Original Article

Recent Advances in Cytogenomics for Genetic Diagnosis in the Center of Excellence for Human Genetics in Egypt

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ABSTRACT

Background: This study aimed to apply the recent advances in cytogenomic techniques in diagnosing genetic disorders for proper genetic counseling. This work was done in the Centre of Excellence of Human Genetics, National Research Centre, Egypt. The center is funded by the STDF (project 5253).

Methods: The total number of referral patients was 2170 from 2016 to 2020. The Cytogenetic Team performed different cytogenomic techniques for four clinical genetics clinics: Neurodevelopment group, Hereditary blood group, Limb Malformation/Skeletal dysplasia group, and Multiple Congenital anomalies group. Karyotype was done for all patients, Fluorescence in situ hybridization (FISH) was performed for 324 patients, multiplex ligation-dependent probe amplification (MLPA) was performed for 160 patients, and array CGH for 90 patients. We followed the European guidelines for constitutional cytogenomic analysis in all applied techniques.

Results: Chromosomal abnormalities were detected in 6.6% of the referred patients (Down syndrome was excluded). Using FISH analysis, 226 patients were diagnosed with microdeletion syndromes and 98 with chromosomal abnormalities or marker chromosomes. MLPA technique was performed for 160 patients who had intellectual disability/multiple congenital abnormalities (ID/MCA). We used a probe mix for MR, subtelomere, and microdeletion syndromes. 26 patients (16%) had positive results. Array CGH was performed for 90 patients, out of them 44(49%) had large copy number variations. Some of these copy number variations involved two chromosomes or complex rearrangements. Negative patients were referred for whole exome sequencing.

Conclusion: The cytogenomic techniques could increase the number of accurately diagnosed patients. The array CGH is the first tier for the diagnosis of the genetic causes of ID/MCA and could allow accurate genotype/phenotype correlation. Proper genetic diagnoses are important for genetic counseling.

Key Words: Amplification, Dysmorphology, Genetic testing, Genotype-phenotype correlations, Multiplex ligation-dependent probe.

Received: 2024-11-07, **Accepted:** 2024-11-09

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ISSN: 2090-8571, 2023

INTRODUCTION

Cytogenomics is a term used to incorporate conventional cytogenetics, Molecular cytogenetics (fluorescence in situ hybridization [FISH] and chromosomal microarray), as well as molecular-based techniques e.g. multiplex ligation-dependent probe amplification (**Claussen, 2005; Liehr, 2019; Silva *et al.,* 2019**). Some authors use instead the term chromosomics (**Claussen, 2005**).

Conventional cytogenetic analysis is the gold standard for chromosomal diagnosis as it gives information about the whole genome. It is the most important tool in the detection of balanced rearrangements (**Mohamed *et al.,* 2015, Hochstenbach *et al.,* 2019**). However, it has a resolution of 5-10 megabases.

DOI: 10.21608/MXE.2024.334495.1034

FISH (fluorescence in situ hybridization) is a reliable and straightforward technique known for its accuracy. It is employed to diagnose chromosomal microdeletions, microduplications, inversions, and translocations. Additionally, it played a crucial role in physical mapping to validate large-scale mapping and sequencing in the Human Genome Project. FISH has extensive diagnostic applications in identifying genetic disorders and cancers (**Ratan *et al.,* 2017**).

MLPA (Ligation-dependent Probe Amplification) targets specific nucleic acid sequences through probe hybridization, followed by ligation and PCR amplification. This high-throughput technique is versatile, allowing for the detection of up to 40 target regions in a single reaction (**Fu *et al.,* 2022**). MLPA has a broad range of applications in diagnosing genetic disorders and cancer, becoming a crucial tool for identifying copy number variations (CNVs). There are various MLPA assays, including methylation-specific MLPA (MS-MLPA), digital MLPA, and array MLPA. These variations enhance MLPA's ability to detect CNVs, single nucleotide polymorphisms (SNPs), and other forms of genomic variation. (**Kravchenko *et al.,* 2015; Moazdarani *et al.,* 2018; Kravchenko *et al.,* 2020**).

