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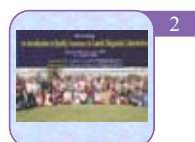
Table of contents:



1

GeNeDit

- Array of genetic tests....
For every genetic disorder



2

GenEvent



3

Clinical Vignette

- Prenatal diagnosis for Freeman Sheldon syndrome:
Dilemmas and decisions?



6

GeNeViSTA

- Nephrotic syndrome:
A genetic perspective



9

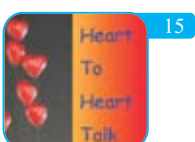
GenEmail



10

GeneFocus

- Ushering in a new era of
molecular diagnostics



15

HearToHearTalk

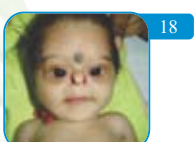
- Looking beyond
the vision of a blind man



16

GeNeXprESS

- Newer advances in prenatal
diagnosis and gene therapy



18

PhotoQuiz 16

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Editorial

Array of Genetic Tests.... For Every Genetic Disorder

The technological advances happening in the twenty-first century are fascinating. Now one can not only look at chromosomes at the molecular level increasing the resolution of chromosomal analysis 1000 times but also sequence each and every nucleotide of the genome. Microarray based cytogenetic analysis and next generation sequencing are the most important technologies mesmerizing the whole world of genetics and genomics. The importance of these techniques has increased multifold as they have found applications in clinical genetics. Chromosomal microarray analysis (CMA) has now become the first step in the evaluation of a child with mental retardation or autism, with or without malformations, replacing the traditional karyotyping-based chromosomal analysis. CMA scans the whole genome in one go and can detect copy number variations as small as 10 kilo bases. This has lead to identification of copy number variations in many new areas in the genome as etiologies of mental retardation (MR) and autism. Most of the patients with mental retardation or autism have subtle dysmorphism and in most cases clinical suspicion of the etiological basis is not possible. For this reason CMA is a handy tool in the evaluation of MR and autism and has a diagnostic yield of 15 to 22%. Of course identification of numerous microdeletion and micro-duplication syndromes will also provide a plethora of genes causing cognitive problems and developmental defects.

Next generation sequencing is another novel technology which has revolutionized genetic testing and research. Now the whole genome or complete coding regions of the genome [exome] can be sequenced and changes identified in the patient to know the genetic basis of the disease in the patient. Such exome sequencing of 2 to four patients with a similar phenotype can identify the causative gene. Every month genes are getting identified for syndromes previously described or new syndromes.

Identification of the causative gene is the first step towards finding the treatment. This has been convincingly proved in a child who had chronic gastrointestinal problems and multiple surgeries. His exome was sequenced and the causative gene was identified. Based on the information hematopoietic stem cell transplantation was done resulting in cure. This proves that exome sequencing can be used for individual cases in clinical settings. Similarly, SNP microarray based homozygosity mapping can be used to identify genes by detecting areas of homozygosity in families with consanguinity. This strategy has been used successfully in mapping many autosomal recessive disorders and has also been shown to be successful in clinical settings. It does not need a visionary to predict that in a decade or less, mysteries of almost all monogenic conditions will be solved.

Next generation sequencing is also being used in providing genetic diagnosis for many phenotypes which can be caused by a number of genes. Deafness, cardiomyopathy, retinitis pigmentosa, Osteogenesis imperfecta and limb girdle myopathy are some such genetically heterogeneous conditions. Molecular diagnosis of these disorders is difficult and very costly as the causative genes are many and each affected individual can have a mutation anywhere in one of these genes. Sequencing one gene after another is costly and time consuming. Now laboratories are using next generation sequencing to sequence multiple genes in one go and this is a successful strategy for providing molecular diagnosis. Molecular diagnosis is not only necessary for confirmation of clinical diagnosis and genetic counseling, but gene based and mutation based therapies are becoming available. Successful gene therapy for retinitis pigmentosa caused by RPE65 gene mutations and efficacy of Ivacaftor in subjects with Cystic Fibrosis who have the G551D CFTR mutation are examples of this.

We are awestruck by these diagnostic advancements and want to convey the excitement of the newer diagnostic techniques to our readers. Dr I C Verma, who has been a witness to these paradigm changes from genetics to genomics, has very succinctly described all the new diagnostic modalities in genetics, their principles and successful applications in his article in this issue. Dr Verma's article is a must read for all clinicians and geneticists of the

twenty-first century. Also, those interested in this field can see the NIH website for exome sequencing <http://www.nih.gov/researchmatters/august2009/08312009exomes.htm>.



Shubha Phadke

1st April, 2012

GenEvent

A quality assurance workshop was organized by the Genetics Division, Department of Pediatrics, AIIMS, New Delhi which was sponsored by the Indian Council of Medical Research (ICMR), The European Molecular Genetics Quality Network (EMQN), Biochemical Genetics EQA (ERNDIM), Cytogenetics European Quality Assessment (CEQA) and Organisation for Economic Co-operation and Development (OECD). This workshop was attended by 90 participants including scientists, technicians and managers from various genetic diagnostic

laboratories from India. This workshop introduced the development and operation of a Quality Management System compliant with international standards. There was a focus on how External Quality Assessment (Laboratory Proficiency Testing) can be used by a laboratory to compare its performance against its peers and improve its practices. It's envisaged that this workshop will help build a foundation for quality assurance programs in India.



QUALITY! QUALITY! QUALITY!

- Quality means doing it right when nobody is looking
- Purpose of accreditation is adding value to the service
- Do not aim for perfection immediately
- Avoid misleading instructions
- Start small: One step at a time
- Quality is a shared responsibility
- Quality is not an extra optional
- Quality should not be an act, it should be a habit
- Everything can be improved

Prenatal diagnosis for Freeman Sheldon syndrome: Dilemmas and decisions?

