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Second Annual Conference of Society for Indian Academy of Medical Genetics

11th - 13th December, 2015

Venue: AIIMS Auditorium, JODHPUR (Rajasthan)

The theme of IAMG 2015 will be,

"New Insights into Therapies for Genetic Disorders"

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Gene Darning for Gene Therapy

Editorial

With the invention of recombinant technology and the ability to cut/ paste genes, the immediate goal was to correct genetic defects at the gene level. In the early 1990s, we started showing hope of gene therapy to families with thalassemia major. Beta globin gene is one of the smallest genes and was considered easy to insert in the genome; but studies showed that it requires well controlled, sustained and a large amount of expression to cure thalassemia major. The hope of gene therapy thus began to appear to be a distant dream. The first real success for gene therapy has been for severe combined immunodeficiency (SCID) in the early 21st century. Till 2012, gene therapy had been attempted for 20 cases of X-SCID and 40 cases of SCID due to adenosine deaminase deficiency (ADA). However 5 of 20 cases with X-SCID developed one type of leukemia, confirming the risk of malignancy due to gene therapy to be true. The malignancy occurred due to insertion of the normal gene into an oncogene disrupting its function, due to the fact that the correct gene was not targeted to a specific location but was supposed to get randomly integrated. This complication of cancer was not reported in mouse models.

Insertion of a large gene, along with controlling regions and continued expression continues to be a challenge. The search for the perfect vector which can carry a large gene and can deliver it to all cells of the required type without causing any adverse effects has still not been successful. The death of a young man, Jesse Gelsinger due to a severe immune reaction to the adenovirus used for a gene therapy trial for ornithine transcarbamylase deficiency was a setback and the norms of Ethics Committees with regards to gene therapy trials became very stringent following this unfortunate episode. Insertion of the beta globin gene leading to stoppage of blood transfusion requirement in a poorly managed, splenectomised thalassemia major patient was first reported in 2010.

The last few years have seen newer developments which can facilitate and speed up the gene therapy trials. These include novel gene editing tools like CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats – Cas9), development of an unlimited number of induced pleuripotent cells (iPCs) from patients' fibroblasts and the ability to transform iPCs into various lineage of cells. CRISPR-Cas9 technology uses a segment of RNA which helps to make a cut at a required site and efficiently replace the defective / mutated part of the gene. This method of homologous recombination is efficient and can replace the defective gene at its normal location and will maintain its relation with gene expression controlling regions of the gene like enhancers and suppressors. This system has been successfully used in a number of gene therapy experiments in cell lines. One such experiment on the 'Seamless gene correction of b-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac' was reported in 2014. This is a really exciting development and such targeted correction reminds me of darning. The continuity of the newly added correct sequence is established with sequences before and after. CRISPR has brought new excitement and hope to scientists working on gene therapy as well as to clinicians waiting for a gene therapy option for patients with monogenic disorders. In this issue, the details of CRISPR technology are given in GeNeViSTA and GeNeXpeSS highlights the recent CRISPR-based publications. Though identification of genetic defects by prenatal genome sequencing and correction by gene therapy still looks a distant dream, CRISPR definitely appears a big step towards gene therapy in clinical practice.

Genetic Clinics aims to bring these latest developments in the field of medical genetics closer to the clinicians. Application of recently available genetic technologies in clinical situations has resulted in a paradigm shift in the care of patients and families with genetic disorders. Learning genetics in this era of molecular medicine sure is exciting and enjoyable!

Dr. Shubha R Phadke
1st July, 2015

Ring Chromosomes 13 and 14 presenting as intractable seizures- report of two cases with unusual features and review of literature

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Abstract

Ring chromosomes (RC) result from terminal deletion of chromosome arms, followed by fusion of the broken ends leading to the loss of genetic material. The phenotype is determined by the chromosome involved and the extent of deletion. Ring chromosome (RC) is a rare cytogenetic abnormality which should be considered in children with intractable seizures and developmental delay. Peripheral blood karyotype can easily identify such cases. We present two cases where chromosome analysis was performed; a girl presenting with intrauterine growth retardation, microcephaly, intellectual disability and early onset difficult to control seizures and a boy with uncontrolled seizures, dysmorphism and craniosynostosis. Chromosome analysis revealed RC13 in the girl and RC14 in the boy. Refractoriness of seizures in RC13 and craniosynostosis in RC14 that are reported here have so far not been described in literature. This report suggests that RC13, invariably leading to group 3 category of deletion 13q syndrome, may be more severe than previously indicated and the case with RC14 expands the phenotype of RC14. Uncontrolled seizures in a child with developmental delay with or without dsymorphism should warrant a chromosomal analysis.

Introduction

Ring chromosomes (RC) usually result from two terminal breaks in both chromosome arms, followed by fusion of the broken ends leading to the loss of genetic material.[1] The most important factor affecting the phenotype of patients with RC is chromosome type and the extent of the deletion of the genomic segments containing crucial genes for a normal development.[2] The large spectrum of clinical phenotypes of RC 13 and 14 is similar to that of large deletions involving the q arms of these acrocentric chromosomes and has been described under "ring 13/14 chromosome syndrome". Though uncommon, these are now considered as well-recognizable chromosomal abnormalities due to a pattern of common dysmorphic features and malformations. Techniques like array comparative genomic hybridization (array-CGH), fluorescence in situ hybridization (FISH) and MLPA (Multiplex Ligation-Dependent Probe Amplification) have allowed improved molecular genotype-phenotype correlations by means of accurate delineations of the deleted regions and precise molecular karyotyping.

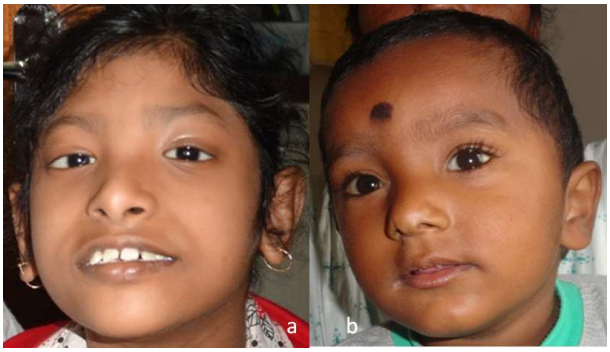
Described by Lejune *et al.* in 1968, RC 13 is relatively uncommon; with an estimated incidence of 1/58,000 live births.[3] Occurrence of RC 14 syndrome also is relatively rare with over 70 cases reported so far.[4] We present here two cases: one with unusual presentation of seizures in a girl with RC 13 and another with additional finding of craniosynostosis in a boy with ring chromosome 14, so far not described in literature.

