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An Overview of Mutation Detection Methods in Genetic Disorders

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Abstract

Genetic disorders are traditionally categorized into three main groups: single-gene, chromosomal, and multifactorial disorders. Single gene or Mendelian disorders result from errors in DNA sequence of a gene and include autosomal dominant (AD), autosomal recessive (AR), X-linked recessive (XR), X-linked dominant and Y-linked (holandric) disorders. Chromosomal disorders are due to chromosomal aberrations including numerical and structural damages. Molecular and cytogenetic techniques have been applied to identify genetic mutations leading to diseases. Accurate diagnosis of diseases is essential for appropriate treatment of patients, genetic counseling and prevention strategies. Characteristic features of patterns of inheritance are briefly reviewed and a short description of chromosomal disorders is also presented. In addition, applications of cytogenetic and molecular techniques and different types of mutations are discussed for genetic diagnosis of the pediatric genetic diseases. The purpose is to make pediatricians familiar with the applications of cytogenetic and molecular techniques and tools used for genetic diagnosis.

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Key Words: Cytogenetics; Chromosomal Aberration; Molecular Diagnostic Technique; Carrier Detection; Mutation

Introduction

The diploid human genome including twenty-three pairs of chromosomes is composed of 20–25 thousand genes; haploid set is estimated to be $3.2*10^9$ base pairs[1]. One member of each chromosome is received from the father, and the other member of the pair is transmitted through maternal lineage. DNA is made up of four base pairs adenine, thymine, cytosine and guanine abbreviated as A, T, C, and G, respectively. Genes consisting of DNA base pairs are located on chromosomes. A gene is a sequence of base pairs that produces a functional product including a RNA molecule or subsequently a peptide (Fig. 1A). An allele is positioned on a locus, the specific location of a gene or DNA sequence on a chromosome; so the diploid genome contains two alleles of each gene. Chromosomes 1 to 22 are called autosomes and the twenty-third pair is the sex chromosomes, i.e. X and Y[2].

Steps in the transmission of genetic information include replication (DNA makes DNA), transcription (DNA makes RNA), RNA processing (capping, splicing, tailing and RNA translocation to cytoplasm), translation (RNA makes protein), and protein processing, folding, transport and incorporation (Fig. 1B). If the DNA sequence is

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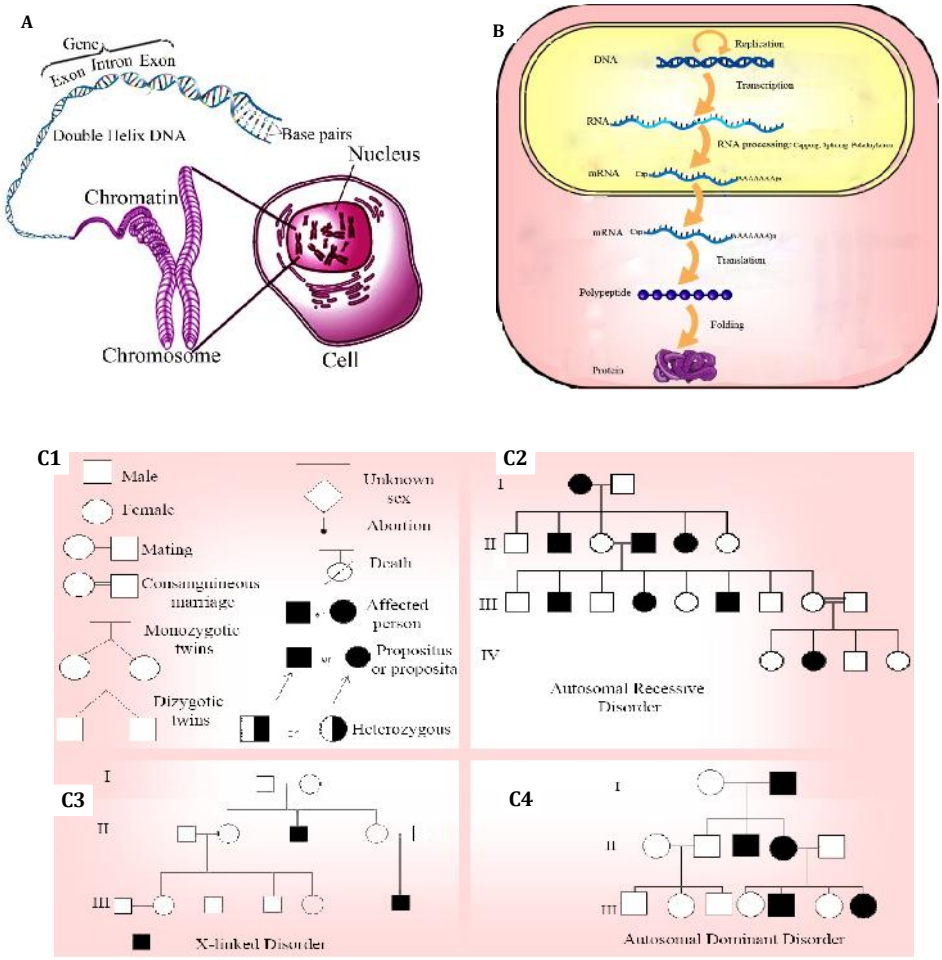

Fig 1: A) Nucleus, chromosomes, gene, exon, intron and base pairs. B) Central dogma. C1) Common symbols for drawing a pedigree. C2) Autosomal recessive mode of inheritance; C3) X-linked recessive inheritance; C4) Autosomal dominant inheritance

mutated and the alteration is not repaired by the cell, subsequent replications reproduce the mutation. A variety of mechanisms can cause mutations ranging from a single nucleotide alteration to the loss, duplication or rearrangement of chromosomes[2]. Genetic diseases are usually categorized into three major classes: single-gene, chromosomal, and multi-factorial disorders. A combination of genes and environmental factors is involved in multifactorial disorders such as congenital heart disease, most types of cleft lip/palate, club foot, and neural tube defects[3].

