, four individuals showed an abnormal karyotype, yielding a frequency of 3%. The abnormalities detected were 46,XX,inv(17)(p13.2:q21.3) (Figure 3.1),
FIGURE 3.1: Karyotype of subject IMR 60: 46,XX,inv(17)(?p13.2:?q21.3). Arrow points to chromosome 17 with a pericentric inversion.

FIGURE 3.2: Karyotype of subject IMR 76: 46,XX,del(18)(p11.2→pter). Arrow points to chromosome 18 with a deletion on the ‘p’ arm.
TABLE 3.1: Demographic details of study objects

<table>
<thead>
<tr>
<th>Total no. of Subjects</th>
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<td>Growth abnormalities</td>
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<td>Family history of ID/seizures</td>
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</table>

46,XX,del(18)(p11.2→pter) (Figure 3.2), 46,XY,del(18)(q21.1→qter) (Figure 3.3) and 46,XY,del(18)(q21.3→qter) (Figure 3.4). The other 126 samples showed an apparently normal karyotype.

**FMR1 gene screening**

All the samples were screened for expansion of the CGG repeats in the *FMR1* gene with two step PCR. PCR 1 was performed on all samples. In the case of males, upon electrophoresis, a single band was observed, indicating a normal allele (Figure 3.5). In the case of females, 31 individuals showed a single band and were further tested with PCR 2, while 2 were found to be heterozygous. Short distinct bands were observed in PCR 2, indicating the absence of expanded alleles (Figure 3.6) in all females. Thus, all
samples were found to have no mutations in the *FMR1* gene. The PCR-based method for fragile X screening was validated by concurrent results obtained for three representative samples tested in another laboratory in a blinded manner.

### 3.4 Discussion

Conventional cytogenetic analysis of G-banded chromosomes at 450 band resolution is the first tier and "gold standard" diagnostic technique that has been adopted traditionally in the evaluation of genetic disorders. Due to the limited resolution of the conventional cytogenetic technique, HRB analysis is preferred for the detection of subtle rearrangements in the genome. The technique involves blocking the cell cycle at the S (synthesis) phase by adding methotrexate, which inhibits thymidine synthesis by blocking the conversion of dihydrofolate to tetrahydrofolate, which is needed to convert uridine to thymidine [14, 15]. In thymidine-depleted cultures, the cells are unable to complete replication. After overnight methotrexate exposure, the block is released with excess
thymidine; thus, cells blocked within S phase proceed in a wave into G2 and then mitosis. By using carefully timed harvests with short mitotic arrest and by adding ethidium bromide, a fluorochrome that interferes with chromosome condensation, a large number of mitotic plates in prophase or prometaphase can be captured.

In this study, while a majority of the subjects showed a normal karyotype, 3% showed abnormalities. An 8-year old female child (IMR 60), second born of a consanguineous marriage showed a pericentric inversion of chromosome 17. The child showed delayed milestones and Treacher-Collins phenotype, with mild ID. Other predominant features included micrognathia, retrognathia, arched eyebrows, prominent forehead, low-set ears, hypertelorism, mild hearing loss and overriding of 5th toe over the 4th on the left foot. Surgeries for mandibular reconstruction, cleft palate and ear malformations had been performed. Since inversions do not result in genomic imbalances, they generally do not result in phenotypic abnormalities. Further analysis of the abnormality
FIGURE 3.5: Gel image showing products of PCR 1, all males showed a single band, indicating absence of mutation in the \textit{FMR1} gene was required for correlation with the phenotype.