Case reports:

- Case 1: A 10 year old girl presented with history of seizures and developmental delay from

early neonatal period. Oligohydramnios and intrauterine growth retardation were detected in the antenatal period. She was the first offspring of non-consanguineous healthy parents. She was born at term gestation with a birth weight of 2 kgs and cried around 10 minutes after delivery, following stimulation. She was on valproate therapy intermittently from day one of her life and had uncontrolled seizures. The parents had noticed significant global developmental delay since early infancy with behavioural issues such as aggressive behaviour, bruxism and abnormal laughter. Her younger brother was healthy. Her height, weight and head circumference were around 3SDs below the mean Indian standards. The dysmorphic profile of the patient is described in Figure 1a. and Table 1. Rest of the physical examination was normal. Her complete blood counts (CBC), metabolic work up, electroencephalogram (EEG) and magnetic resonance imaging (MRI) of brain were normal. Peripheral blood karyotype showed ring chromosome 13, 46,XX,r(13)(p13q34), in all 20 metaphases (Figure 2a). Fluorescence in situ hybridization (FISH) analysis of 200 interphase cells confirmed deletion of the D13S1825 locus on chromosome 13, band q34. Karyotype of the mother was normal. Father's karyotype showed an additional material on the short arm of chromosome 15, 45,XY,add(15)(p13) in all metaphases which was due to a balanced translocation involving chromosomes Y and 15, t(Y; 15)(q12; p11.2). Neither parent had ring chromosomes or any other abnormality involving chromosome 13.

- Case2: A 15 months old boy, first born of non-consanguineous healthy parents, presented with seizures since 1 month of life, requiring multiple antiepileptic drugs for control. The antenatal and perinatal periods were uneventful and the birth weight was 2.72 kg. Developmental delay was present and at 15 months of age he could stand and walk with support but had not attained any language milestones. Physical examination revealed microcephaly, flat occiput, hypertelorism, almond shaped eyes, up slanting palpebral fissures, low set ears, bilateral clinodactyly, and ridging of coronal and metopic sutures (Figure 1b). He had increased muscle tone with symmetrically brisk deep tendon reflexes. Right sided testis was not palpable. Complete blood counts, neurometabolic screening, audiograms, ophthalmologic evaluation, echocardiography and sleep EEG were within normal limits. Premature fusion of metopic suture and lateral aspect of bilateral coronal sutures with brachycephaly were observed in skull x-ray and non contrast computed tomography (CT) bone reconstruction scan. Sagittal and lambdoid sutures appeared normal. Right testis was not visualised in the scrotal sac or inguinal canal by ultrasonography. MRI of the brain at 1 year of age showed mildly dilated temporal horn of the right lateral ventricle, but no focal lesions. Cytogenetic studies from peripheral blood revealed a 46,XY,r(14)(p13q32) karyotype (Figure 2b). Mother's karyotype was normal and the father was not available for chromosomal analysis. The mother came for prenatal diagnostic testing during the subsequent pregnancy; the fetal karyotype was normal and the mother later delivered a normal healthy baby.



a) b)

Figure 1 a) Patient with ring chromosome 13 displaying sloping forehead, hypertelorism, bilateral epicanthic folds, broad nasal bridge, short philtrum, wide mouth and protruding upper incisor. b) Patient with ring chromosome 14 showing hypertelorism, almond shaped eyes with up slanting palpebral fissures and low set ears.

Discussion

Ring chromosomes present commonly as intellectual disability and dysmorphism which may be obvious or subtle depending on the observation and acumen of the clinician.

Deletion 13q has a wide phenotypic spectrum depending on the location of the break point relative to chromosomal band 13q32.[5] This syndrome has been classified into three groups. Deletions

	Clinical Features	Kirchhoff *et al.* (n=14)	Su *et al.*	Lance *et al.*	Present case
1	Break Points	All	13q34	13q33.3	13q34
2	Gender	Both	F	F	F
3	Low Birth weight	90.9%	+	+	+
4	Microcephaly	All patients with terminal deletion	+	+	+
5	Intellectual disability	100%	+	+	+
6	Short Stature	2/9 patients with terminal deletion	+	–	–
7	Facial Dysmorphism ('13q facial appearance' as suggested by Kirchhoff *et al.*)				
	High forehead	78.6%	NK	+	–
	Sloping forehead	78.6%	+	NK	+
	Prominent metopic ridge	71.4%	NK	NK	–
	Deep set eyes	64.3%	NK	NK	–
	Hypertelorism	100%	+	+	+
	Inner epicanthic folds	100%	+	+	+
	Strabismus	92.9%	NK	NK	+
	Ear anomalies	100%	+	NK	+
	Broad and prominent nasal bridge	71.4%	+	+	+
	Prominent columella and a short philtrum	57.1%	Short philtrum	NK	+
	Open-mouth appearance	64.3%	+	NK	+
8	Clinodactyly	57.1%	NK	NK	+
9	Foot anomalies (Club foot, Pes cavus, Pes planus)	57.1%	Club foot	NK	Pes planus
10	Hearing anomalies	20%	+	NK	–
11	Seizures	14.3%	–	+	+
	Onset	NK	NA	4Yrs	Day1
	Type	NK	NA	GTCS	GTCS
	Response to treatment	Lasted till age 3Yrs	NA	Refractory	Refractory
12	Behavioural changes	28.6%	–	–	+

NK - Not Known
GTCS - Generalised Tonic Clonic Seizures

Table 1 Phenotypic profile of RC 13 syndrome patients reported by Kirchhoff *et al.*[6], Su *et al.*[8] and Lance *et al.*[11] in comparison with present case.