Array CGH: Recent developments in array technology have strongly changed the genetic approach to genetic disorders, combining the whole-genome analysis of karyotyping technology and the targeted high-resolution FISH test (molecular karyotype). Genomic microarrays have a resolution 10000 times higher than that of conventional karyotyping. Identifying rare, de novo, submicroscopic interstitial imbalance or CNVs in about 5-20% of cases of idiopathic ID and multiple congenital abnormalities, depending on the clinical selection of patients. The increased identification of novel microdeletion/microduplication syndromes is based on an accurate genotype-phenotype correlation, characterized by the association of similar chromosomal aberrations and overlapped clinical presentations between affected patients. Recently the use of microchips with both CNVs and SNP helped in the analysis of the whole genome and identification of normal and pathological CNVs as well as consanguinity and uniparental disomy (UPD).

Intellectual disability (ID)/Developmental delay (DD), accounts for many cases regularly seen in genetics clinics. Currently, Array Comparative Genomic Hybridization (array CGH) is recommended by the International Standards for Cytogenomic Arrays (ISCA) Consortium as a first-line test in the diagnostics of ID/DD, replacing G-banded chromosome analysis (**Miller *et al.,* 2010, Moeschler and Shevell, 2014**).

The comparative genomic hybridization array (CGH array) associated with the single nucleotide polymorphism array (SNP array) became fundamental for cytogenomics (**Conlin and Spinner, 2010; Iourovet *et al.,* 2019**). Array CGH (chromosomal microarray) and single nucleotide polymorphism array (SNP) is the 1st diagnostic test for patients with multiple congenital anomalies (MCA), intellectual disability (ID), and developmental disorders (DD); (**Lee *et al.,* 2019**). A guideline for using chromosomal microarray in diagnoses was published by the **American College of Medical Genetics (ACMG) (2011)** (**Kearney *et al.,* 2011**) and European guidelines (**Silva *et al.,* 2019**). The International Standard for Cytogenomic Array (ISCA) Consortium has recommended CMA as the first-tier cytogenetic diagnostic test for patients with ID/DD and MCA (**Miller *et al.,* 2010**).

METHODS:

Conventional cytogenetics: GTG-banding was performed, and chromosomal analysis was carried out at 500-550 band level for all patients and some family members when indicated e.g. Cytogenetic studies for the parents of children with chromosomal abnormalities to search for balanced translocation. Cytogenetics technique and nomenclature were done according to **Verma and Babu, (1995)** and International System for Human Cytogenomic Nomenclature (**ISCN, 2020**), respectively.

Fluorescence in situ hybridization (FISH): FISH technique was performed according to **Pinkel *et al.,* 1986**. We used FISH technique to confirm clinical suspicion of microdeletion syndromes, (e.g. Williams syndrome, Velocardiofacial/DiGeorge syndrome, Prader Willi, Rubenstein-Taybi, 1p36 deletion, Wolf-Hirschhorn, Cri-du-Chat, Sotos syndrome, Miller Dieker, Steroid sulfatase deficiency (STS), Kallmann syndrome). Analyzing subtelomere regions of all chromosomes allows the detection of chromosome abnormalities in about 5-10% of ID patients (**Gijsbers *et al.,* 2009**). Also, FISH technique is very potent in the identification of subtelomere deletion and balanced rearrangement. No other technique can accurately detect the subtelomere balanced translocated carriers. We used Subtelomeric FISH studies using ToTelVysion probes (Vysis) according to the manufacturer's procedure (**Riethman *et al.,* 2005**).

Multiplex ligation-dependent probe amplification (MLPA): The method is in the protocol of MLPA (WWW.mlpa.com). The steps involved: were DNA denaturation (day 1), hybridization reaction (day 1), and Ligation reaction (day 2). PCR reaction (day 2), Fragment separation by capillary electrophoresis using ABI 3500 (Applied Bio System).

MLPA results analysis is done using the Coffalyser software for analysis patients against controls. In a normal signal, we obtain a peak value of 1, in case of deletion the peak is lower than 0.7, and in duplication, the peak is higher than 1.3 (**Bunyan *et al.,* 2004**).