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Case Report

A boy born to non-consanguineous Muslim parents from Jharkhand, India was evaluated at 3 months of age for multiple congenital deformities. Antenatal history revealed that mother had a skin rash with fever, which subsided on its own at 2 ½ months and rest of the gestation was uneventful till seven months. Polyhydramnios was noted by ultrasound scan. Active fetal movements were felt throughout the gestation. Baby was born at full term through vaginal delivery (vacuum extraction – for large head). Birth weight was 3.25 kg and the baby cried immediately after birth. Deformities were noted in both upper and lower limbs. Eyes were closed at birth and opened next day but remained partly fused. He had feeding problems- fed by spoon (bottle and breast feeding unsuccessful), regurgitation and delay in development. Family history was unremarkable except for one elder brother who gave history of febrile convulsions. On examination he had gross and fine motor delay, blepharophimosis, hypertelorism, anti-mongoloid slant, deep set eyes, prominent supraorbital ridges, epiblepharon with trichiasis, microstomia –puckered mouth and a H shaped groove over chin, broad base of nose, long philtrum, high arched palate, short

neck, ulnar deviation of hand, camptodactyly, bilateral club feet, dislocated hip joints and bilateral undescended testes. (Figure 1a and 1b). The clinical features were suggestive of distal arthrogryposis. The “H” shaped groove over the chin (whistling face) with camptodactyly was the diagnostic feature for Freeman Sheldon syndrome (Distal Arthrogryposis Type 2A). Investigations-TSH, CPK and complete blood counts were normal. He had normal auditory threshold, EEG, ultrasound abdomen and echocardiography. EMG showed axonal neuropathy. Spine x-ray showed mild thoracic scoliosis. MRI brain showed diffuse cerebral atrophy and hypoplastic corpus callosum. Karyotype was reported as 46, XY. A molecular diagnosis was indicated for confirmation of the diagnosis, prognosis of child and genetic counseling. The genetic confirmation for distal arthrogryposis type 2A was done on a research basis from Professor Michael Bamshad’s laboratory, Seattle. A common mutation 2084G-A transition in exon 17 of the MYH3 gene (R672H) resulting in an arginine to histidine substitution at 672 position (R672H) was detected in proband. Both parents were normal for this mutation. Once the results were obtained the couple was called for genetic counseling to explain about how the gene defect caused the medical problems and pattern of inheritance with risk of recurrence. The parents were counseled about the low risk of recurrence as both parents did not carry the mutation and probably it had arisen as a de novo mutation. The couple wanted to know whether it would be possible to know before hand if the child in the next pregnancy would be affected.



Figure 1a shows the proband at three months

Figure 1b: Facial profile at 2 years of age

They were reassured that despite the low risk of recurrence, it would be possible to provide prenatal diagnosis (PND).

The mother who was then 28 years of age subsequently became pregnant and desired for PND. Though the couple never complained about their affected child, they wished to be anxiety free as they had been in and out of the hospital for this child. The mother did not want to see another child suffer, struggle to feed and undergo repeated investigations like her son.

The couple was anxious about the recurrence. Information about the condition was again provided and the couple were given time to decide on the procedure of CVS (Chorionic villus sampling) and risks involved in it following which they consented to the procedure. The actual molecular test, time taken to get the result and method of informing the result were explained. PND was performed at 11 weeks of gestation by CVS. The consent for chromosomal analysis was willingly given as the couple were keen to do all tests to ensure a healthy baby. The possible implications and outcomes were discussed.

The mutation was confirmed in the proband (Figure 2) and a Chorionic villus Sampling (CVS) was done by the Reproductive Medicine department followed

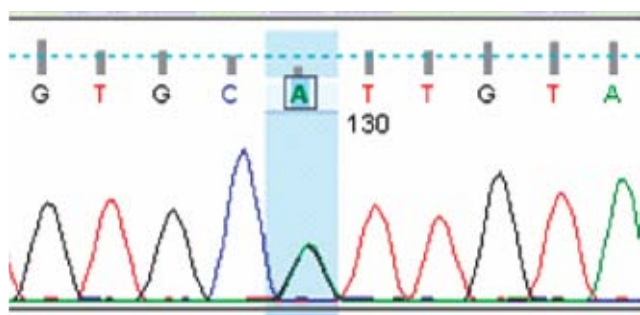


Figure 2. Shows the R672H mutation (2084G-A transition in exon 17 of the MYH3 gene) in the proband

by targeted mutation analysis. This was done along with cytogenetic analysis for chromosomes and maternal contamination by VNTR analysis. The mutation (R672H) and maternal contamination were

ruled out. Long term CVS culture showed a single cell with trisomy 13 of the total 50 cells counted from three primary cultures. The karyotype was reported as 46[49]/47, +13 [1]. A level 1 mosaicism probably from culture artifact was reported.

The parents were counseled about the report. The normal DNA result was reassuring to her. She was informed about the single cell with trisomy 13 and explained the need regarding detailed ultrasound scan and further testing if required. She was reassured of the low risk of the chromosomal anomaly as a culture artifact was likely. Her husband was supportive in all ways and so were her other family members. A morphological scan at week 20 was normal. She decided to continue with the pregnancy and later delivered a baby girl by caesarean section. The baby weighed 3.5 kg and did not have any deformities.

Discussion

Freeman-Sheldon is an autosomal dominant condition. Reduced penetrance has been reported in two kindred.¹ The defect occurs in the MYH3 myosin heavy chain gene. A common mutation 2084G-A transition in exon 17 of the MYH3 gene (R672H) resulting in an arginine to histidine substitution at 672 position (R672H) has been reported in 12/28 patients of Freeman-Sheldon families tested.² This change was predicted to affect ATP binding in the MYH3 gene. This mutation has not yet been reported in the allelic type of distal arthrogryposis 2B (Sheldon-Hall Syndrome).

This family had decided to have prenatal diagnosis soon after their second child was born with multiple congenital problems. The clinical diagnosis was Freeman-Sheldon syndrome with differential diagnosis being Sheldon-Hall, Marden-Walker and Schwartz-Jampel syndrome.³ Since a molecular diagnosis is often required for accurate diagnosis and prenatal diagnosis of genetic syndromes, the mutation analysis was done from a research laboratory since it was not locally available. A known mutation R672H was found which was then



Clinical Vignette



confirmed in the Molecular laboratory of the Clinical Genetics Unit, CMC Vellore. Subsequently PND was carried out in a CVS sample and targeted mutation and chromosomal analysis was performed. The mutation was negative and chromosomal analysis showed presence of a single cell with trisomy 13. During the pretest counselling session the three main areas that were emphasised were information on the condition for which testing is being offered, the characteristics of the tests, implications and possible test results.⁴ The presence of an abnormal cell line is often found in CVS sampling and reported as 1 in 100.⁵ This often challenges the purpose of PND and causes parental anxiety. Here the couple required telephonic counselling followed by appointments to cope up with the uncertainty and planned monitoring of the pregnancy. The repeat normal scan at our hospital reassured the couple to continue the pregnancy and amniocentesis was avoided. The couple took the decision to continue the pregnancy and the outcome was favourable.