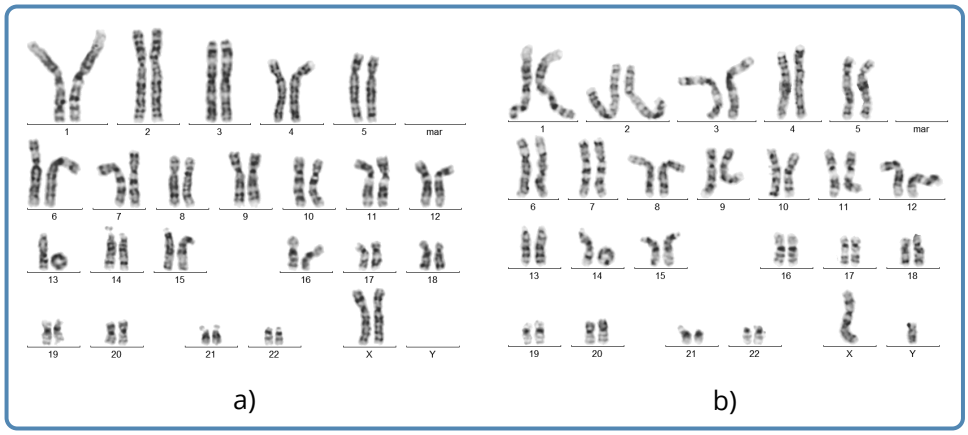


Figure 2 a) Karyotype of patient 1 showing ring chromosome 13; b) Karyotype of patient 2 showing ring chromosome 14.

proximal to q32 and including q32 have been classified under group 1 and 2 respectively, with distinct phenotypes including intellectual disability and growth deficiency, whereas severe intellectual disability, microcephaly with true hypertelorism, frontal bossing, protruding upper incisors and large external ears are frequently found in group 3 with breakpoints at 13q33 and 13q34. Recently, Kirchhoff *et al.* have updated this map by means of high resolution array CGH and have suggested a "13q deletion facial appearance" based on the common dysmorphic features found in a series of 14 patients, regardless of location and size of their deletions [Table 1].[6] Microcephaly is a common feature of individuals with 13q deletions but polymicrogyria is not common.[7] Su *et al.* have recently reported a case of mosaic RC13 [Table 1].[8] Apart from the CNS manifestations, cardiac, renal and genitourinary anomalies are also reported with 13q deletion syndromes.[9–11] Our patient with a deleted band 13q34 presented with early onset epilepsy as the predominant feature. This is a relatively uncommon feature in group 3. With literature review, we could identify only three other cases of group 3 of 13 q deletion syndrome with seizures.[9–11] Two cases reported in the case series by Kirchhoff *et al.* had seizures which lasted until the age of 3 years only.[6] Our patient continued to have seizures at the age of 10. Treatment non-compliance could have contributed to this. On the other hand, our patient bears a close similarity to the case reported by Lance *et al.* who described an 8 year old female child with microcephaly, moderate to severe intellectual disability and uncontrolled epilepsy but without major malformations, harbouring a terminal 13q33.3 deletion.[11] Both cases have a resembling facial profile [Table 1]. We agree with Lance *et al.* in suggesting that the deletion 13q syndrome, group 3 may be more severe than previously indicated and intractable seizures leading to gross cognitive impairment may be considered as a part of the phenotypic spectrum of this group.

Clinically, the RC14 syndrome is characterized by a recognizable phenotype of short stature, distinctive facial appearance, microcephaly, scoliosis, and ocular abnormalities. Almost all patients are intellectually delayed, with aggressive and hyperactive behaviour in some. Seizures occur in all and are usually drug-resistant and predominantly focal type.[12] The facial characteristics include long and sometimes slightly asymmetric face, full cheeks, high forehead, hypoplastic supraorbital ridges, horizontal eyebrows, deep set and down- slanting eyes with short palpebral fissures, hypertelorism, short nose with bulbous tip, long philtrum and small mouth with downturned corners.[12] It is considered that adverse clinical effects like growth retardation, neurologic impairment and facial dysmorphism of RC14 deletions are more pronounced than those of linear 14qter deletions with similar breakpoints.[12–14] Deletion of susceptibility genes during ring formation, position effect of the telomeric end and gene silencing due to spread of inactive state of p arm DNA to q arm are hypothesized mechanisms explaining the clinical manifestations of RC14.[14] One study mentions that in linear terminal 14q deletion syndrome, epilepsy is not included as its component manifestations and seizures are more

likely to be due to ring formation and not the loss of chromosomal material per se.[14] Exact mechanisms pertaining to the severe and drug-resistant seizure disorder are unknown. The reported case had microcephaly, developmental delay, epilepsy and dysmorphic features which are classical presentations of RC14 syndrome but the craniosynostosis seen in the case has not been reported so far with 14q deletion syndrome, to the best of our knowledge. This feature expands the phenotypic spectrum associated with ring 14 syndrome. Since he had the breakpoint at q32 band and 14q32 being the possible region for dysmorphic profile, this unusual trait may be assigned to the 14q32qter region. However delineation of the exact breakpoint by means of FISH or array CGH could not be attempted in our patient.

Ring chromosomes are generally sporadic in occurrence and recurrence in the family is usually low. However, patients with normal reproductive ability should be counselled about the possibility of transmission to the next generation. For our first patient, parental origin of ring 13 was excluded. The paternal karyotype of translocated Y on chromosome 15 was incidental. Most carriers of this translocation have not been reported to have phenotypic consequences and association with reproductive abnormalities is still controversial.[15] Whether this could predispose to ring chromosome formation is at present unknown. Paternal origin of ring 14 could not be excluded in the second patient. However prenatal testing was offered in subsequent pregnancy and the fetal karyotype was found to be normal. Regular follow up was emphasized for both the patients as it is important to assess the natural history, progression of characteristics, and neurodevelopmental achievement.

Conclusion

We have reported here a case of RC13 with unusual presentation of difficult-to-control seizures and a novel case of RC14 with intractable seizures and craniosynostosis. For children with microcephaly, developmental delay and seizures with or without dysmorphism, possibility of ring chromosomes should be kept in mind and karyotype must be an essential part of their evaluation.

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GeNeViSTA

Genetics of Alzheimer disease

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Introduction

Alzheimer disease (AD) is the leading cause of dementia in the elderly. It is estimated to affect more than 5.4 million people in the United States.[1,2] Though the life span has been increasing consistently in highly populous countries like India, no such estimate is yet available on the incidence of this disease in India. The prevalence of the disease increases with age with the incidence of 11-15% over 80 years of age.[3–5]

Definition

Alzheimer dementia is characterized by insidious onset and progressive deterioration of memory and at least one other cognitive domain (language, praxis, executive domain).[6] This is mainly a diagnosis of exclusion and the standard diagnostic criteria have been established by the National Institute for Neurological and Communicative Diseases and Alzheimer's disease and Related Disorders Association (NINCDS-ADRDA criteria) in 1984. The definite criteria for making a diagnosis previously required histopathological evidence along with clinical criteria for probable Alzheimer disease. Now mutation analysis can be done in familial cases.