Array CGH: Array CGH (chromosomal microarray) was performed on 90 patients. DNA was extracted using Qiagen kit. We followed the manufacturer's instructions for Affymetrix microarray. The workflow was through DNA digestion, ligation, PCR amplification, Purification, fragmentation, labeling, and loading to the Cytoscan HD microchip. The microchips were hybridized for 16 hours in GeneChip Hybridization Oven 645 (Affymetrix), washed, and stained in a GeneChip Fluidics Station 450 (Affymetrix). Microchip scanning was done on GeneChip Scanner 3000 (Affymetrix). Data analysis was performed using Chromosome Analysis Suite software (ChAS) (Affymetrix).

RESULTS

Karyotype and nomenclature of chromosomal abnormalities were performed according to the International System for Human Cytogenomic Nomenclature [ISCN] **(2020)**. The total number of referred patients was 2170. They were referred from 4 clinics: Multiple congenital anomalies (MCA), Neurodevelopmental, Limb malformation/Skeletal dysplasia, and Hereditary blood. Chromosomal abnormalities were found in 6.6% of referred patients (Down syndrome patients were excluded). Table (1) and the charts (Figures 1,2) demonstrate the number of referred patients from the 4 clinics and the percentage of chromosomal abnormalities in each group.

Table 1: The number of referred patients from the 4 clinics and the % of the detected chromosomal abnormalities in each patient's group:

	Multiple congenital anomalies group (MCA)	Neurodevelopmental group	Limb malformation/ Skeletal dysplasia	Hereditary blood group	Total
Patients referred for cytogenetics	556	1381	132	101	2170
Chromosomal abnormalities	48	73	5	18	144
% of chromosomal abnormalities	8.6	4	4	18	6.6

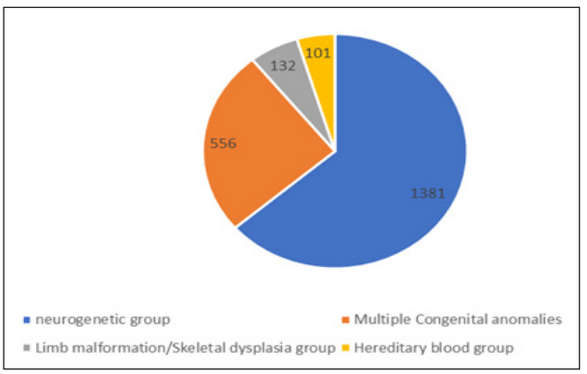

Figure 1: The percentage of referred patients from the four clinics.

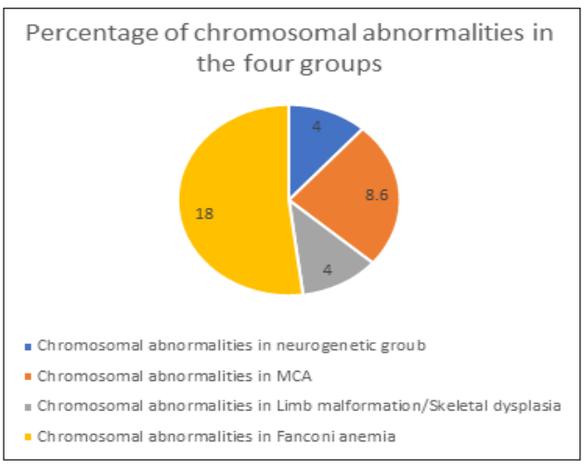

Figure 2: The percentage of the chromosomal abnormalities in each group.

Fluorescence in Situ Hybridization technique (FISH):

FISH was performed for 324 patients, out of 226 patients clinically diagnosed with microdeletion syndromes, 69 had positive FISH results for microdeletions (30.5%). 98 had chromosomal abnormalities or marker chromosomes. FISH was executed using specific identifier probes for clinically diagnosed microdeletion syndrome (Figures 3, 4). Whole chromosome paint or chromosome subtelomere probes were used to identify the breakpoints in patients with chromosomal abnormalities (Figures 5).

Multiplex Ligation-dependent Probe Amplification (MLPA):

160 patients with ID/MCA underwent the MLPA procedure; we employed a probe mix for MR, subtelomere, and microdeletion syndromes. Twenty-six patients (16%) had positive results.

Array CGH:

We established our microarray pre-PCR and post-PCR laboratory.

