2017

National Symposium

September 15 & 16

Genetics in Clinical Medicine

Exploring the Role of Genomic Medicine

JUBILEE CENTRE FOR MEDICAL RESEARCH

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Proceedings

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NATIONAL SYMPOSIUM ON GENETICS IN CLINICAL MEDICINE

15th & 16th September 2017

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GENETICS IN CLINICAL MEDICINE

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PREFACE

It is our pleasure to present the proceedings of the National Symposium on Genetics in Clinical Medicine 2017 before the scientific community. The two days programme held on 15th & 16th September was a well attended symposium with 226 participants across the country. Thirteen invited speakers made presentations in 5 sessions. Twenty one contributed papers were also presented.

The ground on which this symposium organised was to popularize the role of health care providers in the field of genetics. Certainly this was fulfilled. The aim of Genetic Services is to help those affected by, or at risk of a genetic disorder to live and reproduce as normally as possible. The role of medical schools in genetics practice was highlighted through this symposium. Everybody is sure that the deliberations on those two days were a step ahead to achieve this ambitious target. More clinicians and scientists were expected to shoulder this concept to a high reach.

The presentations were of high content and there had fruitful interactions. Senior and young faculty members and researchers freely interacted even in late hours and it was a real festival of Genetics. We hope that this interaction of clinicians and scientists may help to bridge the gap between the present day practice of medicine and the present day knowledge of genetics.

With this level of satisfaction and responsibility we present this proceeding as a credible scientific material for the benefit of future clinical – research interactions.

We are thankful to the co-sponsors (ICMR, KSCSTE, SERB, DBT and CSB) and other benefactors for generously supporting this symposium.

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15th September 2017 9.30 am to 12 noon

Symposium on Genetics of Fertility

Chairpersons

1 Dr. S Kumar
Director, National Institute of Occupational Health (ICMR), Ahmedabad

2 Dr. Sareena Gilvaz
Prof. & Head, Dept. of Obstetrics and Gynaecology, JMMC & RI, Thrissur

Resource Persons (30 minutes including discussion)

1 Dr. Rima Dada
Professor, Lab for Molecular Reproduction and Genetics, Dept. of Anatomy, AIIMS, New Delhi
Male infertility - role of genetic factors

2 Dr. Hema Purandarey
Medical and Reproductive Geneticist, Centre for Genetic Health Care
Hill Road, Bandra West, Mumbai
Importance of genetic studies in female fertility

3 Dr. Anusmitha Andrews
Consultant, Dept. of Fetal Medicine, Lifeline Super Specialty Hospital, Adoor, Kerala
Ultrasound detection of genetic disorders

Contributed papers (7 minutes presentation and discussion 10 minutes at the end)

1 Association of IRS2 gene Gly1057Asp polymorphism with polycystic ovarian syndrome: A hospital based Study
Dr.Vinay Kulkarni: Department of Anatomy, Sri Devaraj Urs Medical College, Karnataka

2 Screening for contiguous gene deletions in chromosome 15q15.3 among infertile males from Chennai - A pilot study
Jeffrey J M , Dr. ALM PG Institute of Basic Medical Sciences, Taramani, Chennai

3 Delineation of a small supernumerary marker chromosome in Turner syndrome
Dr. Suresh Kumar R: Jubilee Centre for Medical Research, JMMC&RI, Thrissur

4 Do product of conception karyotyping alone is mandate to identify the cause of recurrent pregnancy loss??
Dr. Deepika MLN: Institute of Genetics & Hospital for Genetic Diseases, Osmania University, Hyderabad

12 noon Formal Inauguration

Guests Dr. Vedprakash Mishra
Chairman, Academic Committee, Medical Council of India

Dr. M K C Nair
Vice Chancellor, Kerala University of Health Sciences

Dr. Sunil Kumar
Director, National Institute of Occupational Health, Ahmedabad

15th September 2017 2 pm to 5 pm

Symposium on Pediatric Genetics

Chairpersons

1 Dr. Mohandas Nair
Addl. Professor in Pediatrics, Govt. Medical College, Kozhikode
2 Dr. Manoj V C
Associate Professor & Head, Dept. of Neonatology, JMMC & RI, Thrissur