Evidence for the genetic component

Many environmental factors were also studied in association to Alzheimer disease, including the educational status, lead exposure and head trauma. Twin studies done showed a concordance rate of 22-83% in monozygotic twins, suggesting a genetic etiology for Alzheimer disease.

Classification

Depending on the age of onset, Alzheimer disease can be classified into:

1. Early onset Alzheimer disease (EOAD): Onset before 60-65 years of age. This accounts for 5% of cases of Alzheimer disease.[7]
2. Late onset Alzheimer disease (LOAD): Onset after 60-65 years.

Depending on the pattern of inheritance from the family history, the condition can be classified as:

1. Autosomal dominant: 3 individuals affected in 2 or more generations, with 2 of them being first degree relatives of the third. Usually this accounts for less than 5% of cases and is almost exclusively seen in EOAD and hence the terms are used interchangeably.[7]
2. Familial: More than one individual is affected, with at least 2 of them being third degree relatives or closer. Familial segregation accounts for 15-25% in LOAD and 47% in EOAD.[8] Familial clustering can be seen in both EOAD and LOAD.
3. Sporadic: Isolated case in the family or affected individuals separated by more than 3 generations. This constitutes 75% of total cases of Alzheimer disease.

Pathogenesis

The histopathological hall-mark of Alzheimer disease is tangles and plaques. The major component of the plaque is beta amyloid which is the break down product of amyloid precursor protein (APP). Amyloid hypothesis explains the pathophysiology of Alzheimer disease. APP breaks down to beta secretase product or alpha secretase product by

the action of beta and alpha secretase respectively. Beta secretase product is cleaved to A beta 42 toxic product and A beta 40 non toxic product by gamma secretase. The toxic A beta 42 product induces cell dysfunction and neuronal death.

- Genes implicated in EOAD:

1. Amyloid precursor protein (*APP*): This was the first gene to be identified in 1987 and was localized to Chromosome 21. Missense mutation in this gene causes the shift of cleavage of Amyloid Precursor protein towards the more toxic amyloid beta 42 (A β42) which is deposited as neuritic plaques and cause oxidative damage to the neurons.

2. Presenilin 1 (*PSEN1*): This gene was first identified in 1995 and is linked to Chromosome 14. This is the most common cause for EOAD, accounting for 60% of EOAD. Mutation of this gene leads to the most aggressive form of Alzheimer disease with an onset at 40-50 years of age. Presenilin 1 is a component of the gamma- secretase enzyme which forms the break down products of amyloid precursor protein and a mutation in presenilin 1 is associated with an increase in toxic A β42.

3. Presenilin 2 (*PSEN2*): The exact mechanism of action of this gene is not known, but the levels of A β42 are found to be increased in patients with mutation of *PSEN2*. This gene has been mapped to Chromosome 1.

- Genetics of LOAD: LOAD accounts for 95% of Alzheimer disease. Most of the LOAD cases are sporadic and familial forms account for 15-20% of the total LOAD. *APOE* gene which codes for Apolipoprotein E, which is synthesized in astrocytes and involved in the transport of lipids, is the most widely studied susceptibility gene. *APOE* lies on long arm of Chromosome 19. It has three isoforms, Apo E ε2, Apo E ε3 and Apo E ε4, of which Apo E ε4 has the highest penetrance.[6] There is a dosage effect for Apo E ε4, with the age of onset of the disease being determined by the number of alleles of Apo E ε4.

Apo E ε4 allele is associated with higher amyloid plaque density and neurofibrillary tangles. The response to anticholinesterase inhibitors, which are used for treatment of Alzheimer disease, also depends on the number of Apo E ε4 alleles.

- Genetic testing in Alzheimer disease: The use of genetic testing for diagnostic purposes in EOAD is still debatable.[8] Genetic testing could be either symptomatic testing, done in individuals with clinical suspicion of Alzheimer disease or predictive testing done on asymptomatic at-risk individuals. Testing is clinically available for *PSEN1, APP, PSEN2* and Apo E ε4. Before ordering genetic testing, an accurate history and family history should be necessarily obtained. Mendelian forms are rare. Multi-generational involvement and younger age of onset of disease and myoclonus in early stage of disease are pointers towards a genetic etiology. When a genetic etiology is suspected, these genes should be tested in the following order: *PSEN1, APP, PSEN2, APP* duplication. Genetic testing for Apo E ε4 allele is not recommended. The major recommendations made by the American College of Medical Genetics for genetic counseling of Alzheimer disease are as follows[8]:

1. Genetic testing should be done only after proper counseling. For asymptomatic patients, for predictive testing, a protocol based on the International Huntington Association and World Federation of Neurology Research Group on Huntington's Chorea is to be used.

2. Proper risk assessment should be done before categorizing into EOAD and LOAD.

3. The life time risk of Alzheimer disease is 10-12% in the general population in a 75- 80 year life span.

4. In autosomal dominant disease, there is a 50% chance of inheriting the mutation in off springs. *PSEN1* and *APP* have 100% penetrance, which means that the chance of developing the symptoms is 100% if this mutation is present. *PSEN2* has 95% penetrance.

5. In families where the pattern is not consistent with autosomal dominant inheritance, there is a cumulative risk of 20% of developing the disease in first degree relatives, after 75 to 80 years, which is twice the risk in the normal population.

In conclusion, most of the cases of Alzheimer disease are sporadic, but could have genetic factors which influence the onset of disease. Mendelian forms are rare and account for less than 5% of cases. Many more susceptibility loci have been identified in familial cases and newer genes could be implicated in the development of this disease.