We performed all the microarray techniques in the microarray laboratory at the Centre of Excellence for Human Genetics, at the Institute of Human Genetics and Genome Research, National Research Centre, Egypt.

For array CGH Our results were described according to the International System for Human Cytogenomic Nomenclature ISCN (2020).

Array CGH was performed on 90 samples (Table 2). Out of the 90 samples, 44 samples (49%) had large

Table 2: Array CGH detected copy number variation:

No.	Patient complaint	Deleted segment	Duplicated segment
1	Neurodevelopmental disorders	Deletion of 6.5 Mb of 1q43q44	Duplication of 31 Mb of 14q32.33
2	MCA/Id CHD	Deletion of 378 Kb of 14q32.33	Duplication of 34 Mb of 9p24.3p13.3
3	Neurodevelopmental	Deletion of 3.7 Mb of 18q23	Duplication of 22 Mb of 11p15.5p14.3
4	Limb malformation	Deletion of 17.2 Mb of 9p24.3p22.1 Deletion of 1.2 Mb of 9p21.3	
5	Neurodevelopmental disorder		Duplication of 31,7 Mb of 11q13.1q22.1 Interstitial duplication od 1.8 Mb of 4q35.2
6	Neurodevelopmental disorders	Deletion of 18.8 Mb of 9p24.3p22.2	Duplication of 2.47 Mb of 9p22.1p21.3
7	Neurodevelopmental disorders and DSD	Deletion of 2.3 Mb of 9p24.3p24.2	Duplication of 26.8 Mb of 7p22.3p15.2
8	Neurodevelopmental disorder	Deletion of 16.8 Mb of 9p24.3p22.2	
9	MCA/ID CHD	Interstitial Deletion of 26 Mb of 8p12p22	
10	Neurodevelopmental disorder	Deletion of 3.8 Mb of 6p25.3p25.2	Duplication of 18,7 Mb of 7q33q36.3
11	MCA/ID	Deletion of 2.27 Mb of 2q22.2q22.3	
12	Neurodevelopmental disorder	Deletion of 40 Kb of 1q21.1	Interstitial duplication of 46 Mb of 5q21.1q32
13	MCA/ID	Deletion of 1.5 Mb of 7q11.23	
14	MCA/ID	Inv-dup del of chromosome 7 Deletion of 618 Kb of 7p22.3p22.2 Deletion of 1.65 Mb of 7p14.3p14.2 Deletion of 10.2 Mb of 7p21.3p21.1	Duplication of 1.2 Mb of 7q11.2
15	Neurodevelopmental disorder	Deletion of 2.7 Mb of 2q27.3	Duplication of 25 Mb of 12p13.33p12.1
16	MCA/Id CHD	Deletion of 22q11.21letion of 2.5 Mb	
17	MCA/ID and CHD	Deletion of 2.54 Mb of 2q11.21	
18	MCA/ID	Deletion of 1.15 Mb of 14q32.33	Duplication of 34 Mb of 3q26.1q29
19	Neurodevelopmental disorder		Duplication 13q Duplication 18p
20	Neurodevelopmental disorders		Duplications of 573 Kb of 17q21.31
21	Neurodevelopmental disorders	Complex chromosomal rearrangement involved multiple breaks in 4 chromosomes Deletion of 3.7 Mb in 1p22.3 Deletion of 1.8 Mb of 2q24.3 Deletion of 228Kb8p11.22 Deletion 982 Kb 8q21.3 Deletion241 Kb in 8q23.3	Duplication716 Kb 8p23.1
22	MCA/ID		Duplication of 947 Kb 4p16.1 Duplication469 Kb15q13.3 (CHRNA7 gene)
23	Duchenne muscle dystrophy and ID/MCA	Deletion 219 Kb of 1q44	Duplication 219 Kb 13q12.12 Muscular dystrophy, limb girdle
24	Limb malformation?	Deletion of 201 Kb 4q13.3	Duplication of 923Kb of 5q33.2
25	MCA/ID	Deletion 18.9 Mb od 9p24.3p22.1	
26	Neurodevelopmental disorder	Mosaic deletion of 39 Mb of Xq24q28	Mosaic duplication of 87 Mb 14q11.2q32.33
27	Buccal smear	Mosaic deletion of 39 Mb of Xq24q28	Mosaic duplication of 87 Mb 14q11.2q32.33
28	Neurodevelopmental disorders	Deletion of 1.6 Mb 5p15.33	Duplication of 30.5 Mb 10p15.3p11.23
29	MCA/Id	Deletion of 615 Kb of 18q23	Duplication of 56 Mb of 18q11.2q23
30	Neurodevelopmental disorders	Deletion of 10 Mb of 6q25.3q27	
31	Neurodevelopmental disorders	Deletion 10 Mb in 1q43q44	
32	Neurodevelopmental disorder	Deletion of 7 Mb of 5p13.33	Duplication of 37.5 Mb of 9p24.3
33	MCA	Deletion of 597 Kb of 2q24.1	
34	MCA		Duplication 13 Mb of 10p15.3p13
35	Blood clotting disorder		Gain of 912 Kb in 19p13.3 Blood group, complement factor, neutropenia
36	Father of patient 37	Deletion 1.27 Mb in 4q35.2	
37	Neurodevelopmental disorder	Deletion 1.35 Mb in 9q34.3 Deletion of 1.27 Mb of 4q35.2	