Resource Persons (30 minutes including discussion)

1 Dr. Prajnya Ranganath
Head, Department of Medical Genetics, Nizam's Institute of Medical Sciences, Hyderabad
Handles of dysmorphology
2 Dr. Sankar V Hariharan
Additional Professor & Consultant Geneticist, Department of Pediatrics,
SAT Hospital, Government Medical College, Trivandrum.
Single gene disorders- diagnosis and management
3 Dr. Sreelata B Nair
Geneticist, Dept. of Genetics & Fetal Medicine, Lifeline Hospital, Adoor, Kerala
Importance of genetic counseling

Contributed papers (7 minutes presentation and discussion 10 minutes at the end)

1 Potential use of next generation sequencing (NGS) in the diagnosis of monogenic disorders
Sherrin T Alex: Child Development Centre, Dept of Paediatrics, SAT Hospital, Trivandrum
2 Down syndrome with Autism Spectrum Disorders: A subject of concern
Dr. Alka Anilkumar: Institute for Communicative and Cognitive Neurosciences, Palakkad
3 A rare case of two distinct autosomal dominant disorders in a newborn - familial maternal Di-George Syndrome along with paternal neurofibromatosis
Meera M: Lifeline Genetics and Research Centre, Adoor, Kerala
4 Chromosomal aberrations and *IRF6* gene polymorphism in orofacial clefts
Soumya Raj: Jubilee Centre for Medical Research, JMMC&RI, Thrissur
5 Genetic and other etiological associations in children with PDD (Pervasive Developmental Disorders)
Dr. Mini Kariappa: Department of Anatomy, Amala Institute of Medical Sciences, Thrissur
6 Genetic soft markers in fetal neurosonogram
Dr. Srinadh B: Asian Institute of Fetal Medicine and Reproductive Genetics, Hyderabad

16th September 2017 8.30 am to 11 am

Symposium on Genetics of Metabolic Disorders

Chairpersons

1 Dr. K G Raghu
Principal Scientist, NIIST- CSIR, Thiruvananthapuram
2 Dr. P T Annamala
Professor & Head, Dept. of Biochemistry, JMMC & RI, Thrissur

Resource Persons (30 minutes including discussion)

1 Dr. Kannan Vaidyanathan
Head, Dept. of Biochemistry, PIMC & RC, Thiruvalla, Kerala
Biochemical basis of metabolic disorders

2 Dr. Kausik Mandal
Associate Professor, Department of Medical Genetics
Sanjay Gandhi Postgraduate Institute of Medical Sciences, Raibareilly Road, Lucknow
Newer techniques in the molecular diagnosis of metabolic disorders

Contributed papers (7 minutes presentation and discussion 10 minutes at the end)

1 Uncovering novel disease genes in mendelian disorders by exome sequencing
Dr. Sudhesna Mohapatra: SRL Diagnostics, Bannerghata Road, Bangalore
2 Contribution of GNPTAB and GNPTG mutations to persistent stuttering
G Nandhini Devi: Dr. ALM PG Institute of Basic Medical Sciences, Taramani, Chennai
3 Prevalence of T2DM, hypertension and hyperlipidemia association study with SNP among different populations of Palakkad municipality, Kerala.
Dr. Jayasree S: Department of Zoology, Mercy College, Palakkad
4 Study of DRD2 TaqA1 polymorphism in developmental stuttering
*M Kalaimath*i: Dr. ALM PG Institute of Basic Medical Sciences, Taramani, Chennai

16th September 2017 11 am to 1 pm

Symposium on Diagnostic Genetics

Chairpersons

1 Dr. Rima Dada
Professor, Lab for Molecular Reproduction and Genetics, AIIMS, New Delhi
2 Dr. Susheela J Innah
Prof. & Head, Dept. of Transfusion Medicine, JMMC & RI, Thrissur

Resource Persons (30 minutes including discussion)

1 Dr. Rema Devi
Head, Division of Human Genetics, Pondicherry Institute of Medical Sciences, Pondicherry
How, when and which test in chromosomal disorders