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GeNeImage

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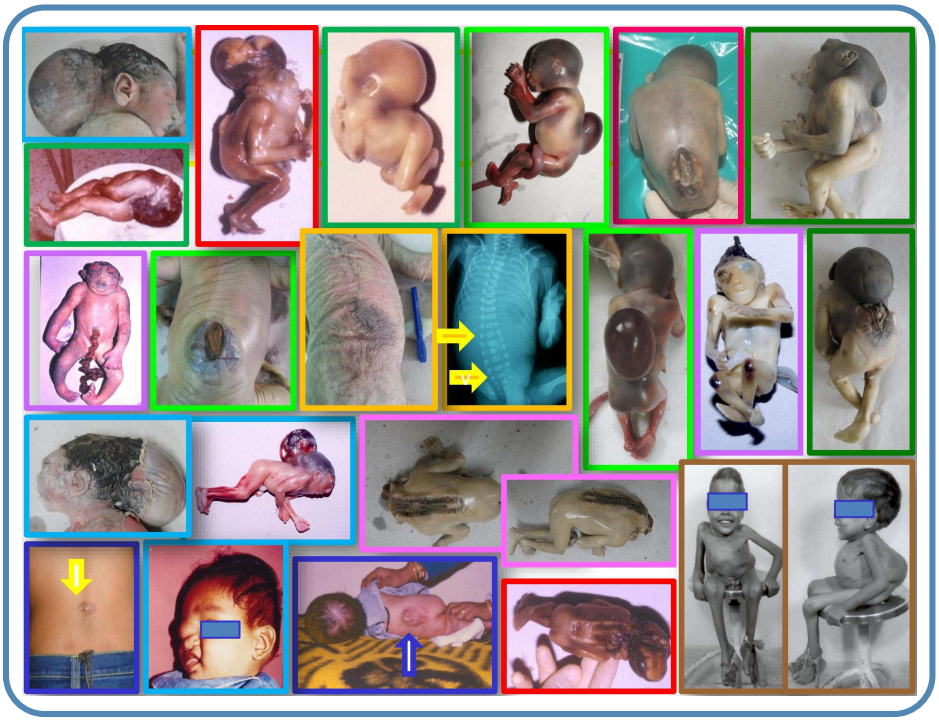
Types of Neural Tube Defects
Periconceptional FOLIC ACID for Prevention of One & All

Color code for images:
Encephalocele, Iniencephaly clausus, Anencephaly, Acrania, Meningomyelocele, Open spina bifida, Closed spina bifida, Iniencephaly apertus, Craniospinal rachisisis, Healed spina bifida, Operated spina bifida, Meningomyelocele.



Genome Editing: Precise and "CRISPER"

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Introduction

Creation of a desired change in the genome either by gene addition/ gene insertion/ gene correction at targeted sites is known as genome editing. The various methods that are being conventionally used for genome editing typically include a nucleotide or amino acid sequence which identify the targeted genomic site and a nuclease which produces double stranded DNA breaks (DSBs). These DSBs are then repaired by using cellular intrinsic DNA repair machinery. If these broken DNA ends are joined end to end it leads to either insertion or deletion of a few nucleotides which often disrupts the reading frame and leads to frame shift mutation. This method of DNA repair which is called as nonhomologous end joining (NHEJ) is more commonly used but being error prone, it often causes the knocking down of a functioning gene. The other method for repair of DSBs, homology directed repair (HDR), wherein breaks are repaired by complementary pairing to a template strand, is more accurate and by providing an already synthesized DNA template strand, desired gene modifications are possible.[1,2]

Various Methods for Genome Editing

Zinc finger nucleases (ZFNs), Transcription activator like effector nucleases (TALENs) and Clustered regularly interspaced short palindromic repeats-Cas9 (CRISPR-Cas9) are the three most common genome editing technologies.[1,3] All these methods are based on a combination of mechanisms to identify genome targets with great specificity and creating DSBs. They mainly differ in the mechanism of recognising target genomic sequences, multiplexing to various genomic targets, potential off-targeting effects and ease of in vitro genetic engineering. A comparison of the characteristics of these genome editing technologies is presented in table 1.

Potential Uses of Genome Editing Methods

These genome editing technologies have a vast range of utilities from research in understanding gene function to gene therapy and drug discovery. The potential uses include:

1) For gene functional studies- by producing gene disruption, can produce knock out models to study functions of genes. Conventionally these forward genetic screen methods used random chemical mutagenesis in model organisms like yeast, fruit fly, zebra fish, nematodes and rodents. However the process was labour intensive and random. Moreover the technique had few other limitations like incomplete knock down of the genes and identification of causative mutation was difficult. By use of genome editing technologies, specific genes can be targeted with minimal off targeting effects on global/ irrelevant genes and high efficiency, strong phenotypic effects and high validation rates. These technologies seem to be of immense value in the era of whole genome sequencing where establishing the functional significance of a genetic variant seems to be of utmost importance.[4]

2) For gene expression studies - nucleases which are used in genome editing methods can be made inactive by introducing a mutation in one of their functional domains which in turn can just tag at the desired genomic sites. These deactivated nucleases can control expression of endogenous genes and also are an important source to learn epigenetic modification of highly repetitive loci like telomeres and chromatin remodelling in live cells,

Character	Zinc finger nuclease (ZFN)	Transcription activator like effector-nucleases (TALEN)	Clustered regularly interspaced short palindromic repeats (CRISPR-Cas9)
Type of nuclease	FokI nuclease	FokI nuclease	Cas9
DNA identifying mechanism	Based on DNA- protein interaction. DNA binding domain is composed of repeats of 30 amino acids. 3-6 repeats per ZFN, each domain recognises 3 base pairs.	Based on protein DNA interaction. Repeats of 34 amino acids recognizing one amino acid.	Based on Watson crick base pairing between RNA and target DNA. 20 nucleotide long crRNA which typically consists of specific nucleotide sequences flanked by repetitive sequences. Target sequence should be preceded by NGG sequence called as protospace adjacent motif (PAM).
Mechanism of producing DNA double strands repair	Two ZFNs bind to opposite sides of the targeted DNA sequence with space of 5-7 nucleotides where Fok1 nucleases dimerise and produce double strand break in the space.	Same as ZFN	crRNA hybridizes with tracrRNA and activates cas9 nuclease which produces DSB.
Efficiency	0-12%	0-75%	0-80%
Off target effects	+	+	+++
Multiplexing	Difficult	Difficult	Easy
Ease of construction	Difficult	Difficult	Relatively easy

Table 1 Comparative features of the three important genome editing systems. [1,3]

a technique known as dynamic imaging.

3) For large scale high throughput gene disruption in drug discovery.

4) For gene therapy by using targeted gene disruption or gene correction methods.

5) For agricultural and live stock genomic modification.

CRISPR–cas9 System

CRISPR-Cas9 system is a Clustered, Regularly Interspaced, Short Palindromic Repeats-Cas9 (CRISPR-associated) system. This method of genome editing has been modified from the adaptive immune system of bacteria and archea against invasion of foreign viruses. Almost all bacterial genomes have CRISPR-Cas9 loci. As the name suggests these loci consist of clustered direct palindromic repeats which are spaced by interspersed nucleotide sequences. The repeats are typically 21-47 nucleotides long and identical in a single locus. The interspersed nucleotides sequences are called as spaces and are derived from foreign viruses. Spacer sequence is transcribed into Crispr RNA (crRNA). CRISPR locus also contains DNA sequences which code for a complementary transactivating Crispr RNA (tracrRNA) and various Crispr Associated genes (cas) which code for nucleases. These crRNA hybridize with complementary transactivating Crispr RNA (tracrRNA) and together, as a double strand, they recognise the complementary foreign nucleotide sequences. The diagrammatic representation of structure of CRISPR/Cas9 system is

given in fig 1.