Conclusions

This case highlights the fact that prenatal diagnosis may lead to uncertainty in the outcome. The parents may be exposed to unnecessary anxiety and adequate pretest and post-test genetic counseling are the prerequisites for any prenatal diagnosis. This case also highlights that though PND may be an easy choice for many couples who have a previous child with a genetic abnormality, it needs to be accompanied with detailed genetic counselling at

the outset to explain the implications of the test and its possible results. Genetic counselling is a new evolving area in India which practitioners have to face and expert opinion is called upon to deal with such situations. Genetic counselling is often done by clinical geneticists due to lack of trained genetic counsellors. The primary aim of genetic counselling is to help a couple seeking prenatal diagnosis to make an informed decision. Every prenatal diagnosis could be a distressing event and more so if unexpected results come out from it. Therefore genetic counselling is an integral part of prenatal diagnosis in health care and awareness is warranted in common practice.

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Join Us!

The application form for membership of Genetics Specialty Chapter of Indian Academy of Pediatrics can be downloaded from the following link:

http://www.iapindia.org/proforma/IAP_genetics_chapter_application_form.pdf

Nephrotic Syndrome: A Genetic Perspective

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Introduction

Nephrotic syndrome (NS) is a clinical entity resulting from consequences of increased permeability of the glomerular filtration barrier. It is characterized by hypoalbuminemia, dyslipidemia and edema. Although the typical clinical and laboratory features are sufficient enough to establish the diagnosis of nephrotic syndrome, further investigations are necessary to find out the basic patho-physiology in order to determine whether it is familial, whether there is a risk of recurrence in the family and the prognosis and outcome with treatment.

Nephrotic syndrome has two broad categories namely Steroid sensitive nephrotic syndrome (SSNS) and Steroid resistant nephrotic syndrome (SRNS). Ninety percent of cases are SSNS. These and few SRNS respond well to immunosuppressive therapy, probably due to underlying immune mechanisms. Most familial forms of nephrotic syndrome are steroid resistant. Some of the familial forms have syndromic etiology with extra renal manifestations.

Genes responsible for the familial forms of nephrotic syndrome

Genes identified to cause familial forms of nephrotic syndrome code for parts of the glomerular filtration barrier (GFB). The GFB consists of three distinct layers: the porous capillary endothelium, the glomerular basement membrane, and the layer of inter-digitating foot processes with filtration slits in between, known as the slit membrane. Three main structural components of the slit diaphragm are nephrin, podocin and CD-2AP encoded by NPHS1, NPHS2 and CD-2AP respectively (Figure 1).¹ The slit membrane represents a porous proteinaceous

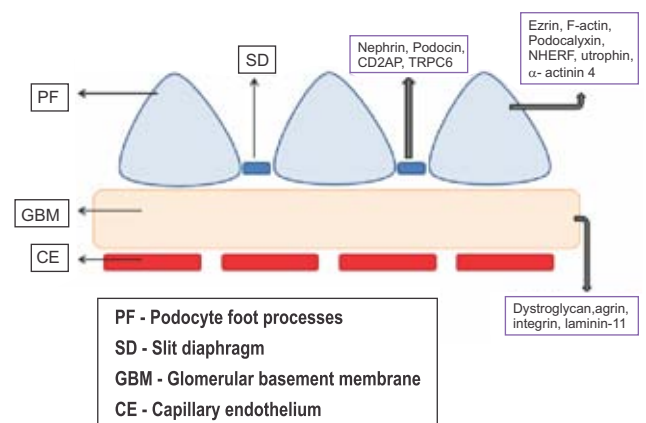


Figure 1: Diagrammatic representation of the glomerular filtration barrier. The main proteins/ molecules involved in the composition of each layer are mentioned.

membrane primarily composed of nephrin. In addition to its role as a structural component, nephrin also acts as a signaling molecule. The slit membrane proteins are joined to the cytoskeleton via various adaptor proteins including podocin, zonula occludens protein-1, CD-2 associated protein and catenins. TRPC6, a calcium channel associated with podocin at the slit membrane, is responsible for cell-matrix interaction. Other genes involved are ACTN4, INF 2, PLCE-1 and PTPRO, which code for Alfa-actinin-4, formin (actin regulating protein), Phospholipase C epsilon and Protein tyrosine phosphatase receptor type O respectively. Integrated functioning of all these genes and some more yet to be discovered genes is responsible for proper functioning of the glomerular filtration barrier and a defect in any of these can result in a non-syndromic form of nephrotic syndrome. Table-I lists some of the non-syndromic forms of nephrotic syndrome.

Syndromic forms of nephrotic syndrome may be



due to mutations in genes coding transcriptional factors (WT1, LMX1B), glomerular basement membrane components (LAMB2, ITGB4), lysosomal (SCARB2) and mitochondrial (COQ2, PDSS2, MTTL1)

proteins or a DNA nucleosome restructuring mediator (SMARCA1). Table-II lists some of the syndromic forms of nephrotic syndrome and their associated phenotypic features.

Table I: Non-syndromic forms of nephrotic syndrome ^{1,2}

Phenotype	Mode of inheritance	Gene	Protein	Function of protein
1) Congenital nephrotic syndrome(CNS) - Finnish type 2) Early -onset SRNS, (in cases carrying at least one mild mutation)	AR	NPHS1	Nephrin	Main component of the slit diaphragm. Anchors the slit diaphragm to the actin cytoskeleton. Modulates signaling events.
1) Congenital nephrotic syndrome 2) Early and late onset SRNS. 3) Juvenile and adult SRNS (in cases bearing the R229Q variant in compound heterozygous state with a pathogenic mutation)	AR	NPHS2	Podocin	Scaffold protein linking plasma membrane to the actin cytoskeleton. Modulates mechano-sensation
Early onset SRNS with DMS and FSGS	AR	PLCE1	Phospho-lipase C epsilon	Involved in cell junction signaling and glomerular development.
Adult-onset SRNS with FSGS	AD	TRPC6	Transient receptor potential cation channel 6	Receptor activated non-selective calcium permeant cation channel. Involved in mechano-sensation.
Childhood onset SRNS with FSGS and MGC	AR	PTPRO	Protein tyrosine phosphatase receptor type O	Tyrosine phosphorylation of tight junction proteins plays a major role in controlling paracellular permeability, cell signaling, and actin cytoskeleton remodeling.