Here, Mendelian patterns of inheritance and chromosomal disorders are reviewed and a brief summary of genetic methods in genetic disorders is presented to make the pediatricians familiar with the basics of the cytogenetics and molecular methods of mutation detection as well.

Mendelian Disorders

Gregor Mendel discovered a set of principles of heredity in the mid-19th century; characteristic patterns of inheritance are determined on the basis of these principles. Single gene disorders arising from errors in DNA sequence of a gene are categorized into autosomal dominant (AD), autosomal recessive (AR), X-linked recessive (XR), X-linked dominant and Y-linked (holandric) disorders[3].

In autosomal dominant disorders (Fig 1C1 and 4), damage in one allele of a pair of the gene leads to the deficiency (Table 1)[3], e.g. a mutation in FGFR3 gene can cause achondroplasia[4,5]. A parent with an autosomal dominant disorder has a 50% chance of transmitting the disease to her/his child[2]. The range of signs and symptoms of some diseases in different people vary widely (variable expressivity), e.g. some people with Marfan

Table 1: Modes of inheritance and their properties

Inheritance pattern	Description	Examples
Autosomal Dominant	One mutated allele causes the disease Each affected person usually has one affected parent Appears in every generation of an affected family (Vertical)	Marfan syndrome; Achondroplasia; Huntington disease; Myotonic dystrophy
Autosomal Recessive	Two mutated alleles needed to cause the disease Parents are usually unaffected heterozygotes Not typically seen in every generation (Horizontal).	Beta thalassemia; Cystic fibrosis; Homocystinuria
X-linked Dominant	Females are more frequently affected than males no male-to-male transmission	Rett syndrome; Hypophosphatemia
X-linked Recessive	Males are more frequently affected than females Both parents of an affected daughter must be carriers Fathers cannot pass X-linked traits to their sons	Hemophilia; Duchenne Muscular Dystrophy
Mitochondrial	Only females can pass on mitochondrial conditions to their children (maternal inheritance) Both males and females can be affected Can appear in every generation of a family	LHON: Leber's hereditary optic neuropathy

syndrome (due to mutation in FBN1) have only mild symptoms (such as being tall and thin with long, slender fingers), while others have life-threatening complications involving the heart and blood vessels as well[6]. Furthermore, some individuals exhibit signs and symptoms of a given disorder while others do not, even though they have the disease-causing mutation (i.e. a proportion of people with a particular mutation show the condition in this type of disorders), e.g. many people having mutation of the BRCA1 gene will develop breast cancer during their lifetime, while some people will not[2,7]. In other words, in a pedigree a healthy individual has at least one affected parent and one affected child (skipped generation)[2].

Mutations in both alleles (loss of function) of a gene are required to cause the defect to appear in an autosomal recessive disorder (Table 1 and Fig. 1C2), i.e. an affected person has got one abnormal allele from one heterozygous parent. In this type of disorders, there is a 25% chance of having an affected offspring for heterozygous parents. In case of common autosomal recessive disorders or traits (sickle cell anemia due to a specific mutation in HBB gene encoding beta globin[8] or nonsyndromic autosomal recessive hearing loss due to mutations in GJB2 gene encoding connexin 26[9-14]), sometimes a homozygous affected person marries a heterozygous carrier; such an example, in which apparently dominant transmission of this disorder occurs, is called pseudodominant inheritance[2].

In an X-linked disorder (Fig. 1C3 and Table 1), the mutated gene is located on the X chromosome. A recessive mutation can lead to the disease. The gene in chromosome X should be mutated to cause the condition; hence, an X-linked recessive disorder is carried by females, while usally affects males.

Some of genetic conditions follow none of the mentioned patterns of inheritance; mitochondrial diseases, trinucleotide expansion disorders and genomic imprinting defects have non-Mendelian or nontraditional pattern of inheritance[2,15].

Chromosomal Disorders

Typically, somatic cells proliferate via division called mitosis while germ cells are produced through meiosis division. Meiosis involves a reductional division followed by an equational division, Meiosis I and II, respectively.

Oogenesis begins in the female fetus at 12 weeks, but it is stopped in a stage of meiosis I (when the homologous chromosomes have replicated and paired as bivalents or tetrads) at about 20 weeks[16]. At puberty usually only one oocyte is released per month; a primary oocyte completes meiosis I and produces one secondary oocyte and one polar body. Chromosomal aberrations including numerical (due to errors at chromosome pairing and crossing-over) and structural damages lead to chromosomal disorders (Table 2, 3 and 4; Fig. 2A and B). Aneuploidy is usually due to failure of segregation of chromosomes in meiosis I or meiosis II (non-disjunction, premature disjunction or anaphase lag) [17]; examples of numerical aberrations include Down syndrome (trisomy 21), Edwards syndrome

Table 2: Examples of numerical aberrations (aneuploidies)

Aneuploidy	Karyotype	Incidence	Main features
Down syn. Trisomy 21	47, XX or XY, +21	1/700 live births	Epicanthal folds, hypotonia, flat occiput, Brushfield spots in irides, single transverse crease, clinodactyly, etc.
Edward syn. Trisomy 18	47, XX or XY, +18	1/3000 live births	Clenched fists, rocker bottom feet, low-set, malformed ears, micrognathia, cardiac and renal abnormalities, etc.
Patau syn. Trisomy 13	47, XX or XY, +13	1/5000 live births	Microcephaly, holoprosencephaly, rocker-bottom feet, microphthalmia, anophthalmia, cyclopia, cryptorchidism, heart defects; cleft lip and palate, etc.
Klinefelter syn.	47, XXY (48, XXXY; 49, XXXXY)	1/500 male births	Gynecomastia, small genitalia and infertility
Turner syn.	45, X	1/5000 female births	Fail to mature sexually, lymphedema, webbed neck, low posterior hairline, cubitus valgus, etc.
XYY syn.	47, XYY	1/1000 male births	Tall stature, large teeth; fertility is normal
Triple X syn.	47, XXX	1/1000 female births	Some learning problems

Syn: syndrome

(trisomy 18), Patau syndrome (trisomy 13), Klinefelter syndrome (XXY syndrome), Turner syndrome (monosomy X) and trisomy X. Chromosomal errors in oocytes are increased dramatically with maternal age. Non-disjunction or chromosome lag during mitosis can lead to mosaicism[2].