No.	Patient complaint	Deleted segment	Duplicated segment
38	Neurodevelopmental disorder	Deletion 395 Kb in 16p11.2 (16p11.2 deletion syndrome)	
39	ID/MCA	Deletion of 11.4 Mb in 9p24.3p21	Duplication of 19.4 Mb in 8p23.3p21.3
40	ID and CHD		LOH on chromosome 21
42	ID/MCA	Deletion 2q31.1	
42	ID/MCA and DSD	Deletion Xp22.33-p22.31	
43	MCA/ID	Del(4)(p)	
44	MCA		Dup10q

copy number variations, deletion, and/or duplication segments. Some of these copy number variations involved two chromosomes or complex rearrangements Table 2 represents the copy number variations detected by the array CGH. Figure (6) represents the percentage of the detected chromosomal abnormalities.

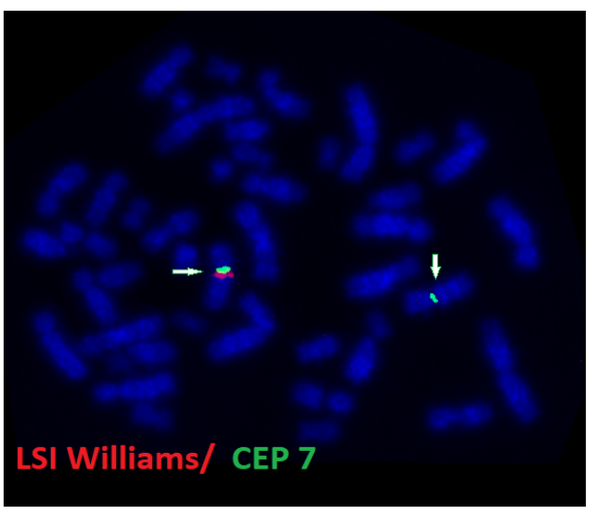

Figure 3: Deletion of William syndrome specific locus (Green signal is the centromere of chromosome 7 as a control and the red signal is the Williams syndrome specific locus).

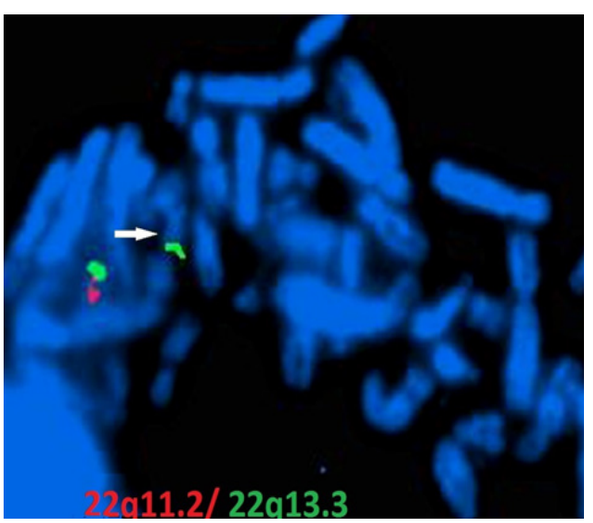

Figure 4: Deletion of the Di-George syndrome specific locus (The green signal is the terminal 22q13.3 locus as a control, the red signal is the Di-George syndrome specific locus).