Note: DMS- Diffuse mesangial sclerosis, FSGS- Focal segmental glomerulosclerosis, MGC- Minimum change disease

Table II: Syndromic forms of nephrotic syndrome ³

Syndrome	Mode of inheritance	Gene/ Protein	Function	Phenotype
Nail-patella syndrome	AD	LMX1B/ LIM/ homeobox protein	Podocyte and GBM development and maintenance	Dysplasia of nails, absent or hypoplastic patellae, iliac horns, nephrotic syndrome
Schimke immuno-osseous dysplasia	AR	SMARCA1/ hHARP	ATP dependent annealing helicase that rewinds stably unwound DNA	Spondyloepiphyseal dysplasia(SED), numerous lentigines, slowly progressive immune defect, immune complex nephritis
Denys-Drash Syndrome, WAGR syndrome	AD	Wt1/ Wilms tumor 1	Zinc finger transcription factor that functions both as a tumor suppressor and as a critical regulator of kidney and gonadal development	Wilms tumor, genital abnormality, and renal disease

Clinical approach ⁴

Routine diagnostic work up of nephrotic syndrome includes clinical evaluation including family history, urine dip stick, random urine protein/creatinine ratio, serum creatinine, serum albumin, and serum lipid profile. Additional studies including HIV screening test, hepatitis serology panel, ANA or complement level depend on the patient's history. Renal biopsy is often recommended in persons with nephrotic syndrome to establish the pathological subtype of the disease, to assess disease activity, or to confirm the diagnosis of etiology, such as amyloidosis or systemic lupus erythematosus. There are, however, no clear guidelines on when renal biopsy is indicated or whether it is needed in all persons with nephrotic syndrome.

When to suspect a genetic etiology

Genetic testing is a complex task and needs to be based on clinical information including the family history, age of onset, associated extra-renal manifestations and the type of renal histopathology lesion. Based on the renal histopathology, the different types of NS are minimum change disease(MGC); diffuse mesangial sclerosis (DMS), a particularly severe renal lesion characterized by

mesangial expansion and sclerosis that evolves towards obliteration of capillary lumen and contraction of the glomerular tuft; and focal segmental glomerular sclerosis (FSGS), a lesion characterized by sclerosis and foot process effacements in only some of the glomeruli and only a part of each entire glomerulus.

Identification of the disease causing mutation is important as it may enable the treating physician to avoid (or discontinue) prescribing immunosuppressive therapies for types of NS known to be unresponsive, thereby sparing the patient the significant side effects associated with drugs. Genetic testing has now become more easily accessible than in the past.

Genetic testing and Genetic counseling

Based on observations of the association of gene mutations involved in various structural components of the slit diaphragm and other genes involved in renal development, with the age of onset, renal histology and extra-renal abnormalities, a systematic approach has been described for mutation detection (Table-III).

Table III: Approach for mutation detection in Nephrotic syndrome

Onset	Renal Histology	Gene
Congenital	Radial dilatation of PT	NPHS1
	MGC/FSGS	NPHS2 → NPHS1
	DMS	WT1/PLCE1
Infantile	MGC/FSGS	NPHS2 → NPHS1 → WT1 → PLCE1
	DMS	WT1/PLCE1
Childhood	MGC/FSGS	NPHS2 → NPHS1 → WT1 → PLCE1
	DMS	WT1/PLCE1
Juvenile and adult	FSGS	AR or Sporadic → NPHS2(p.R229Q)
		AD → TRPC6/ACTN4/INF2

Note: If no mutation is detected in the first tested gene, the next gene is tested

Source: Review by Benoit et al.⁵



Genetic counseling should be offered to any patient affected with an inherited disorder and his/ her family. Early genetic consultation for suspicion of inherited disorder based on family history, renal histo-pathology, refractoriness to steroid treatment or extra-renal manifestations, provides opportunity of mutation detection in the proband. The clinician can then discuss specific aspects of the disorder related to the mutation, with the family. Prenatal diagnosis should be offered to families with familial forms of nephrotic syndrome, especially for those known to have an early onset and greater severity.

Most patients with inherited nephrotic syndrome are refractory to steroid treatment and are at high risk for end stage kidney disease, therefore renal transplantation may be the treatment of choice in these cases. Recurrent nephrotic syndrome develops in 30-50% of transplant recipients with FSGS. Risk of recurrence is also mutation specific and is more in patients with NPHS1 mutations than in those with NPHS2 mutations. Renal graft from obligatory

carriers of NPHS2 mutation, such as the parents of an affected individual, should be avoided because of the higher risk of recurrence of FSGS disease post-transplantation.⁵

In summary, genetic disorders account for most of the cases of nephrotic syndrome that start within the first year of life and a considerable proportion of those with childhood-onset nephrotic syndrome. Genetic work up is important for many aspects like counseling, therapy guidance, risk of recurrence after renal transplant and prenatal diagnosis to prevent further recurrence in the family.

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GenEMail



I sincerely congratulate Dr Neerja Gupta and Dr Madhulika Kabra for highlighting a very pertinent issue in fetal care in India. The option of possible late terminations would actually save more fetuses than kill them. At present, many terminations are done in haste to stick to the legal deadline of 20 weeks especially in cases with unsure prognosis like borderline abnormalities of vital organs (e.g. mild ventriculomegaly). Indeed the 20 week cut off affects clinical decision making for terminations and invasive procedures which are not always evidence-

driven. It is heartening to see experts from an apex medical institute raise this very valid point in this forum. I hope the law makers see the wisdom in this suggestion. Happy New Year to the Newsletter team.

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Suggested reading

Late termination of pregnancy for fetal abnormalities: the perspective of Indian lay persons and medical practitioners. Phadke SR, Agarwal M, Aggarwal S. Prenat Diagn 2011; 31: 1286-91.