There are 3 types of CRISPR-Cas immune systems. Out of them only the type II system (adapted from Streptococcus pyogenes), which uses Cas9 as a nuclease, is used as a method of genome editing, which is why it is known as the CRISPR-Cas9 system. Cas9 nuclease has HNH nuclease domain and the RuvC-like domain which generate DSBs.[1–3]

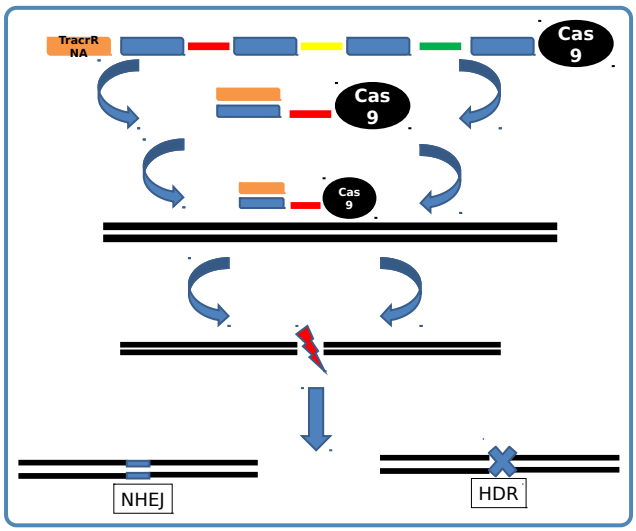

Figure 1 Schematic representation of the CRISPR-Cas9 system. Blue bars represent the array of identical repeats. Red, yellow and green bars represent the various spaces/interspersed elements. Black oval is Cas9 and blue bar is transactivating crRNA (tracrRNA). Mature crisper RNA (spacer with repeats) hybridises with tracrRNA and along with cas9 makes a ribonucleoprotein complex. This complex binds to targeted 20 nucleotide genomic sequence and produces double strand breaks (DSBs). These DSBs are repaired by either error prone nonhomologous end joining (NHEJ) or precise Homology directed repair (HDR).

CRISPR–Cas9 System Construction and Modifications

In the laboratory the original CRISPR-Cas9 system is slightly modified for use as a genome editing tool. crRNA and transcrRNA can be constructed together as a single guide RNA (gRNA). Multiple gRNA can be multiplexed in a single construct to target multiple genomic sequences in one experiment. Also these gRNA can be combined with another DNA template which can be used for homology directed repair to induce desired genetic changes. To reduce the off target effects of the CRISPR-Cas9 system instead of a single gRNA combination, a combination of 2 gRNAs can be produced which have modified nucleases. These nucleases can be modified at their functional domain so that they produce a nick only in a single strand (known as nickase). Two gRNAs bind to adjacent genomic regions on the sense and antisense strands and paired nickases produce independent breaks. Hence an off target effect break will be there only in one strand which will be repaired by the base excision repair method using the homologous strand as a template. It would be very rare and unlikely for two adjacent genomic regions to have off target effects. This modification thus decreases the likelihood of off target effects by 50-100 folds (Fig 1).

Various Applications of CRISPR–Cas9 System

Genome editing technology, especially the CRISPR-Cas9 system has emerged as the new platform to study correlation of DNA sequences with its functional significance i.e. reverse genetics. Apart from the research interest, it is also being used as a newer approach to treat human genetic disorders and for research in chromatin modification. Following are the few success stories of the CRISPR-Cas9 system.

- *Role of CRISPR/Cas9 in repairing of CFTR defect*: A recent study performed by Schwank *et al.* used the CRISPR-Cas9 genome editing approach to correct the d508 mutation in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*). They isolated and expanded adult intestinal stem cells from two patients affected with cystic fibrosis.[5] Genomic editing was done using CRISPR-Cas9 system and homology mediated repair of d508 mutation. The authors also demonstrated functionality of the gene in in vitro epithelial organoid system. This article provides proof of concept that the CRISPR-Cas9 system can be used as an effective gene therapy method in various

single gene disorders where no curative treatment is available.

- *Role of CRISPR in HIV therapy*: After the introduction of highly active antiretroviral therapy (HAART), human immunodeficiency viral (HIV) infection is now considered a chronic disease which requires prolonged treatment. However HAART is associated with relapse of disease as soon as the treatment is stopped, high cost of treatment, commitment of the family and treating clinicians and potential of emergence of HIV resistance. CCR5 is a co-receptor on CD4+ cells which is essential for HIV entry inside these cells. A 32 base pair deletion in single exon of CCR5 gene produces a frame shift and disrupts the gene function. The famous patient from Berlin who had HIV with lymphoma was cured by bone marrow transplantation from a person who was harbouring this homozygous deletion in CCR5 gene. Hence disruption of CCR5 gene by gene silencing methods appears to be an attractive model of gene therapy for HIV.[2]

The first reported genome editing method was based on using ZFN and then other methods such as TALEN and CRISPR-Cas9 system began to be used. Genome editing is heritable and therefore does not require repeated therapy like short interfering RNA (siRNA) and only one time therapy is sufficient. Trials are going on using gene editing of patient derived CD4+ /CD34 +/ hematopoietic stem cells and induced pluoripotent stem cells followed by autologous transplantation back in patients. Early results are positive in terms of persistence of genetically modified cells in the circulation and no serious side effects. However the true efficacy can only be determined after interruption of HAART and after the final results of these trials. Also genome editing system has been used to eliminate already integrated HIV proviral DNA. Ebina *et al.* in 2013 in their study targeted the long terminal repeats (LTR) of the integrated HIV viral genome using CRISPR-Cas9 and showed the blocked expression of genes.[6]

- *Role of CRISPR-Cas9 in curing liver disorder*: In a recent study published by Yin *et al.*, the CRISPR-Cas9 genome editing approach has been used to cure hereditary tyrosinemia in mouse models.[7] The genetic correction was observed in 1/250 liver cells. Strong positive selection and further expansion of genetically corrected cells also contributed to the success story.