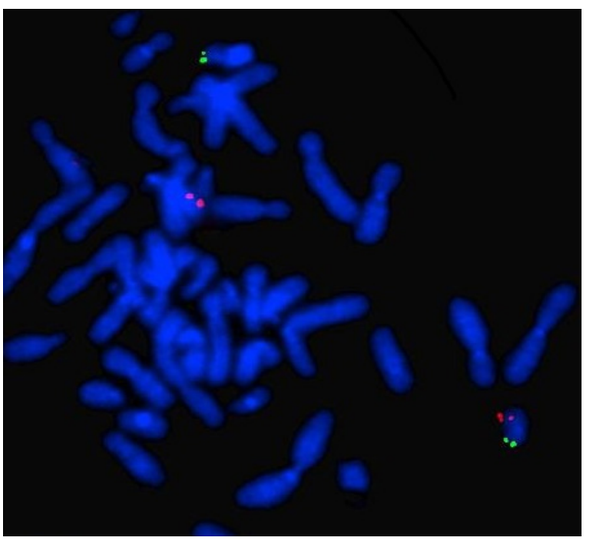

Figure 5: translocated 20p subtelomere (the green signals are 20p subtelomere, the red signals are the 20q subtelomeres) one of the 20p subtelomere is translocated to another chromosome.

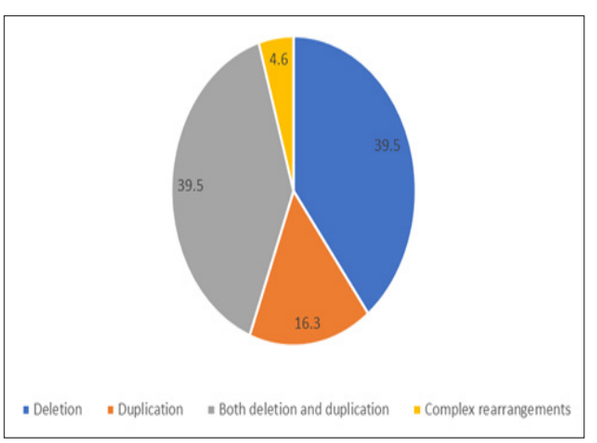

Figure 6: The percentage of the detected chromosomal abnormalities.

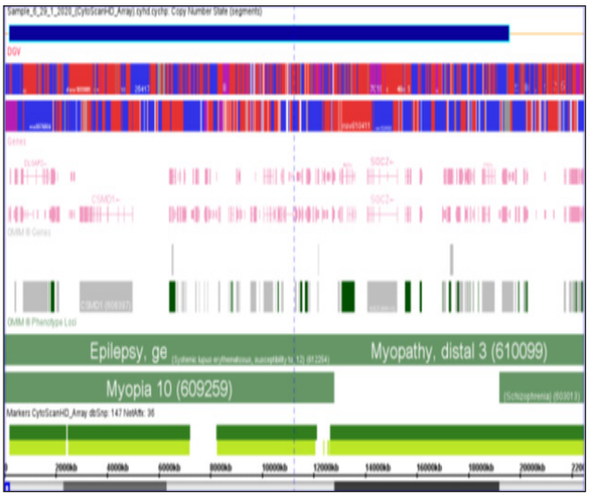

Figure 7: Chromosome 8 terminal duplication (The blue bar at the top represents the duplicated segment).

DISCUSSION

Exploring the mechanisms causing genetic disorders holds the promise for diagnosis, prediction, prevention, and treatment. Depending on resources and an extensive track record in genetic disorders studies in Egypt, the Scientific Technology Development Fund, Academy of Science and Technology Egypt, hosted by the Institute of Human Genetics and Genome Research, National Research Centre established the Genetic Disorders Centre of Excellence. The Human Cytogenetic Department is a partner in the Centre of Excellence for Human Genetics Our aim is the application of different cytogenomic techniques in the diagnosis of various genetic disorders.