Most of structural aberrations, including translocations, deletions, inversions, duplications, ring chromosomes and isochromosomes (Table 3, 4 and Fig. 2B) result from unequal exchange between chromosomes or enzymatic misrepairing of two chromosome breakages; examples of structural aberration are cat cry syndrome (5p-), Williams syndrome (7q11.2 deletion), DiGeorge syndrome (22q11.2 deletion), etc.

Types of Mutations

A mutation is a change in the nucleotide sequence in coding portions of the DNA which may alter the amino acid sequences of proteins, or a change in noncoding regions of DNA which has the potential for changing expression of the gene, for example by altering the strength of a promoter. There are many mutations which are classified to chromosomal and DNA-based mutations (Table 3 and 4). Mutations can also be categorized on the basis of the function: 1) The loss-of-function mutations cause a decrease or a loss of the gene product or the activity of the gene product; 2) The gain-of-function mutations cause an increase in the amount of gene product or its activity, and sometimes create a new property, leading to a toxic product responsible for a pathological effect.

Three types of mutations usually observed in dominant disorders are gain-of-function, haploinsuficiency and dominant negative. Mutations may act as dominant or recessive when the amount of product from one allele is not sufficient for a complete function (Haplo-insufficiency), e.g. mutations in LDLR leading to haploinsufficiency in familial hypercholesterol-emia[18]. If the product of the defective allele interferes with the product of normal allele (Dominant negative) it affects the function of normal protein; basically collagen mutations are dominant negative ones[19]. A mutated allele may gain a new or excessive activity (Gain of function) e.g. mutations of FGFR3 in achondroplasia[4,5].

Mutation Detection

With the development of new technologies for more accurate understanding of the genome and potential gene therapies, the detection of mutations has an increasingly central role in various areas of genetic diagnosis including preimplantation genetic diagnosis (PGD), prenatal diagnosis (PND), presymptomatic testing, confirm-ational diagnosis and forensic/identity testing. Two groups of tests, molecular and cytogenetic, are used in genetic syndromes. In general, single base pair mutations are identified by direct

sequencing, DNA hybridization and/or restriction enzyme digestion methods. However, there are two approaches for genetic diagnosis; indirect approach depends on the results from a genetic linkage analysis using DNA markers such as STR(short tandem repeat) or VNTR (variable number tandem repeat) markers flanking or within the gene[20-22]. The direct approach for diagnosis essentially depends on the detection of the genetic variations responsible for the disease.

Cytogenetics and Molecular Cytogenetics

Conventional Karyotyping: Chromosome studies are advised in the following situations: suspected chromosome abnormality, sexual disorders, multiple congenital anomalies and/or developmental retardation, undiagnosed learning disabilities, infertility or multiple miscarriage, stillbirth and malignancies[2]. Traditionally, the microscopic study of chromosomes is performed on compacted chromosomes at a magnification of about 1000 at metaphase.

Preparation of a visual karyotype (Fig 2C) is done by arresting dividing cells at metaphase stage with a microtubule polymerization inhibitor such as colchicine; the cells, then, are spread on a glass slide and stained with Giemsa stain (G-banding). Chromosomes are studied by making a photograph or digital imaging and subsequent assembling of chromosomes. Human chromosomes are categorized based on position of centromere; in metacentric chromosomes, centromere is located in the middle (chromosome-

Table 3: Chromosomal aberrations in human disorders

Mutation	Definition	Example (Gene)	Disease/ condition	Ref
Structural aberrations				
Deletion	A part of a chromosome is deleted	46,XX,del (5p15.2-pter)	Cat Cry Syn.	[2]
Duplication	A portion of a chromosome is duplicated	46, XX, dup (22q11.2)	Cat Eye Syn.	[2]
Translocation	An interchange of genetic material between nonhomologous chromosomes	46, XX, t (9; 22) (q34; q11)	CML	[2]
Reciprocal T.	An interchange of genetic material between two nonhomologous chromosomes	46, XX, rcp (9; 22) (q34; q11)	CML	[2]
Robertsonian T.	The fusion of the long arms of two acrocentric chromosomes and loss of their short arms	45, XX, rob (14q21q)	Normal	[2]
Inversion	A portion of a chromosome is inverted			
Pericentric Inv.	The inverted segment includes the centromere	46, XX, Inv (9) (p11q13)	Normal?	[2]
Paracentric Inv.	The inverted segment is located on one arm of the chromosome	inv(14)(q13q24)	Microcephaly	[51]
Ring	Both arms of a chromosome have fused together as a ring	46, Xr (X)	Turner syn.	[2]
Isochromosome	A chromosome that has two identical arms because of duplication of one arm of the chromosome; a mirror-image of one arm of a chromosome	46, Xi (Xq)	Turner syn.	[2]
Dicentric chromosome	An abnormal chromosome that has two centromeres	46, X, psu dic (Y) (pter→q11.2::q11.2→pter)	Azoospermia	[52]
Numerical aberrations				
Aneuploidy	An abnormal number of chromosomes			
Monosomy	The presence of only one of two homologous chromosome in a diploid organism (e.g. Human)	45, X	Turner Syn.	[2]
Uniparental disomy	Inheritance of two pairs of a homologous chromosome from one parent and no copy from the other parent (Fig 2)	46, XX, upd (15) mat	Prader-Willi syndrome	[2]
Trisomy	Existence of three copies of a homologous chromosome	47, XX, +21	Down syn.	[2]
Tetrasomy	Existence of four copies of a homologous chromosome	48, XXXX	X tetrasomy	[2]
Monoploidy	The state of having a single (non-homologous) set of chromosomes	23X		
Triploidy	Having three sets of chromosomes instead of two	69, XXX	Abortion, Hydatidiform mole	[2]