2 Dr. Jayarama S. Kadandale
Professor and Head, Clinical and Molecular cytogenetics
Centre for Human Genetics, Biotech Park, Electronic city phase I, Bangalore
Clinical applications of FISH, PRINS, SKY, mFISH and mBAND in detecting genetic disorders

Contributed papers (7 minutes presentation and discussion 10 minutes at the end)

1 Correlation of Cytogenetic and Radiological Evaluation of Fetal Cystic Hygroma to Determine the Recurrence Risk– A Short Study
Sruthi S. D: Lifeline Genetics and Research Centre, Adoor, Kerala
2 Cytogenetic and molecular study on ring chromosome 9 in a child with developmental delay
Sharifa M M : Jubilee Centre for Medical Research, JMMC & RI, Thrissur
3 Modulation of the Expression of miR-572 and miR-663a by Fluoxetine in Human Neuroblastoma Cell Lines
Dr. Mahesh Mundalil Vasu: Institute for Communicative and Cognitive Neurosciences, Palakkad
4 Parental Karyotype in unravelling the Structural Abnormalities in children with chromosomal aberrations
Dr Santhi S: Child Development Centre, Dept. of Paediatrics, SAT Hospital, Trivandrum

16th September 2017 1.30 pm to 4 pm

Symposium on Cancer Genetics

Chairpersons

1 Dr. Sree Kumar Pillai
Head, Dept. of Surgical Oncology, JMMC & RI, Thrissur

2 Dr. Mobin Paul
Consultant, Department of Clinical Hematology & Hemato Oncology,
Rajagiri Hospital, Aluva

Resource Persons (30 minutes including discussion)

1 Dr. Prasanna Kumari
Assoc. Professor, Dept. of Pathology, KIDWAI Memorial Institute of Oncology, Bangalore
Conventional cytogenetics in cancer diagnosis and prognosis

2 Dr. Hariharan S
Associate Professor, Division of Cancer Research, Regional Cancer Centre, Trivandrum
Molecular cytogenetics in cancer diagnosis and prognosis

3. Dr. Cornelia M. Wilson
Senior Lecturer & Academic Laboratory Director, Canterbury Christ Church University, UK
Role of exosomes in cancer diagnostics

Contributed papers (7 minutes presentation and discussion 10 minutes at the end)

1 Identification of Different Cytogenetic Groups in Acute Myeloid Leukemia: Combined Conventional And Molecular Cytogenetic Study
Akhila Raj T V: Regional Cancer Centre, Trivandrum

2 Telomere signal gain and loss-A progressive mode of genomic instability
Dr. Alex George: Jubilee Centre for Medical Research, JMMC & RI, Thrissur

3 The Importance of Cytogenetics and Fluorescent *In situ* Hybridization (FISH) In the Detection of BCR-ABL Fusion Gene in CML Patients
Priya G: Regional Cancer Centre Trivandrum

4 Genomic analysis of miRNAs in the diagnosis and management of prostate cancer
Dr.Vidyalakshmi S: PSG College of Technology, Coimbatore

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SYMPOSIUM ON

GENETICS OF FERTILITY

GENETIC FACTORS IN MALE INFERTILITY: DIAGNOSTIC AND PROGNOSTIC IMPLICATIONS

Shilpa Bisht, Manoj Kumar and Rima Dada
Laboratory for Molecular Reproduction and Genetics, Department of Anatomy, AIIMS, New Delhi

INTRODUCTION

Male subfertility or decreased fecundity solely or in combination with the female factor constitutes the major cause (approximately in 50% cases) of infertility which affects nearly 8-12% couples (in some regions peaking up to nearly 30%) in the reproductive age group [1]. Despite of the tremendous research which has been already done in the context of male factor infertility, nearly 30-40% of male infertility cases are found to be idiopathic where no male infertility associated factor is found (according to the latest guidelines on male infertility published in 2015 by the European Association on Urology). Male infertility could be a result of anatomical defects, immunological factors, ejaculatory failure, urogenital tract infections, gametogenesis dysfunction, increased scrotal temperature, environmental toxicity, gametogenesis dysfunction and genetic defects [2]. According to the World Health Organization (WHO) Laboratory Manual for the Examination and Processing of Human Semen (2015), the diagnostic evaluation of infertile men includes routine estimation of basic semen parameters (motility, morphology and count) [3]. The diagnosis based on WHO (2015) manual is a poor predictor of the cause of decreased fecundity in case of "normozoospermic infertile men" and also couldn't predict cases with idiopathic or unexplained infertility as they are present with or without abnormal semen parameters, respectively [4]. Oxidative stress (OS) plays a major role in sperm DNA damage as it causes damage to both mitochondrial and nuclear DNA and also affects the sperm epigenome, resulting in infertility, recurrent pregnancy loss, poor pregnancy outcomes and an increased disease burden in the offspring including childhood cancers and neuropsychiatric disorders.