The subject (IMR 76) was a six-and-a-half year old female child with an 18p deletion. The child was of short stature, had a triangular face, low posterior hairline, upslanting eyes, hypertelorism, strabismus and stammering and performed poorly in school and clinically evaluated and found to exhibit features of Turners syndrome. Family history of MR was positive: a deceased paternal uncle was affected. It is reported that about 1 in 50,000 babies is born with a deletion of 18p; however, there is a great deal of variability between those with an 18p deletion. Most reports suggest that 18p deletions affect girls more often than boys. Although numerous cases with microscopically visible 18pter deletions have been reported, none of them is submicroscopic [16]. Some people with 18p- and short stature have been found to have growth hormone deficiency which may warrant growth hormone treatment. Most children have mild to moderate learning difficulties and exhibit delays in many developmental domains. However, a
few children have no problems at all while a small minority have severe or profound learning disabilities and ID. Facial features include short neck, round face, flat, broad nasal bridge, ptosis, hypertelorism and epicanthal folds, large, protruding low-set ears. About 10% of individuals with 18p- have brain malformations as part of the holoprosencephaly spectrum. Vision problems are common; including strabismus, long sight and short sight. Children with 18p– may have more frequent ear infections and thyroid disturbances [17].

Chromosome 18 represents about 2.5% of the total DNA in cells and has been estimated to contain between 300 and 500 genes, around 100 of which are located on 18p. One study has identified a preferential breakpoint or “hotspot” on 18p. This hotspot is located close to the centromere and within region 18p11.1. The HPE4 gene that is thought
to be the cause of holoprosencephaly has been mapped to 18p11.3. Four missense mutations in the \textit{TGIF} gene have been reported in 268 patients with holoprosencephaly [18]. Determining the exact size of the deletion is necessary for identifying critical regions and candidate genes that may contribute to the features of 18p- syndrome. Techniques such as FISH or array-CGH can more precisely define the deleted region and lead to a more accurate delineation of 18p- syndrome.

Two subjects showed a deletion of 18qter (IMR 105 and IMR 126); both the children were male and showed global developmental delay, hypertelorism, dysmorphism of the mouth and feet, and bilateral single palmar crease. One child showed partial corpus callosal agenesis on MRI, while no MRI information was available for the other child (however, microcephaly was observed). The 18q- syndrome, also known as de Grouchy syndrome, is a well-described disorder resulting from a partial deletion on the long arm of chromosome 18. Despite the fact that the breakpoints vary greatly among reports, with the deletions categorized as proximal or distal, the spectrum of clinical features of this syndrome are common, including short stature, hypotonia, hearing impairment, dysmorphic features, foot deformities, low levels of immunoglobulin -A and growth hormone deficiency. Global developmental delay and varying levels of cognitive impairment are reported. They may have low muscle tone (hypotonia), poor reflexes, or tremors. About 10% of people with distal 18q- have seizures. Two genes on 18q23, myelin basic protein (\textit{MBP}), and the galanin receptor (\textit{GALR2}), are candidate genes for the growth hormone insufficiency phenotype [19].

Screening for FXS is highly recommended in the diagnostic evaluation of idiopathic ID [20]. Therefore, the study subjects were screened for \textit{FMR1} mutation using newer methodology [13], by which expanded alleles in both male and female samples can be accurately flagged, reserving laborious experimentation such as Southern blotting only for those cases that require further testing. The absence of \textit{FMR1} mutations in any of the study subjects may be due to the low frequency of the mutations. The prevalence
of FXS is estimated to be 1 in 5000 males and 1 in 4000 to 6000 females. Inter-lab comparison of a few samples tested by different approaches yielding concurrent results confirmed the validity of the assay employed in the present study; thus the absence of a mutation was not due to technical limitation. Hence, this technique presents an excellent screening tool for FXS in children with unexplained ID, as its variable expression and complexity make fragile X testing absolutely essential in all cases with unknown etiology. Using HRB, a frequency of 3% of chromosomal abnormalities was observed in the study population, delineating the cause for ID in three individuals. Molecular genetic screening of FMR1 mutations confirmed the absence of mutations in the target loci investigated. Thus, in the rest of the study subjects, screening of more genomic hotspots using further advanced detection methods was warranted.
References