We followed the European guidelines for constitutional cytogenomic analysis (**Silva *et al.*, 2019**). For array CGH, our results were given using the most recent International System for Human Cytogenomic Nomenclature [ISCN] (**McGowan Jordan *et al.*, 2020**). The application of cytogenomic testing in the diagnosis of genetic disorders is essential for the accurate detection, characterization, and management of these conditions. Various techniques, including karyotyping, multiplex ligation-dependent probe amplification (MLPA), and array comparative genomic hybridization (array CGH), offer unique advantages and serve specific purposes in clinical genetics.

Karyotyping can identify large chromosomal abnormalities such as numerical chromosome abnormalities e.g., trisomy 13, 18, 21 and structural abnormalities specifically balanced translocation which can be propagated to the next generations. It targets the whole genome. It offers direct Visualization of chromosomes, aiding in understanding complex genetic issues. The limitation is its lower resolution compared to molecular techniques thus cannot detect microdeletions or duplications smaller than 5-10Mb (**Hochstenbach *et al.*, 2019; McGowan-Jordan *et al.*, 2020**). In our study karyotyping could detect chromosomal abnormalities in 6.6% of referred patients (Excluding Down syndrome).

FISH is used to visualize specific DNA sequences on chromosomes, allowing for the detection of specific genetic abnormalities, including microdeletions and translocations. It targets particular genes or regions, providing precise information that can identify smaller genetic abnormalities that karyotyping might miss. However, it requires prior knowledge about the specific regions of interest and does not provide a genome-wide view (**Chrzanowska *et al.*, 2020**). Positive results were detected in 30.5% of our patients with microdeletion syndromes and other chromosomal abnormalities.

MLPA technique has many applications, the most important is the detection of copy number variations (CNVs) of specific genomic regions. It is especially useful for assessing deletions or duplications in genes associated with a large number of genetic disorders. It has high sensitivity and specificity and it is capable of detecting small CNVs that karyotyping may miss. It is a targeted Analysis that focuses on specific regions of interest, making it efficient for certain genetic conditions (e.g., Duchenne muscular dystrophy). It is Limited to known target sequences; and cannot detect unknown variants (**Kravchenko *et al.*, 2020**). In our study, we could detect 16% of patients with positive results.

Array comparative genomic hybridization (array CGH) enables genome-wide analysis of copy number variations (CNVs) and is particularly useful for diagnosing genetic disorders with unknown etiology. Its high resolution allows the detection of submicroscopic CNVs that may be missed by karyotyping and MLPA, providing a comprehensive view of chromosomal imbalances. However, one limitation of array CGH is that while it detects CNVs, it does not identify point mutations or balanced rearrangements (**Iourov *et al.*, 2019**). In our patients, array CGH yielded positive results in 49% of the tested patients. This high percentage is due to the application of array CGH mainly for patients who already have chromosomal imbalances. We performed array CGH to determine the exact breakpoints and the genes involved in the detected CNVs, which is crucial for establishing genotype/phenotype correlations.

Cytogenomic testing plays a vital role in diagnosing genetic disorders, each technique offering distinct advantages. Karyotyping remains foundational, while FISH, MLPA, and array CGH provide advanced capabilities for detecting smaller genetic abnormalities.

CONCLUSION

The concept of a center of excellence for genetics is crucial for fostering collaboration among researchers

at the institute. It provides an opportunity to equip the facility with essential equipment and materials. This, in turn, maximizes the chances for patients to receive proper diagnosis, counseling, and management. Additionally, it creates strong connections among all researchers within the institute. By introducing the latest technologies, the center facilitates advancements in the field of Human Genetics.

ACKNOWLODGEMENT

We would like to express our deepest gratitude to the soul of Professor Samia Temtamy, the principal investigator, for her unwavering support and guidance throughout the progression of our projects. Her dedication to overseeing patient results and coordinating efforts among all departments within the Human Genetics Institute has been invaluable. Professor Temtamy organized numerous scientific meetings to ensure optimal collaboration across the institute, all aimed at providing the best possible service for our patients. Her commitment to advancing diagnosis, counseling, and management in human genetics has greatly benefited Egyptian patients through the latest developments in the field.

CONFLICT OF INTEREST

There are no conflicts of interest.

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