Genetic testing in idiopathic male infertility patients holds an important aspect to decipher the etiology of male infertility and to develop clinical measures to improve the prognosis in these patients which could not be possible otherwise via routine semen analysis only [5]. Over 2000 genes are involved in normal spermatogenesis and fertility which is remarkably a very high percentage of the total human genome complement [6]. Most of the genes regulating spermatogenesis are present on the autosomes and approximately 30 genes are present on the Y chromosome [Table 1]. Spermatogenesis in mammals is a complex hormone-regulated process where numerous developmental programming took place to ensure that germ cells reach their proper developmental stage at the proper time. This developmental programming is regulated by the tempero-spatial expression of various transcription factors that regulate these events [7]. Various animal studies have deciphered candidate genes regulating male infertility phenotype. Telomerase enzyme has the potential to restore the telomere length and plays a crucial role in sperm telomere length maintenance in the male germ cells during spermatogenesis. Telomere- the ends of the chromosomes are the first site in the sperm genome that responds to the oocyte for pronucleus functioning and helps in synapsis, homologous recombination and segregation [8]. Telomeres which are evolutionary conserved hexameric tandem repeats are present in the sperm nuclear periphery and hence are more prone to OS induced injury [9]. Telomere also play an essential role in chromosome localization within sperm nucleus and oxidative stress injury in sperm genome results in DNA fragmentation, abnormal telomere-telomere interaction and abnormal embryonic development [10].

Sperm with longer telomeres help in the maintenance of species-specific telomere length in the next progeny and hence, compromised or shortened telomere length may be one of the causative factor for male factor infertility [11]. A number of intrinsic and extrinsic factors may help to regulate OS but they must be maintained at moderate levels for optimal sperm function and the maintenance of cellular homeostasis and redox-sensitive signal-transduction pathways. Simple lifestyle modifications and interventions can substantially reduce levels of testicular inflammation, OS and oxidative DNA damage and improve the quality of life of infertile couples.

Causative factors for male infertility

1. Chromosomal abnormalities: Chromosomal abnormalities (structural and numerical) are the most commonly evident (approximately 5%) genetic abnormalities in male infertility patients therefore, cytogenetic analysis (karyotyping) is most commonly used genetic test for evaluation of men with azoospermia and oligozoospermia (spermatozoa <10 million/ml) [12]. Sex chromosomal aneuploidy (Klinefelter syndrome; 47,XXY and its variants; 46,XY/ 47,XXY mosaic karyotype) are the most common and accounts for nearly 2/3rd of all the sex chromosomal aneuploidy found in infertile men [13]. It is generally associated with impaired spermatogenesis, impairment in Leydig cell function, small testicles, elevated serum FSH, normal or elevated estradiol levels and azoospermia. Sperm Fluorescence *In Situ* Hybridization (FISH) studies showed an increased frequency of sex chromosomal abnormalities and increased incidence of autosomal aneuploidy (disomy for chromosomes 13, 18 and 21) and therefore, concerns have been raised to perform

Table 1: Candidate genes for male infertility and the associated genotype/ phenotype