Ushering In a New Era of Molecular Diagnostics

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It has become necessary for practicing clinicians to be acquainted with advances in genetics. Firstly, with the lowering of the infant mortality rate in India resulting from control of infectious and nutritional disorders, pediatricians encounter more and more children with genetic disorders. Secondly, treatment is now available for many genetic disorders. Not diagnosing a treatable disorder is a misfortune for the patient, and in this day of consumer courts can end in misfortune for the clinician too. Thirdly, the recent advances have made possible precise genetic counseling and prenatal diagnosis. Therefore, it is imperative that genetic disorders be included as differential diagnosis in the evaluation of all children, and they should no longer be a diagnosis of exclusion.

The recent advances in genetic technologies were fuelled by the human genome project that involved determining the entire human sequence. The use of these technologies led to the discovery of new genes at an unprecedented rate. Researching the whole genome resulted in improved understanding of the pathways of disease causation, recognition of genes predisposing to common genetic disorders, identification of markers to monitor the progress of disease and the discovery of new drug targets. What is remarkable is that the time for the application of these discoveries from the bench to the bedside has become progressively shorter. This article will describe briefly the recent advances that have taken place in genetics and show their application in clinical practice. The addition of microarrays for detecting small copy number variations in chromosomes has been a spectacular advance for evaluating dysmorphic children as well as those with autism. Microarrays have also proved useful for studying gene expressions in cancer and other

disorders. SNP microarrays have been helpful to identify areas of homozygosity in the genome and led to discovery of many new genes in consanguineous families. Whole genome sequencing has become possible, although the cost is rather high, and the analysis of the data obtained is a real challenge. Exome sequencing of the coding regions of the genome has been successfully used to identify the mutant gene in many children with obscure disorders. Next generation sequencing has been found to have clinical utility in finding the specific gene carrying the mutation in disorders where a similar phenotype results from many genes e.g. in life threatening cardiac arrhythmias, familial cardiomyopathies, limb girdle muscle dystrophies etc.

Advances in Clinical Examination of Dysmorphic Children:

In spite of all the technological advances clinical examination has remained paramount to determine the phenotype, because this is crucially important in order to delineate the function of genes. For evaluation of children with malformations defining and recognizing various dysmorphic features has always been tricky and difficult. Human structural malformations (anomalies or birth defects) have an enormous and complex range of manifestations and severity. The description of these findings can be challenging because the variation of many of the features is continuous and only some of them can be objectively assessed by measurement. An international group of clinicians working in the field of dysmorphology have standardized the terms used to describe human morphology, and have reached consensus regarding their definitions. This will increase the utility of descriptions of the human phenotype and facilitate reliable comparisons of



findings among patients. Discussions with other workers in dysmorphology and related fields, such as developmental biology and molecular genetics, will become more precise. These definitions along with illustrations have been set forth in a series of articles that have been published in the American Journal of Medical Genetics and these are freely accessible, and should be consulted to clarify the precise definitions. Areas covered are ears, hands and feet, head and face, nose and philtrum, and peri-orbital region.^{1,2}

It is now apparent that advancing technologies like next generation sequencing threaten to marginalize clinical skills of dysmorphologists. This is certainly not a new phenomenon. Earlier physicians would perform a detailed and sophisticated examination to localize a neurologic disorder, or the valves and chambers involved in a congenital heart defect. Now, these skills have been devalued in the era of MRIs and echocardiograms. Surprisingly however, it has been observed that these new diagnostic technologies in fact improved diagnostic capabilities of the physicians and did not make them obsolete. It will be the same for the clinical geneticist. Referrals for the evaluation of a child with developmental delay, or with a 'funny look' are likely to decrease as microarrays and tandem mass spectroscopy become commonplace. The general pediatrician will have ordered these tests and detected the abnormality. However, the clinical geneticist will still have an important role, to assist in the interpretation of the abnormal results, correlate the abnormality with the patient's phenotype, and explain the clinical significance to the patient and the family. Indeed, clinical acumen and skills in dysmorphology will help save money by reducing the number of patients for whom advanced tests are ordered.³

Microarray technology

One of the greatest advances in recent times has been the development of microarrays. The word "array" simply means to "place in an orderly

arrangement". The underlying principle is the same for all microarrays, no matter how they are made. Each array has thousands of different DNA probe sequences arranged in a defined matrix on a glass or silicon support. An unknown sample is then hybridized to these immobilized DNA molecules. The main advantages of this technology are its sensitivity, specificity and scale as it enables data for thousands of relevant genomic regions of interest to be generated rapidly in a single experiment. Lastly, but important for precious clinical samples, the amount of input sample material required is generally low, usually 0.1 mg.

DNA microarrays or 'chips' are currently applied to a wide range of applications in medicine. Originally developed for gene expression profiling, they are now commonly used to unmask copy number changes (array-based CGH), for single-nucleotide polymorphism (SNP) genotyping, DNA methylation, alternative splicing, miRNAs and protein-DNA interactions. Expression profiling has been extremely useful in classifying various leukemias and cancers, and based on these a prediction can be made regarding therapies that would be useful. Comparative genome hybridization (CGH) is a new and powerful high-resolution molecular cytogenetic approach that can detect imbalances of chromosomes (microduplications or microdeletions) in much finer detail than achievable by conventional karyotyping. This can now be performed in a single test. With the use of CGH the yield of abnormal results in cases of dysmorphic children has improved by about 8-17%.⁴ Gijsbers et al reported abnormalities in 22.6 % of 318 cases.⁵ Hayashi et al recruited 536 patients with clinically uncharacterized multiple congenital anomalies/mental retardation (MCA/MR), whose karyotypes were normal according to conventional cytogenetics.⁶ They observed abnormalities in 23.9 % of cases using a genome-wide high-density array at intervals of approximately 0.7Mb. There is a general consensus that in dysmorphic children and those with autism the first line diagnostic test should be a

cytogenetic microarray analysis. Microarray-based clinical tests also enable the simultaneous detection of multiple genotypes in various disorders that have genetic heterogeneity. For example, a gene chip has been developed to sequence 60 genes related to deafness, and this may in future replace sequencing GJB2 gene as a first-line test in a child with sensorineural deafness. Microarrays are expected to become a significant part of clinical diagnostic testing in the future, and hold promise in improving disease diagnosis, risk stratification, and selection and optimization of drug regimens.