Syn: Syndrome; Ref: Reference; T: Translocation; Inv: Inversion; psu dic: Psuedodicentric; rcp: Reciprocal; rob: Robertsonian; upd: Uniparental Disomy

Table 4: Gene-based Mutations in human disorders

Mutation	Definition	Example (Gene)	Disease/ condition	Ref
Point Mutation	A single base pair alteration; it includes transition: purine (A,G) to purine (G,A) or pyrimidine (C,T) to pyrimidine (T,C), and transversion: purine (A,G) to pyrimidine (T,C) or pyrimidine (T,C) to purine (A,G)	A>G, A>T		[2,45]
a. Missense (Nonsynonymous)	A single nucleotide resulting in a codon that codes for a different amino acid	A82P (HSD3B2)	3βHSD deficiency	[46]
b. Nonsense	A single nucleotide resulting in a premature stop codon	G23X (HBB)	Beta Thalassaemia	[47]
c. Synonymous	A single nucleotide that changes a codon to an amino acid with similar properties e.g. Lysine to Arginine	V153I (GJB2)	Hearing loss	[10]
d. Silent	A single nucleotide which does not alter amino acid sequences e.g. GCT, GCC, GCA and GCG all code for alanine; any change in the third position of the codon (e.g. GCA>GCG), does not alter the amino acid sequence	I69I (GJB2)	Hearing loss	[10]
e. Neutral	A single nucleotide which does not have any harmful or beneficial effect on the organism, it usually occurs at noncoding DNA regions			
Duplication	A region of a sequence is duplicated; if the number of nucleotides is not evenly divisible by three from DNA sequence it is called a frameshift mutation	920dupTCAG (LDLR)	Familial hyper-cholesterolemia	[48]
Deletion	A portion of a sequence is deleted; if the number of nucleotides is not evenly divisible by three from DNA sequence it is called a frameshift mutation	delE120 (GJB2)	Hearing loss	[11]
Insertion	Addition of one or more nucleotide base pairs into a DNA sequence; if the number of nucleotides is not evenly divisible by three from DNA sequence it is called a frameshift mutation	3524insA (FBN1)	Marfan syndrome	[49]
Splice mutation	A sequence change in the site splicing of an intron; it may result in one or more introns remaining in mature mRNA.	IVS1+1G>A (GJB2)	Hearing loss	[10]
Dynamic mutation	An unstable mutation in which the number of copies of a sequence is changed during meiosis division e.g. trinucleotide expansions	(CGG)n>200 (FMR1)	Fragile X syndrome	[50]

Syn: Syndrome; Ref: Reference

-s 1, 3, 16, 19 and 20), chromosomes 13, 14, 15, 21, 22 and Y are acrocentric (the centromere near one end), and other chromosomes are sub-metacentric. Chromosome arms are defined by region number (from centromere), band, sub-band and sub-sub-band numbers, e.g. 12q13.12 refers to chromosome 12, long arm, region 1, band 3, sub-band 1, sub-sub-band 2 (read chromosome 12, q, 1, 3, point, 1, 2). High resolution banding needs fixation before the chromosomes are fully compacted. The convenient methods of chromosome banding are G-(Giemsa), R-(reverse), C-(centromere) and Q-(quinacrine) banding.

Fluorescence in situ hybridization (FISH): FISH is applied to provide specific localization of genes on chromosomes. Rapid diagnosis of trisomies and microdeletions is acquired using specific probes. Usually a denatured probe is added to a metaphase chromosome spread and incubated overnight to allow sequence-specific hybridization. After washing off the unbound probe, the bound probe is visualized by its fluorescence under UV light; thus, the site of the gene of interest is observed as in situ (Fig 2D)[2,23]. This technique is used to check the cause of trisomies, microdeletion syndromes, etc.

Comparative genomic hybridization (CGH): CGH, a special FISH technique (dual probes), is applied for detecting all genomic imbalances. The basics of technique is comparison of total genomic DNA of the given sample DNA (e.g. tumor DNA) with total genomic DNA of normal cells. Typically, an identical amount of both tumor and normal DNAs is labeled with two different fluorescent dyes; the mixture is added and hybridized to a normal lymphocyte metaphase slide. A fluorescent microscope equipped with a CCD camera and an image analysis system are used for evaluation[24]. Technical details have been described in numerous CGH publications[25,26]. Copy number of genetic material (gains and losses) is calculated by evaluation software[27]. CGH is used to determine