Candidate Gene	Genotype/ Phenotype
DAZL (Deleted in azoospermia like)	Linked to severe spermatogenic failure and infertility in males
USP9Y (ubiquitin-specific protease 9, Y chromosome)	Azoospermia
DBY (DEAD box on the Y)	Encodes for a putative ATP-dependent RNA helicase and involved in spermatogenic disruption
UTY (Ubiquitous TPR motif Y)	Contains 10 tandem TPR motifs that may be involved in protein-protein interactions and regulation of spermatogenesis
ADAM3 (a disintegrin and metallopep tidase domain 3)	Linked to impaired migration of sperm into oviduct
PRM 1 and *2* (Protamine 1 and 2)	Polymorphism is associated with poor chromatin compaction and abnormal spermatogenesis
TRF2 (TATA-binding protein (TBP) related factor 2)	Component of the core promoter complex required for gene/tissue-specific transcription of protein-coding genes by RNA polymerase II
TAF7 (TATA-binding protein (TBP) associated factor 7)	Associated with structurally abnormal sperm, reduced sperm count, weakened motility, and compromised fertility
SMCP (Sperm mitochondria associ ated cysteine rich protein)	Associated with reduced sperm motility and impaired fertilization
CREM (*c*AMP-responsive element modulator)	Linked to decreased postmeiotic gene expression and defective spermiogenesis

high-resolution karyotype in case of infertile men with Non-obstructive azoospermia or severe oligozoospermia before using their sperm to perform Intra cytoplasmic sperm injection (ICSI) due to the significant increase of sex chromosomal and autosomal abnormalities in the embryos of Klinefelter's patients [14]. Follow-up (possibly every year) of men with Klinefelter's syndrome is required and androgen replacement therapy should be started after fertility issues have been addressed and when testosterone level is in the range of hypoandrogenism. Autosomal translocations such as Robertsonian translocations, reciprocal translocations, paracentric inversions, and marker chromosomes are also 4-10 times more common in infertile men as compared to the general population and associated with an increased risk of aneuploidy or unbalanced chromosomal complements in the fetus [15]. Sex chromosome translocations is also associated with severe damage to spermatogenesis and hence, infertility [16]. Translocations involving a sex chromosome and an autosome are more capable of causing infertility than translocations involving autosomes. FISH, Pre-Implantation Genetic Diagnosis (PGD) and amniocentesis provides valuable information when IVF/ICSI is planned to be carried out for men with autosome or sex-chromosome translocations [17].

2. Y-chromosome microdeletions: Y chromosome (Yq) microdeletions designated as azoospermia factor (AZFa, AZFb and AZFc) are one of the most potent (one in every 2000-3000 infertile men) cause of male infertility and found in 10-15% of the infertile men with severe oligozoospermia or azoospermia. Males with Yq microdeletions usually have no obvious symptoms but physical examination may reveal small testes, cryptorchidism and varicocele. In each AZF region, there are several spermatogenesis candidate genes that play one or more essential roles during various stages of spermatogenesis and most of the deletions that cause azoospermia or oligozoospermia occur in non-overlapping regions of the long arm of the Y chromosome (Yq11) [18,19]. AZFa (proximal) region comprises two genes namely *DBY* (dead box on the Y) and *USP9 Y* (ubiquitin-specific protease 9, Y chromosome). *DBY* plays an important role in spermatogenesis and is involved in the development of pre-meiotic germ cells where as *USP9 Y* is expressed in spermatogonial stem cells and has a role in the efficiency of protein turnover in spermatogenesis. AZFb (central) region comprises two genes, the first one is *RBMY* (RNA binding motif on Y) which encodes for RNA binding proteins involved in splicing, metabolism and signal transduction pathways and the second one is *HSFY1* and *HSFY2* (Heat shock transcription factor, Y-linked) which has essential role during spermatogenesis. AZFc (distal) region harbors two essential genes namely *PRY* and *PRY2* which regulates apoptosis to remove abnormal sperm and *DAZ* (Deleted in azoospermia) which regulates translation, code for germ cell specific RNA binding proteins, controls meiosis, and maintains primordial germ cell population [17]. Deletions of AZFa and AZFb are usually associated with Sertoli-cell-only syndrome (SCO) and maturation arrest, respectively [20]. Partial or complete loss of AZFc region is associated with a highly variable heterogeneous phenotype ranging from severe oligozoospermia to azoospermia, and testicular histology showing Sertoli cell-only syndrome, maturation arrest or hypospermatogenesis. This is mainly due to presence of multiple copies of DAZ gene in AZFc and the presence of an autosomal homologue on 3p24 (*DAZLA*). Y-chromosome microdeletions is inherited in a Y-linked manner and any Y microdeletions are transmitted imperatively to the male offspring which makes genetic counselling very essential for male infertility patients with Yq microdeletions as they show higher risk of transmitting such microdeletions and hence, infertility to their male offspring [21]. The extent of spermatogenic failure (still in the range of azoo-/oligozoospermia) cannot be predicted entirely in the son, due to the different genetic background and the presence or absence of environmental factors with potential toxicity for reproductive function. When ICSI is used in the presence of a Y microdeletion, long-term follow-up of any male children is needed with respect to their