Advances in Sequencing of DNA

In the early years finding new genes and the mutations therein depended upon sequencing of the DNA fragments. The sequencing technology developed by Fredrick Sanger proved most useful in these discoveries. It made use of chain terminating dideoxynucleotides, which are prepared by removing a hydroxyl group from the usual bases that comprise a DNA strand. Once a dideoxynucleotide is incorporated in the synthesis of DNA this prevents the addition of further nucleotides. In practice, labeled primers are added to single stranded DNA whose sequence is to be determined. DNA polymerase adds free bases to the single strand, using complementary base pairing. The process results in fragments of varying length which can be separated by electrophoresis. The process has been automated by using fluorochrome labeled primers and capillary electrophoresis. This has been termed as the first generation sequencing technology. However, it remains costly and time-consuming. For example, using Sanger technology it took 13 years and an estimated cost of \$ 2.7 billion to sequence the first human genome. Next generation sequencing dramatically reduced the cost and the time taken so that the human genome was sequenced in 2008 over a 5-month period for approximately \$ 1.5 million. The technology has advanced even further, and currently four platforms are available for next generation sequencing, each using a slightly different design. In most

applications of NGS, each region is sequenced many times, the reads are aligned against the reference genome, and variations observed. Analysis of the large amount of data generated by NGS is a major challenge. It is now possible to sequence the entire human genome at a cost of US \$ 7,500 or so. The lofty aim of the scientific community is to sequence the human genome for US \$ 1000.⁷ Next generation sequencing has been used in clinical and research settings in a number of ways – whole genome and exome sequencing.

Whole-genome sequencing (WGS) has been applied for discovering the interaction of various genes in cancer, or other complex disorders likes diabetes mellitus, coronary artery disease, inflammatory bowel disease etc. It is the key technology being used in the International Cancer Genome Project, which aims at determining whole genomes of the commonest cancers around the world. This will yield crucial information about how cancer is caused, how it progresses and pathways to be targeted to cure it. Indian scientists have joined the International project and are undertaking sequencing of patients with oral cancer. Whole genome sequencing has also revolutionized medical diagnostics through rapid identification of alleles that cause disease.⁸ For example, Lupski, a famous molecular scientist and his four siblings were affected with an autosomal recessive form of Charcot Marie Tooth (CMT) disease. The family had previously screened negative for alterations of some common Charcot-Marie-Tooth genes, including PMP22, MPZ, PRX, GDAP1, and EGR2. He and his colleagues carried out whole genome sequencing of the family members, identified all potential functional variants in genes likely to be related to the disease, and genotyped these variants in the affected family members. The group identified and validated the two causative alleles in SH3TC2 gene.⁹ Others have used this technology for identification of a gene defect responsible for severe hypercholesterolemia, a novel TP53 cancer susceptibility mutation of a patient with therapy-



related AML, a variant in DHDDS in retinitis pigmentosa, etc.^{10,11,12} Currently, whole genome sequencing has great promise in clinical practice, but it has to overcome several barriers (cost, availability, limited experience) before it is ready for widespread use.

Whole Exome sequencing (WES) targets the subset of the human genome that is protein coding. It is a powerful and cost-effective new tool for dissecting the genetic basis of diseases and traits that have proved to be intractable to conventional gene-discovery strategies. Over the past 2 years, experimental and analytical approaches relating to exome sequencing have established a rich framework for discovering the genes underlying unsolved Mendelian disorders. The cost is now considerably reduced and it is possible to carry out exome sequencing for US \$ 3000. Additionally, exome sequencing is being adapted to explore the extent to which rare alleles explain the heritability of complex diseases like diabetes mellitus. Exome sequencing is beginning to be used to facilitate clinical diagnosis and personalized disease-risk profiling.^{7,13} For example, WES has identified a new genetic etiology for familial hypobetalipoproteinemia. WES has also been used to identify the breakpoints in balanced chromosome translocations and inversions. This is of great practical value in clinical cases as it permits the identification of a gene or genes connected with the phenotype in cases with de-novo chromosomal rearrangements.

NGS is now used to identify the molecular defect when multiple genes cause a similar phenotype. For example, in X-Linked intellectual disability a Next-Generation Sequencing for panel of 92 genes is available. Indeed the whole exome of the X chromosome has been sequenced leading to the discovery of many new genes causing X-linked mental retardation. A NGS test for 46 genes causing hypertrophic cardiomyopathy has been developed. Many commercial laboratories have introduced tests

for panel of genes causing long QT interval, microcephaly, Seckel syndrome, Meckel Gruber syndrome etc. Recently, NGS was employed in the care of a child with a severe Crohn's disease-like illness, in which other testing had not been able to establish a diagnosis. WES was able to identify a mutation in XIAP. This case was important because the diagnosis led to the choice of an effective treatment, hemopoietic stem cell transplant. In another case, WES resulted in an important treatment decision. An infant with acute liver failure was found to have a recessive disorder due to mutations in C10orf2 (TWINKLE) resulting in mitochondrial DNA depletion. With this definitive diagnosis, the parents could be confidently counseled that the infant would not be an appropriate candidate for liver transplantation. Ng et al identified MLL gene as the cause of Kabuki syndrome.¹⁴ Using exome sequencing in a child originally suspected to have a diagnosis of Bartter syndrome, a novel homozygous missense (Asp652Asn) variant was discovered in the SLC26A3 gene (cause of congenital chloride diarrhea). These examples provide proof-of-concept that exome sequencing can be used as a clinical tool for evaluating patients with an undiagnosed genetic disorder.^{7,13}

Recently, next-generation sequencing has been successfully applied for diagnosing Down syndrome from cell free fetal DNA isolated from maternal plasma from pregnant women. This will reduce markedly the invasive procedures that are currently employed for definitive diagnosis of Down syndrome in the fetus. However the test is still considered an advanced screening test, and confirmation by an invasive test is recommended before decisions are made regarding termination of pregnancy.