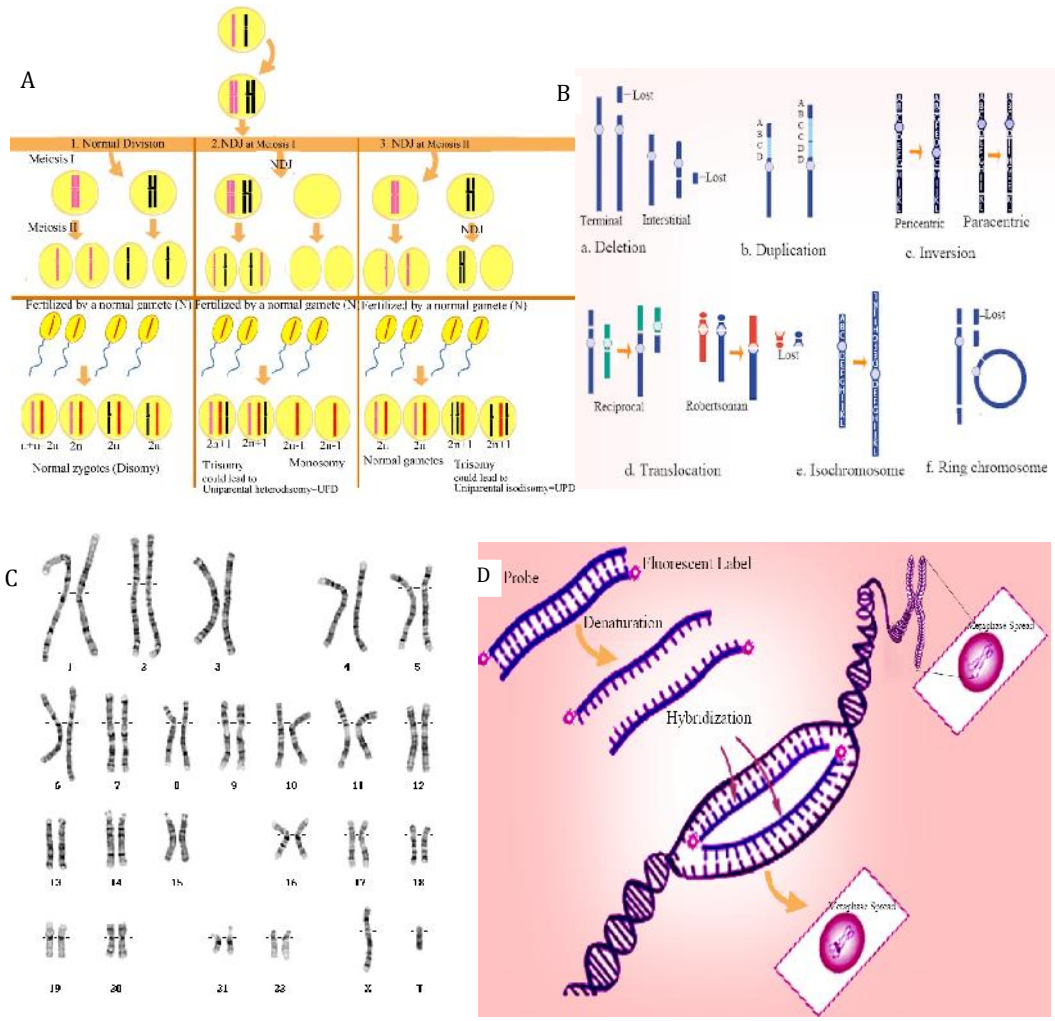

Fig. 2: A) Chromosome segregation during meiosis, nondisjunction and its consequences. Nondisjunction (NDJ) at meiosis I and II leads to uniparental heterodisomy and uniparental isodisomy, respectively. B) Structural aberrations of human chromosomes. C) Human karyotype. Chromosomes in a typical male. D) Fluorescence in situ hybridization (FISH).

copy number alterations of genome in cancer and those cells whose karyotype is hard or impossible to prepare or analyze. In array-CGH, metaphase slide is replaced by specific DNA sequences, spotted in arrays on glass slides[28]; so its resolution is increased.

Molecular Diagnostics

In addition to genetic causes of disorders, predisposition to a disease or treatment options could be revealed by determining DNA variations. Molecular diagnostics provide a way for assessment of the genetic makeup of human; it combines laboratory medicine with molecular genetics to develop DNA/RNA-based analytical methods for monitoring human pathologies. A wide range of methods has been used for mutation detection. Molecular methods for identification of the disease-causing mutations could be classified as methods for known and methods for unknown mutations. Several criteria, however, have to be met for choosing a suitable method; for example the following points should be considered: type of nucleic acid (DNA or RNA), kind of specimen (e.g., blood, tissues, etc.), the number of mutations, and reliability of the method. The pediatricians need to be noticed when prescribing these tests to provide an accurate diagnosis for the patients.

Table 5: Cytogenetics and molecular methods for mutation detection

Method		Application	Advantage/disadvantage	Known mutation	Unknown mutation
Cytogenetics	**Karyotype**	Detecting numerical and gross structural aberrations	Low resolution Time consuming and labor requirements	+	+
	FISH	Detecting trisomies, monosomies and microdeletions	Detects mosaicism	+	-
	CGH	Detects copy number variations of genetic material	Used only for losses and gains	+	+
Molecular	**RFLP**	Restriction fragments are separated by electrophoresis	Requires mutation in restriction site	+	-
	ARMS PCR	Allele-specific amplification of mutant and normal allele, determination of the genotype of an individual	Highly sensitive Possible to detect any known mutation May increase time and costs	+	-
	Multiplex PCR	Amplification of more than one target simultaneously	Reduces time and labor requirements Lower sensitivity and specificity	+	-
	Nested PCR	Amplification using external and internal primer sets	More sensitive Decreases nonspecific amplification	+	-
	RT-PCR	Amplification of RNA	Amplification of all RNA types May increase time and costs	+	+
	Real-time PCR	Amplification, detection, and quantification of target	Increased specificity Usually eliminates postamplification analyses More expensive	+	-
	MLPA	Deletions and duplications	A multiplex technique Identifies very small single gene aberrations (50-70 nt)	+	+
	DGGE	Based on migration within gradient gel electrophoresis	Detects close to 100% of point mutaions	-	+
	SSCP	Based on migration within gel electrophoresis	Detects about 80–90% of point mutations	-	+
	Hetero-duplex analysis	Based on homoduplices and heteroduplices motilities in gel electrophoresis	Detects nearly 80% of mutations	-	+
	CCM	DNA: DNA or DNA:RNA heteroduplices are cleaved by piperidine	All possible mutations are detectable Uses toxic substances	+	+
	PTT	It is based on a combination of PCR, transcription, and translation	Detects translation-terminating mutations Missense mutations are not detected.	-	+
	OLA	It is based on ligation of two flanked primers annealed with target sequences	Detects all base exchanges	+	+

FISH: Fluorescence in situ hybridization; CGH: Comparative genomic hybridization; RELP: Restriction fragment length polymorphism; ARMS; Amplification refractory mutation system; PCR: Polymerase chain reaction; RT: Reverse transcriptase; MLPA; Multiplex ligation-dependent probe amplification; DGGE: Denaturing Gradient Gel Electrophoresis; SSCP: Single Strand Conformational Polymorphism; CCM: Chemical cleavage of mismatch; PTT: Protein truncation test; OLA: Oligonucleotide ligation assay

A) Known Mutations

Many different approaches have been used for identifying known mutations. Usually starting with the polymerase chain reaction (PCR), additional assay steps are performed based on the type of mutation. Table 5 shows examples of some of the frequently used techniques as well as their advantages and disadvantages. Here, a brief view of some of these techniques is presented with focus on their applications.