fertility status, and cryopreservation of spermatozoa at a young age can be considered [22].

3. Cystic fibrosis (CF) gene mutation: Cystic fibrosis transmembrane conductance regulator (*CFTR*) gene is having its cytogenetic location at 7q31.2 spanning a region of 190 kb with 27 exons associated with over 1500 mutations reported so far [23]. *CFTR* gene encodes for a glycosylated transmembrane protein which is a cAMP regulated chloride channel that regulates viscosity through sodium and chloride exchange in epithelial secretions of exocrine tissues such as the lungs, pancreas, sweat glands and vas deferens. *CFTR* gene mutations affects multiple organs, including pancreas, intestine, sweat gland, and the respiratory tract and associated with cystic fibrosis (CF) which is characterized by recurrent pulmonary infections, elevated sweat chloride, pancreatic failure, hepatic insufficiency, and other glandular defects [24]. The exact etiology of congenital bilateral absence of the vas deferens (CBAVD) is not known yet but various studies has revealed a well-established connection between CBAVD and CF as nearly 98% of CF males exhibit Wolffian duct abnormalities such as absence of the vas deferens, epididymis, seminal vesicles, or ejaculatory ducts [25]. With numerous *CFTR* gene mutations already reported in the literature, the most common one which is found in nearly 65-70% of the *CFTR* associated mutations is the deletion of a single amino acid, phenylalanine, at position 508 (ÄF508) of the *CFTR* gene product [26]. Even with complete *CFTR* gene screening, mutations may still be undetectable in 25% of CBAVD patients. Comprehensive analysis of the *CFTR* gene with DNA sequencing is reserved for patients with CF history or CBAVD. In CBAVD, which is classified as surgically unreconstructable obstructive azoospermia, microsurgical epididymal sperm aspiration is the optimal treatment with subsequent assisted reproductive technology [27]. If the male partner has CBAVD, it is important to test his female partner also for *CFTR* gene mutations. If the female partner is found to be a carrier of *CFTR* mutations, the couple must consider very carefully whether to proceed with ICSI using the male's sperm, as the couple has a very high risk of having a child with CF or CBAVD (nearly 50%), depending on the type of mutations carried by the parents. If the female partner is negative for the known *CFTR* mutations, the risk of being a carrier of unknown mutations is approximately 0.4% [28].

4. Kallman syndrome: Kallman's syndrome (OMIM 308700) or idiopathic hypogonadotropic hypogonadism is characterized by anosmia due to agenesis of the olfactory bulb. It is a rare disorder and found in 1 in every 10,000-60,000 live births [29]. Patients with classic Kallmann's syndrome may or may not experience puberty or may experience incomplete puberty and have symptoms associated with hypogonadism such as decreased libido, erectile dysfunction, decreased muscle strength, and diminished aggressiveness and drive. Kallman syndrome is inherited in an X-linked manner, is caused by mutation in the *KAL1* gene which encodes a cell adhesion molecule [30]. Autosomal dominant inheritance in Kallman's syndrome is caused by mutations in *FGFR1*, *FGFR8*, *PROKR2* and *PROK2* genes [31]. All patients with Kallmann syndrome have either anosmia or severe hyposmia and may exhibit symptoms of associated conditions including those of congenital heart disease (eg. fatigue, dyspnea, cyanosis, palpitations, syncope) or neurologic manifestations (eg. color blindness, hearing deficit, epilepsy, paraplegia) [32]. As many as 30% of the mutations found in *FGFR1* might be *de novo* mutations, certainly a possibility to be considered before assessing recurrence risk of this genetic form in a family. Genetic testing in Kallmann syndrome is based on patient's gender, familial history (if any) and putative mode of disease inheritance, and the presence of additional clinical anomalies that may direct the geneticist towards a particular disease gene or occasionally a contiguous gene syndrome. Treatment of Kallman's syndrome is that of the hypogonadism. There is currently no treatment for olfactory deficit. In both sexes, hormone replacement therapies are used to stimulate the development of secondary sexual characteristics at the time of puberty, and later to induce fertility [29].