Homozygosity mapping

Consanguinity (union between related individuals) is common in many communities in India. The degree of inbreeding is computed as a percentage of

chances for two alleles to be identical by descent. This percentage is called "Coefficient of inbreeding". It varies from 6.25 % in offspring of first cousin marriages to 12.5 % in offspring of uncle-niece or aunt-nephew marriages. It is well established that consanguineous marriages increase the risk of autosomal recessive disorders in the offspring. The same phenomenon increases the probability of disease-causing mutations to reside in blocks of homozygosity. This has made it possible for researchers to identify disease-causing mutations by pursuing genome wide search for blocks of homozygosity.¹⁵ This is becoming increasingly easier and cheaper to perform—thanks to the advent of single nucleotide polymorphism (SNP) chip-based genotyping platforms. Of course in affected patients many areas of homozygosity are observed. One has to filter out the homozygous regions leaving a few genes for downstream mutation analysis. This has led to the identification of many new genes in consanguineous Pakistani families in UK, in the Amish in USA and other consanguineous communities around the world.

Genome wide association studies

Genome-wide association studies are a relatively new way for scientists to identify genes involved in human disease. This method searches the genome for small variations, called single nucleotide polymorphisms or SNPs (pronounced "snips"), that occur more frequently in people with a particular disease than in people without the disease. Each study looks at hundreds or thousands of SNPs at the same time. Researchers use data from this type of study to pinpoint genes that may contribute to a person's risk of developing a certain disease. Because genome-wide association studies examine SNPs across the genome, they represent a promising way to study complex, common diseases in which many genetic variations contribute to a person's risk. This approach has already identified SNPs related to several disorders such as diabetes, coronary artery disease, inflammatory bowel disease etc. Researchers hope that future genome-wide

association studies will identify more SNPs associated with chronic diseases, as well as variations that affect a person's response to certain drugs and influence interactions between a person's genes and the environment.

Conclusion

With the ability to study any and each nucleotide of the genome, diagnostics has taken a big leap in the twenty first century. All these advances are now becoming available to clinicians and it is hoped that soon it would be possible to determine the exact molecular basis for every genetic disorder. The causative genes for all monogenic conditions will soon be identified, lending help not only in diagnostics but also in better understanding of the pathophysiology and molecular biology of these disorders. New treatment strategies are coming up and rapid advancements in the field of medical genetics are expected in this decade. Clinicians need to keep themselves updated about these new ongoing developments.

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Looking beyond..... the vision of a blind man

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Big things come in small packages. And the most wonderful surprises spring up where we least expect. As doctors we usually have patronizing attitudes towards our patients. We are always trying to help them, trying to communicate so much of medical jargon, all in good faith and all the time thinking we know what's best for them. But we all have moments when we are humbled by some of our patients. I had the fortune of being the genetic resident in charge of one such very special family. This family consisted of a Class IV government employee, his two sons and his wife. His courteous and smiling demeanor was very endearing. He had been blind since the age of 18 years, and in the absence of medical services, had no clue about the cause of his visual handicap. He had been happily going on with his life, taking his handicap in his stride, till his elder son was born. The child had congenital nystagmus which made the parents suspect that something was wrong with his eyes. After many inconclusive consultations with local doctors, the child was taken to an ophthalmologist where a diagnosis of aniridia was made. The younger brother and the father were both also diagnosed to be suffering from the same condition. The father had become blind due to superimposed complications like glaucoma, corneal opacification and cataract. This child, who was in his teens at that time, luckily had no other associated problems and his vision was correctable with glasses. His younger brother however was not so fortunate and had associated problems in the form of corneal opacification and subsequently developed blindness. This family had come to our department in 2002 with the diagnoses of aniridia for genetic consultation. They were told about the genetic nature of this condition and were informed about the 50% risk of transmission to the progeny of the two boys. They were also informed that a genetic test may be possible from outside India and this can help in providing prenatal diagnosis. After this first consultation, they returned after a gap of 7 years when the elder son was married. It is to their credit that they had remembered all the scientific information correctly and understood the need for timely genetic consultation. They requested us to arrange for genetic testing of the elder

son. Their earnest request made us search for laboratories across the globe which was doing the PAX6 gene testing and finally the test was done in a laboratory in Thailand. The family made no pretensions of being poor or helpless, and without much ado arranged for the amount needed. They followed up sincerely and luckily a mutation was identified in all the affected members of the family. Next, they came when the elder son's wife was 8 weeks pregnant. We told them that prenatal diagnosis would be possible. Then we worked to devise a PCR based test to detect the point mutation which was found in that family. After hard work over 2-3 weeks, we were able to devise a simple RFLP-PCR based test for the particular mutation. But surprisingly, there was no communication from the family. We waited a little more, finally curiosity got the better of us and we called them up. The father sounded very apologetic and said that he would come to meet us. So, next morning he turned up with his wife in tow. His hands were folded as he said "We are sorry for the inconvenience caused to all of you. We talked to our son and daughter-in-law and the daughter-in-law says she's not interested in getting the test done. We've tried talking to both of them but we can't force our decision on them." We were surprised at the stand taken by this man. In a country where the falling gender ratio and endless atrocities on women across all socio-economic strata bear testimony to the disadvantage that being a woman is, the respect given to the newly wedded daughter-in-law in this poor family was indeed amazing. This family with a disease associated with such a debilitating handicap as blindness had such a positive attitude that they were ready to take the 50% risk of similar illness in the unborn child. The daughter-in-law had the courage to say no, the parent-in-laws had the wisdom to let their children make their own decisions. The family was such an inspiring example of the happy Indian family we all like to boast about. They let love and respect rule their lives. And that truly was the secret of their happiness, their togetherness against all odds. Their resilience and willingness to take on all challenges that life threw at them was indeed very moving. It has continued to inspire me in my difficult moments till date.