Polymerase chain reaction (PCR) and its versions: In 1980s, Dr Mullis introduced a method for amplifying DNA fragment to a large number of fragments in only a few hours; this method, named polymerase chain reaction (PCR), was a critical point in molecular biology[29,30]. Essential components of polymerase chain reaction are template DNA, primers (a pair of synthetic oligonucleotides complementary to the two strands of target DNA), thermostable DNA polymerase enzyme (e.g. Taq), divalent cations (usually Mg^{2+}), deoxynucleoside triphosphates (dNTPs) and buffer solution. PCR, consisting of 25-40 repeated cycles, has three discrete steps of temperature changes (Fig. 3A); after a single temperature step at a high temperature (>90°C), a series of cycles of denaturation, annealing of primers and extension are performed and followed by a single temperature step called final product extension or brief storage. These steps are as follow:

Initial denaturation step includes heating the reaction to a temperature of 92–96°C for 1–9 minutes.

1) Denaturation step includes heating the reaction to 92–98°C for 20–30 seconds. The hydrogen bonds between complementary bases are disrupted and DNA molecules are denatured, yielding single-stranded DNA molecules (DNA melting).

2) Annealing step is performed by decreasing temperature to 50–65°C for 25–40 seconds; so the primers are annealed to their targets on single stranded DNAs by hydrogen bonds and a polymerase can bind to the primer-template hybrid and begin DNA polymerization in next step.

3) Extension step includes polymerization of the bases to the primers; a thermostable such as Taq polymerase extends a new strand complementary to the DNA template strand by adding matched dNTPs in 5' to 3' direction at a temperature of 72°C. A series of 25-40 repeated cycles of denaturation, annealing of primers and extension is performed to amplify the template fragment (Fig. 3A). Subsequently, a final elongation is sometimes done at 70–74°C for 5–15 minutes after the last PCR cycle to ensure full extension of any remaining single-stranded DNA[2].

Checking the PCR products, electrophoresis (agarose or polyacrylamide gel electrophoresis) is employed for sizing the PCR products by comparison with a DNA ladder (a molecular weight marker). Here, applications of some PCR versions are mentioned.

1) Reverse transcriptase PCR (RT-PCR): In this version, a strand of RNA molecule is transcribed reversely into its complementary DNA (cDNA) using the reverse transcriptase enzyme. This cDNA is then amplified by PCR. RT-PCR is applied to study the mutations at RNA level.

2) Multiplex PCR: In this technique, multiple selected target regions in a sample are amplified simultaneously using different pairs of primers.

3) Nested PCR: It includes two successive PCRs; the product of the first PCR reaction is used as a template for the second PCR. This type of PCR is employed to amplify templates in low copy numbers in specimens. It has the benefits of increased sensitivity and specificity.

4) Amplification refractory mutation system (ARMS) PCR: Allele-specific amplification (AS-PCR) or ARMS-PCR is a general technique for the detection of any point mutation or small deletion[31]. The genotype (normal, heterozygous and homozygous states) of a sample could be determined using two complementary reactions: one containing a specific primer for the amplification of normal DNA sequence at a given locus and the other one containing a mutant-specific primer for amplification of mutant DNA. ARMS-PCR has been used to check the most common mutation in GJB2 gene, 35delG mutation among deaf children.

5) Real time PCR: In this technique, the amplified DNA is detected as the PCR progresses. It is commonly used in gene expression studies and quantification of initial copy number of the target[2].