- *Role of CRISPR-Cas9 in Duchenne muscular dystrophy*: Duchenne muscular dystrophy is one of the common and severe genetic disorders of muscles with onset in childhood. The condition is relentlessly progressive and there is no curative treatment available. Because of the large size of the causative gene (Dystrophin), delivery of a smaller gene coding for a small but functional protein (microdystrophin or microutrophin) has been tried but results are not very promising and in preclinical trials. The other methodologies which are being explored are exon skipping by using oligonucleotides which, by blocking expression of one or more exons, restores the reading frame of the coding part and produces a functional protein. The limitations of this approach are the requirement of repeated delivery of oligonucleotides and development of separate oligonucleotides for each type of deletion. Recently genome editing technologies have also been explored that insert the deleted exons, help in exon skipping or produce small indels, thereby producing a full/small transcript. Li *et al.* isolated fibroblasts from a patient with DMD having deletion in exon 44 of the dystrophin gene.[8] These fibroblasts were programmed into induced pluoripotent stem cells (iPSCs) and by using TALENs and CRISPR-Cas9 system, 3 gene correction methods were tried. These methods were exon skipping, insertion of exon 44 and inserting small indels and hence restoring the reading frame. The authors found minimal off -target effect and concluded that exon knock-in approach was the most promising. These experiments hold the promise for future patient-specific mutation correction and autologous ex vivo gene therapy using iPSCs.

- *CRISPR-Cas9 in treating beta Thalassemia major*: Hematopoietic stem cell transplantation from a HLA matched donor is the curative treatment for beta Thalassemia major. In absence of an HLA matched donor, hyper-transfusion and iron chelation therapy remain the mainstay of therapy for these patients. Recently, Song *et al.* have created iPSCs from patients suffering from beta thalassemia major and by using CRISPR-Cas9 system have corrected the mutation in the human beta globin gene (HBB).[9] The authors have shown that the differentiation capacity of these corrected iPSCs is great with minimal off-targeting effects. These experiments further raise the hope and potential for using CRISPR-Cas 9 technology in genetically modified iPSCs transplantation.

Conclusion

CRISPR-Cas9 system has revolutionised the area of genome editing mainly because of ease of laboratory construction, targeting multiple genomic sites simultaneously and application in broad areas. Future challenges involve invention of efficient delivery systems, reducing off-target effects and increase in efficient homology mediated repair.

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GeNeXpreSS

Genome editing technologies: Future of functional and therapeutic genetics!

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Minimal risk of miscarriage by invasive prenatal testing: Review and meta-analysis[1,2]

All over the world, the procedure related risk of miscarriage following an invasive prenatal testing is quoted to be 1-2%. Akolekar *et al.* have performed a meta-analysis of articles available on MEDLINE, EMBASE, CINHAL and Cochrane library in the period between 2000 and 2014.[1] The weighted pooled risk was estimated by 324 losses in 42,716 women undergoing amniocentesis and 207 losses in 8,899 women undergoing chorionic villous sampling. The authors concluded that the risk of procedure related risk of miscarriage after amniocentesis is 1:1000 and 1:500 after chorionic villous sampling which is not significantly different from the miscarriage rate in women who have not undergone the invasive testing. These figures can be used for counseling women who opt for invasive prenatal testing and help them in make informed choices.

Private mutations to private gene therapy: Beta thalassemia as an example[2]

Beta thalassemia major is one of the most common monogenic disorders worldwide. Hematopoietic stem cell transplantation from a histocompatible donor is the mainstay of therapy. However correction of mutation in the human beta globin gene (*HBB*) will be the ideal situation. Though few successful experiments of gene transfer using viral vectors have been performed, they have their own disadvantages including random insertion in the genome and the potential of insertional mutagenesis. Recently Xie *et al.* created induced pluoripotent stem cells from somatic cells of patient with beta thalassemia major harbouring compound heterozygous mutations in HBB gene.[2] These mutations in iPSCs were corrected using CRISPR/Cas9 system along with the piggybac system leading to a global switch to the normal gene and preserving the normal promoter sequences. The future in vivo use of this genome editing technology can lead to mutation specific gene therapy for the patients.

Restoring the reading frame in more than half of DMD patients: genome editing technology[3]

Various cell and gene based therapies are in preclinical/phase I trials for Duchenne muscular dystrophy (DMD). In majority of the patients, the disease is caused by deletion mutations which cause a shift in the reading frame, which leads to dysfunctional dystrophin protein production. The milder phenotype (Becker muscular dystrophy) is caused by mutations in the same gene, but these mutations are in-frame, which result in the formation of an abnormal but functional protein. Skipping of exon 51 by oligonucleotide-based therapy to restore the reading frame, has the potential to be used in 13% of DMD patients. If skipping of multiple exons is performed between 45-55 exons, which is the mutational hotspot, about 60% of all DMD patients can be offered therapy. However designing, the short half life and requirement of life-long injections of oligonucleotides are limiting factors. Recently Ousterout *et al.* have used CRISPR/Cas9 based genome editing technology to generate a 336 kb deletion across the 45-55 exons of dystrophin gene in skeletal myoblasts taken from DMD patients.[3] These edited cells were grafted in immunodeficient mice and expression of dystrophin protein was

observed. This study is a proof of the concept that CRISPR/Cas9 technology, being very versatile, can be used to correct mutations in 60% of DMD patients.

Promising curative therapy for HIV by genome editing technology: More than 30 years after first case of AIDS[4,5]

The first case of AIDS was described in 1981. Since then various forms of antiretroviral therapy (ART) are the main-stay of treatment. But antiretroviral treatment needs to be continued lifelong and is associated with significant side effects. CCR5 receptor is established to be important in entry of the HIV1 virus in CD4+ positive human T cells. Persons homozygous for a 32 base pair deletion in CCR5 (CCRΔ32) gene are naturally resistant to HIV1 virus and allogenic bone marrow transplantation from a homozygous CCRΔ32 donor is also shown to be curable in patients with AIDS. In recent years, knock down of CCR5 gene by using various technologies including ribozymes, short interfering RNA (SiRNA) and Zinc finger nuclease have been shown to be promising in acquiring resistance for HIV1 infection. CRISPR/Cas9 technology being rapid, efficient and high throughput, offers many advantages over existing genome editing technologies. Wang *et al.* have constructed a lentivirus vector coexpressing single guide RNA and Cas9 targeting CCR5 and transduced human CD4+ cells and showed the high frequency of CCR5 disruption, minimal off-target effects and stable transduction in cell lines.[4]

Another approach which is being explored is the eradication of the integrated HIV viral genome in CD4+ cells which subsequently cause viral reactivation and viremia as soon as 50 days of stopping the ART. This integrated viral genome is not treatable by existing ART. Strong *et al.* have used transcription activator like effector nucleases (TALENs) to show that this genome editing technology can cleave integrated viral genome with high specificity and efficiency in vitro and in living cells.[5] The authors also constructed the TALEN variants which can recognise wild type and triple mutant viral sequences which can escape from other genome editing technologies included CRISPR/Cas9.