Newer advances in prenatal diagnosis and gene therapy

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Cell free fetal DNA for prenatal diagnosis¹

A definitive prenatal testing for Down syndrome is done by invasive tests to obtain fetal samples. These invasive tests have at least some risk of fetal loss. Development of an accurate, safe, rapid and non-invasive test for prenatal diagnosis is an area of interesting and active investigation. Fetal genetic material can be found in maternal circulation. Intact fetal cells are not a reliable source of genetic material, as they are very few and may persist for years after previous pregnancies. But fetal cell free DNA and RNA are unique to the present pregnancy and offer an alternative. Primary source of fetal cell free DNA in maternal circulation is by apoptosis of syncytiotrophoblast. In 1997 it was discovered that 3-6% of cell free DNA in maternal plasma was of fetal origin. That formed the basis of studies by shotgun sequencing. Palomki et al enrolled a total of 4,664 antenatal women at high risk for Down syndrome (34% in the first trimester) from 27 prenatal diagnostic centers in their study.¹ Samples were drawn immediately before invasive testing. Circulating fetal cell free DNA fragments were isolated from maternal plasma and quantified to determine fetal fraction. Massively parallel shotgun sequencing was used. This technique sequences the first 36 bases of millions of DNA fragments to determine their specific chromosomal origin. If the fetus has a third chromosome 21, the percentage of chromosome 21 fragments is higher. A positive detection rate of 98.6% and false positive rate of 0.25% was documented. Among high risk pregnancies, sequencing of circulating cell free DNA detected nearly all cases of Down syndrome. Single molecule sequencing appears to be an attractive, easy to use and reliable method. This can potentially

reduce invasive diagnostic procedures and related fetal losses. It may replace the so far gold standard of amniocentesis and chorionic villus sampling for fetal karyotyping.

End of a long winding road, gene therapy for hemophilia B²

Drawbacks of classical therapy for genetic disorders have fueled the interest in alternative treatments, especially gene therapy. In vivo gene replacement therapy for the treatment of inherited disease is one of the most compelling concepts in modern medicine. AAV-adenovirus associated virus vectors have been extensively used and shown therapeutic efficacy in a range of animal models. Successful translation to clinical use has been slow but long term expression of donated gene at therapeutic levels has now been achieved in patients of hemophilia. Gene therapy is ideal for hemophilia, as it is a monogenic disorder and a relatively modest increase in clotting factor can result in significant therapeutic benefit. Recently Nathwani et al successfully treated six patients with Hemophilia B. All six patients included in the study had Factor 9 activity < 1%. Single dose of serotype -8-pseudotyped, self complementary adenovirus associated virus vector (AAV) expressing a codon optimized human FIX transgene was injected in a peripheral vein. Six participants were divided in 3 groups. Each group received low, intermediate and high dose of vector, without immunosuppression. All were followed for 6-16 months. AAV mediated expression of F9 increased to 2-11% of normal levels. Four out of six discontinued Factor 9 prophylaxis and remained free of spontaneous hemorrhage. Two patients who received high dose of vector had transient, asymptomatic elevation of serum aminotransferase



and increase in liver enzymes. Both of them responded to a short course of glucocorticoids and maintained FIX levels of 3-11%. Resultant gene expression of FIX, persistent at sufficient levels gave a significant therapeutic benefit. Early failures had dismissed gene therapy as over hyped, but this clinical success has bolstered new optimism. So from Royal genes to gene therapy, Hemophilia has come a long way!

Prenatal diagnosis: Is aCGH ideal?³

Prenatal diagnosis demands fast, precise and cost effective technology. So far gold standard has been microscopic karyotype analysis of cultured cells, which accurately detects abnormalities larger than 5Mb. Need for rapid testing method which does not require culture led to the development of FISH –fluorescent in situ hybridization and QF-PCR—Quantitative fluorescent polymerase chain reaction. However both FISH and QF-PCR have the disadvantage that they are difficult to scale to a comprehensive genome wide screen. Array CGH (comparative genomic hybridization) is a comprehensive genome wide screening strategy for detecting copy number imbalances. Array CGH can be rapid, less labor intensive than karyotyping banding analysis and highly amenable to automation. Fiorentino et al did a prospective study to assess feasibility of offering array based comparative genomic hybridization test for prenatal diagnosis as a first line test. A total of 1037 prenatal samples were processed, using aCGH and G-Banding for standard karyotyping. Specimens included 89% amniotic fluid, 9.5% chorionic villus samples and 1.5% cultured amniocytes. Chromosomal abnormality was found in 34 samples. In 9 out of 34 aCGH detected pathogenic copy number variations, not found in standard karyotype. In the rest of the samples complete concordance was seen. Advantages of aCGH are high throughput analysis, minimal amount of DNA requirement, rapid turnaround time and avoidance of culturing fetal cells. Array CGH has the potential to deliver a higher resolution result as compared with G-banded

chromosome analysis. It enables detailed characterization of submicroscopic copy number variants (CNVs). Rearrangement of CNVs represents an increasingly recognized cause of genetic disorders. Limitations of aCGH could be inability to isolate sufficient quantities of fetal DNA from amniotic fluid samples, inability to detect low levels of mosaicism, and an increase in results of unclear relevance that may cause difficulties in case counseling and management. Array CGH is an improved diagnostic tool. However feasibility of introducing aCGH as a first line diagnostic test in routine prenatal diagnosis needs to be evaluated further.

Treatment for cystic fibrosis^{4,5}

A randomized, double-blind, placebo-controlled trial to evaluate ivacaftor (VX-770), a CFTR potentiator, in subjects 12 years of age or older with cystic fibrosis and at least one G551D-CFTR mutation was conducted by Ramsey et al. The main parameter measured was the estimated mean change (in percentage) in the forced expiratory volume (FEV) from baseline to 24 weeks after starting the drug. The drug was tested in 84 subjects and 83 received a placebo. Ivacaftor- administered patients showed improvements in lung functions. Substantial improvements were also observed in the risk of pulmonary exacerbations, patient-reported respiratory symptoms, weight, and concentration of sweat chloride. However Flume et al did not find encouraging results with this drug in patients who are homozygous for the F508del-CFTR Mutation.

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16

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This 3 months old male child was referred for evaluation of failure to thrive, recurrent loose stools and steatorrhoea, and dysmorphic facies. Identify the condition.



Answer to the PhotoQuiz 15 of the previous issue

Osteogenesis imperfecta type 2 (OMIM 166210)

Osteogenesis imperfecta type 2 is a lethal skeletal dysplasia caused by mutations in COL1A1 and COL1A2 genes. The condition is characterized by short and bent long bones, fractures, narrow thorax and blue sclera. Most of them die in utero and immediately after birth. The condition has to be differentiated from other lethal skeletal dysplasias. The condition is inherited in autosomal dominant form. As the parents are usually normal, the risk of recurrence is very low. In view of the gonadal mosaicism, the risk is estimated to be around 6%. Prenatal diagnosis can be attempted by antenatal ultrasonography. However, a definitive diagnosis would require molecular testing.



Correct response to PhotoQuiz No. 15 was given by

Ravi Goyal, Kota



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