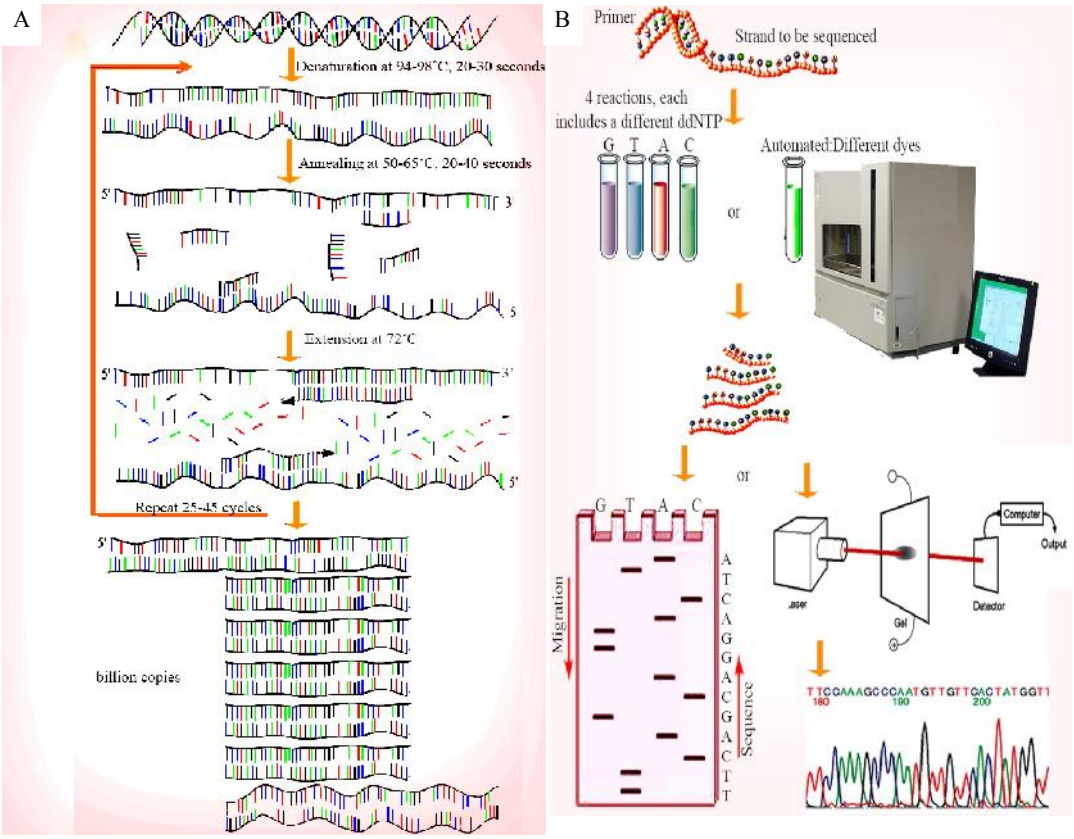

Fig. 3: A) Polymerase chain reaction. B) DNA sequencing

DNA microarray: DNA "chips" or microarrays have been used as a possible testing for multiple mutations. In this technology, single DNA strands including sequences of different targets are fixed to a solid support in an array format. On the other hand, the sample DNA or cDNA labeled with fluorescent dyes is hybridized to the chip (Fig. 4E)[32]. Then using a laser system, the presence of fluorescence is checked; the sequences and their quantities in the sample are determined.

DNA Sequencing: As a powerful technique in molecular genetics, DNA sequencing provides analysis of genes at the nucleotide level. The main aim of DNA sequencing is to determine the sequence of small regions of interest (~1 kilobase) using a PCR product as a template. Dideoxynucleotide sequencing or Sanger sequencing represents the most widely used technique for sequencing DNA[33]. In this method, double stranded DNA is denatured into single stranded DNA with NaOH. A Sanger reaction consists of a single strand DNA, primer, a mixture of a particular ddNTP with normal dNTPs (e.g. ddATP with dATP, dCTP, dGTP, and dTTP). A fluorescent dye molecule is covalently attached to the dideoxynucleotide. ddNTPs cannot form a phosphodiester bond with the next deoxynucleotide so that they terminate DNA chain elongation. This step is done in four separate reactions using a different ddNTP for each reaction (Fig. 3B)[2]. DNA sequencing could be used to check all small known and unknown DNA variations.

***Multiplex ligation-dependent probe amplification* (MLPA):** MLPA is commonly applied to screen deletions and duplications of up to 50 different genomic DNA or RNA sequences. Altogether gene deletions and duplications account up to 10%, and in many disorders up to 30% of disease-causing mutations[34,35]. In this technique, briefly, the probe set is hybridized to genomic DNA in solution. Each probe consists of two halves; one half is composed of a target specific sequence and a universal primer sequence, and other half has other more sequences, a variable length random fragment to