Together these experiments raise the hope for effective curative treatment to be available for patients infected with HIV1.

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PhotoQuiz - 29

Contributed by: Dr. Prajnya Ranganath

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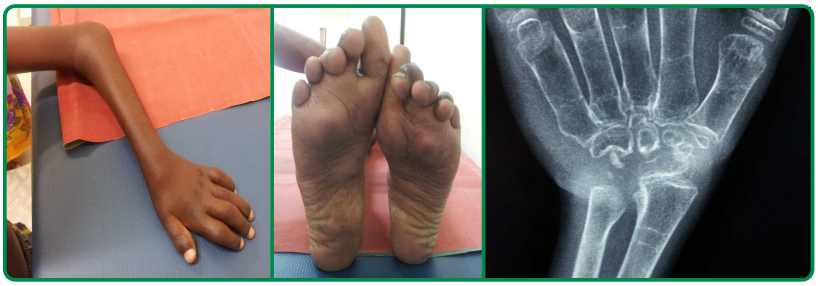
This 6 year old boy, the second offspring of third degree consanguineous parents, presented with progressively increasing deformity of bilateral elbow and interphalangeal joints and nodular swellings on the plantar aspect of both feet, of 3 months duration. Radiograph of the hand and wrist showed typical findings as shown below. Identify the condition.

Please send your responses to editor@iamg.in
Or go to http://iamg.in/genetic_clinics/photoquiz_answers.php
to submit your answer.

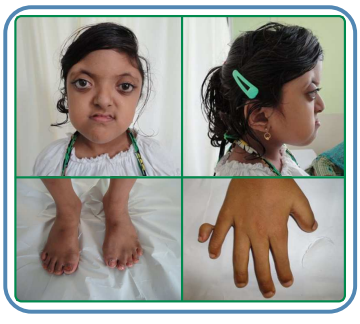


Answer to PhotoQuiz 28

Carpenter syndrome 1 (OMIM #201000)

Carpenter syndrome 1 is also known as acrocephalopolysyndactyly II. It is characterized by craniosynostosis involving the sagittal, lambdoid and coronal sutures in variable proportions. The facies is characteristic with acrocephlay/turricephaly or sometimes clover leaf skull, telecanthus and midface hypoplasia. The intelligence is very variable with an IQ range of 50-100. The limb abnormalities include brachydactyly, syndactyly, clinodactyly and pre- and post-axial polydactyly. Brachymesophalangy or agenesis of middle phalanges is observed on radiography. It is an autosomal recessive condition caused by mutations in the *RAB23* gene. Carpenter syndrome 2 has features of Carpenter syndrome 1 as well as right-left laterality defects and occurs due to mutations in the *MEGF8* gene.

Correct responses were given by:
1. Rekha Gupta, Jaipur
2. Mala Misra, Amsterdam
3. Bharat Ramamurthy, Bengaluru
4. Ravi Goyal, Kota, Rajasthan
5. Atanu Kumar Dutta, Vellore
6. Arun Prasad, Chennai



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- Causing hepatosplenomegaly, anemia, thrombocytopenia and bone involvement
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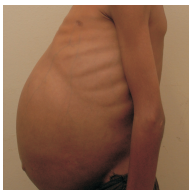
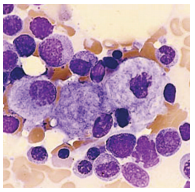
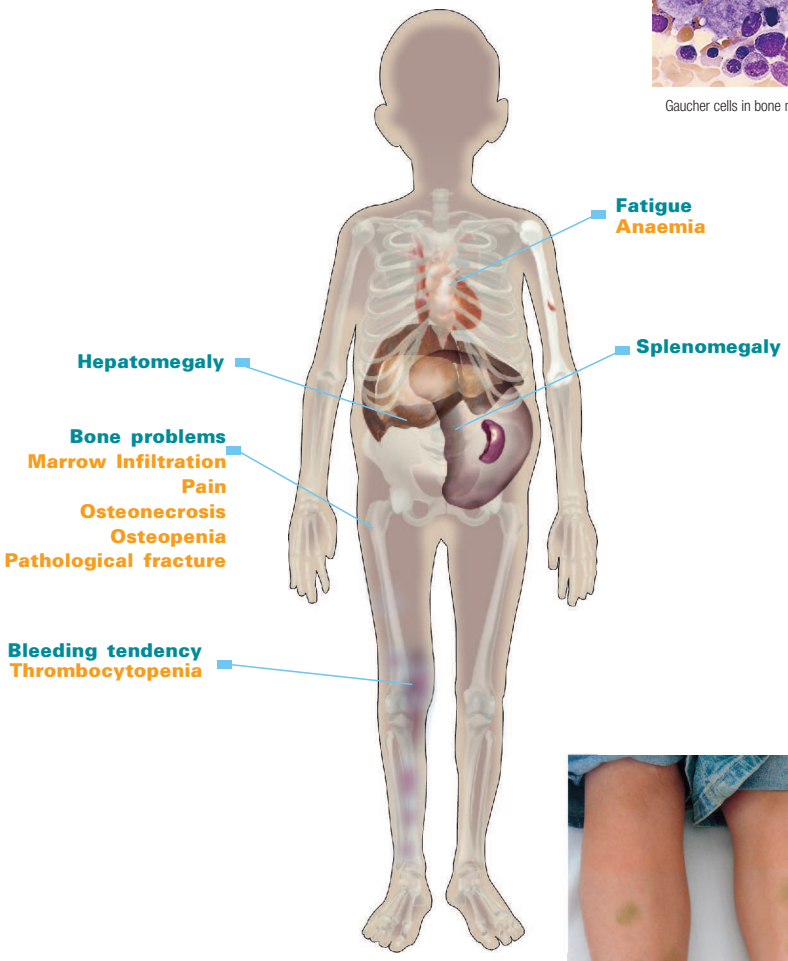


Enlarged Liver and Spleen



Gaucher cells in bone marrow



Hematoma[6]

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