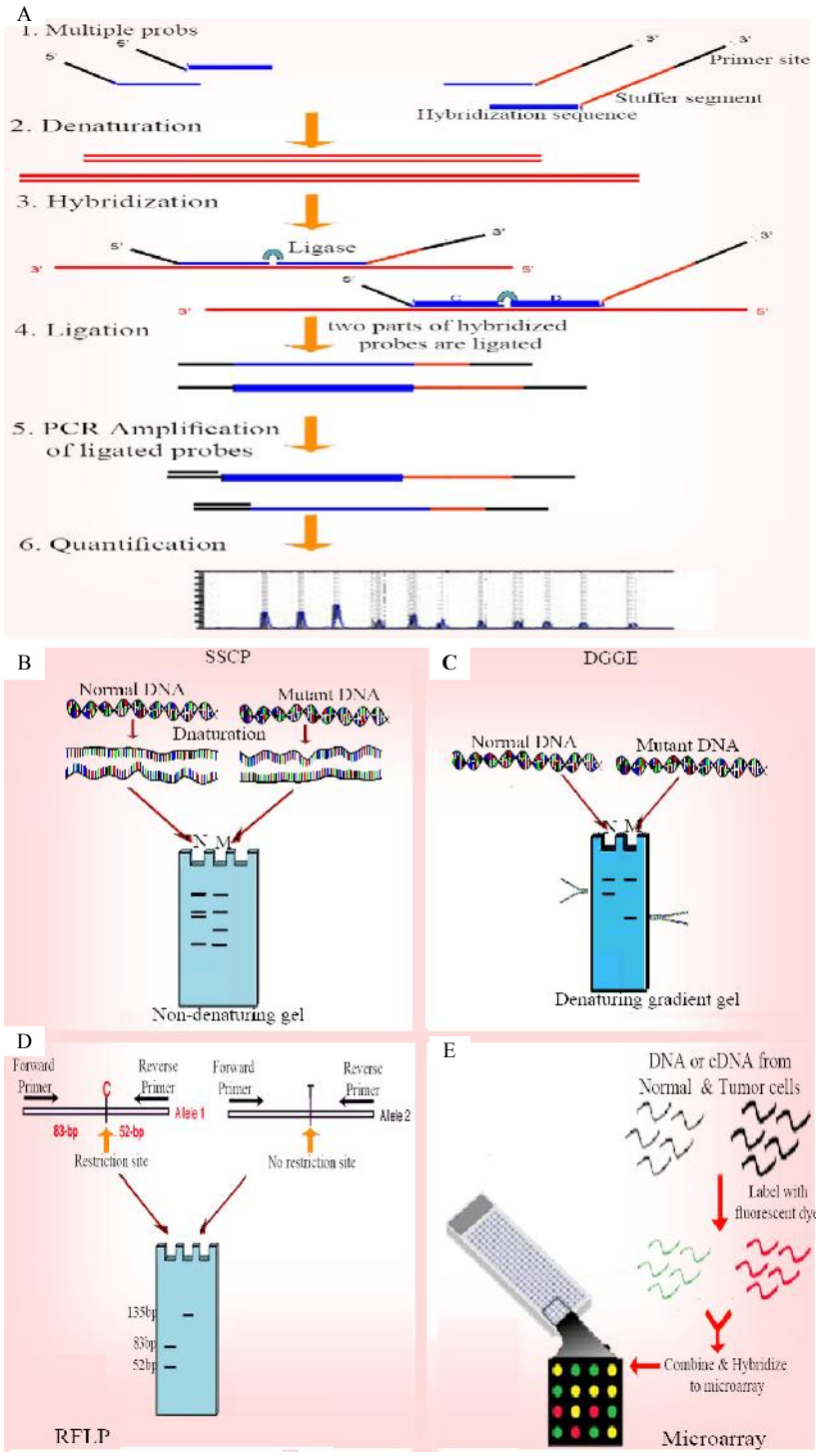

Fig. 4: A) Multiplex ligation-dependent probe amplification (MLPA). B) Single-strand conformation polymorphism (SSCP). C) Denaturing gradient gel electrophoresis (DGGE). D) Restriction fragment length polymorphism (RFLP). E) Microarray.

provide the size differences for electrophoretic resolution. A pair of probes is hybridized on the target region adjacently so that they can then be joined by use of a ligase; the contiguous probe can be amplified by PCR[36]. After PCR amplification, the copy number of target sequence i.e. deletion or duplication of target sequence can be determined and quantified using the relative peak heights (Fig. 4A).

B) Unknown Mutations

Single Strand Conformational Polymorphism (SSCP): SSCP is one of the simplest screening techniques for detecting unknown mutations (microlesions) such as unknown single-base substitutions, small deletions, small insertions, or microinversions. A DNA variation causes alterations in the conformation of denatured DNA fragments during migration within gel electrophoresis. The logic is comparison of the altered migration of denatured wild-type and mutant fragments during gel electrophoresis[37]. In this technique, briefly, DNA fragments are denatured, and renatured under special conditions preventing the formation of double-stranded DNA and allowing conformational structures to form in single-stranded fragments (Fig. 4B). The conformation is unique and resulted from the primary nucleotide sequence. Mobility of these fragments is differed through nondenaturing polyacrylamide gels; detection of variations is based on these conformational structures. PCR is used to amplify the fragments, called PCR-SSCP, because the optimal fragment size can be 150 to 200 bp. About 80–90% of potential point mutations are detected by SSCP[37,38].

Denaturing Gradient Gel Electrophoresis (DGGE): DGGE has been used for screening of unknown point mutations. It is based on differences in the melting behavior of small DNA fragments (200-700 bp); even a single base substitution can cause such a difference. In this technique, DNA is first extracted and subjected to denaturing gradient gel electrophoresis. As the denaturing condition increases, the fragment completely melts to single strands. The rate of mobility in acrylamide gels depends on the physical shape of the fragment (Fig. 4C). Detection of mutated fragments would be possible by comparing the melting behavior of DNA fragments on denaturing gradient gels. Approximately less than 100% of point mutations can be detected using DGGE. Maximum of a nearly 1000 bp fragment can be investigated by this technique[39].

Heteroduplex analysis: A mixture of wild-type and mutant DNA molecules is denatured and renatured to produce heteroduplices. Homo-duplices and heteroduplices show different electrophoretic mobilities through nondenaturing polyacrylamide gels. In this technique, fragment size ranges between 200 and 600 bp. Nearly 80% of point mutations have been estimated to be detected by heteroduplex analysis[40].

Restriction fragment length polymorphism (RFLP): Point mutations can change restriction sites in DNA causing alteration in cleavage by restriction endonucleases which produce fragments with various sizes (Fig. 4D). RFLP is used to detect mutations occurring in restriction sites[41].

Next Generation Sequencing

In recent years, newer technologies for DNA sequencing in a massive scale have been emerged that are referred to as next-generation sequencing (NGS). High speed and throughput, both qualitative and quantitative sequence data are allowed by means of NGS technologies so that genome sequencing projects can be completed in a few days[42,43]. NGS systems provide several sequencing approaches including whole-genome sequencing (WGS), whole exome sequencing (WES), transcriptome sequencing, methylome, etc. The coding sequences compromises about 1% (30Mb) of the genome. More than 95% of the exons are covered by WES; on the other hand, 85% of disease-causing mutations in Mendelian disorders are located in coding regions. Sequencing of the complete coding regions (exome)[44], therefore, could potentially uncover the mutations causing rare, mostly monogenic, genetic disorders as well as predisposing variants in common diseases and cancer.

Conclusion

Any change in DNA sequence could be pathogenic if it has abnormal effect on biologic pathways within the cell. Characterization of the genetic basis of the disease is required for an accurate diagnosis. PCR as a powerful and sensitive technique can amplify very small amounts of DNA. This technique has many applications in various areas of biology and it has been used for diagnosis

of inherited diseases on the DNA level. Examining DNA would demonstrate the changes in the genes that may cause disease. Molecular diagnosis of genetic disorders is noticed as the detection of the pathogenic mutations in DNA and/or RNA samples. It could facilitate fine subclassification, prognosis, and therapy of disorders. Since most hereditary disorders affect people at childhood ages, it is important for pediatricians to be familiar with genetic testing methodology as well as applications of these tests in clinic to get an accurate diagnosis. The clinicians should be able to recognize and categorize genetic disorders and affected patients on the basis of symptoms and signs to a subtype of chromosomal or single gene disorders, so that they could offer an appropriate genetic test for diagnosing the disease. They could also discuss and consult with a medical geneticist.

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