5.Androgen receptor gene: The *androgen receptor (AR)* gene contains a polymorphic trinucleotide repeat that encodes a polyglutamine tract in its N-terminal transactivation domain. Different factors including environmental and genetic factors may cause alteration in sperm production, while several genetic studies have been recently conducted in this regard. The role of androgen as the main male hormone in determination of male sexual differentiation and male secondary sexual characteristics is well known [33]. Also, the initiation and maintenance of spermatogenesis is due to well action of this hormone. It is clear that androgen act on target cells with the help of AR. Locus of the *AR* gene is on X chromosome at position Xq11-12. The *AR* gene with 8 exons can produce AR with three following domains: i) Exon 1 encoding transactivation domain ii) Exon 2 and 3 encoding DNA-binding domains and iii) Exon 4-8 encoding C-terminal ligand-binding domain. Exon one has two polymorphic sequences known as CAG and GGN that contain three nucleotide repeats. The CAG repeats encode polyglutamine residues with different length in transactivation domains of the receptors. The CAG repeats are unstable and the number of their repeats may change during meiotic division. Many mutations in *AR* gene cause various degree of androgen resistance [34,35]. The functional consequences of variations in the GGC repeat are less clear, even if deletion of the polyglycine tract reduces AR transcriptional activity in transient transfection assay [36]. Epidemiological investigations on the association between the number of GGC repeats and prostate cancer risk have produced inconsistent results. Even if short GGC repeat length seems to increase the risk of the disease [37,38], no clear conclusions could be made. Only two studies reported the distribution of GGC lengths among the infertile men and found no difference from that in the general population [39,40]. Moreover, the effect of combined CAG and GGC repeats in spermatogenesis is largely found to be unknown.

6. Mitochondrial DNA mutations: Mitochondrial DNA (mtDNA) encodes for 37 genes which regulates oxidative phosphorylation. mtDNA is unique and differs from the nuclear DNA with respect to replication, repair mechanism, genome packaging and position. However, unlike nuclear DNA, mtDNA is not protected by histones and is physically associated with the inner mitochondrial membrane, where highly mutagenic oxygen radicals are generated as by-products of oxidative phosphorylation (OXPHOS) in the respiratory chain [41]. The leakage of the free radicals via mitochondrial OXPHOS makes mitochondria a major intracellular source and also a potent target of reactive oxygen species (ROS) induced free-radical damage. The unique features of mitochondria are probably due to 10-17 times faster accumulation of polymorphisms and mutation in mtDNA than in nuclear DNA. Sperm possess about 70-80 mitochondria in the midpiece [42]. Any mutation in mtDNA disrupts adenosine triphosphate (ATP) production and thus, result in an impaired spermatogenesis and impaired flagellar movement. Sperm mid piece has few mtDNA copies, thus enhanced number of mutant mtDNA results in early phenotypic defect which manifest as spermatogenic arrest or asthenozoospermia. OS and mtDNA mutations are positively correlated and mutations in mitochondrial genome are implicated in the lowered fertilizing capacity of the sperm and affects the reproductive potential of an individual [43]. Mutation and depletion of mitochondrial DNA (mtDNA) are associated with poor motility and diminished fertility of human sperm. Lack of an efficient repair system in mitochondria and abnormal mitochondrial metabolism may accelerate the rate of mitochondrial DNA mutation and therefore, higher amount of 8-hydroxy-2'-deoxyguanosine (8-OHdG) have been reported in mitochondrial DNA than in nuclear DNA of infertile patient [41]. Several single nucleotide polymorphisms in mtDNA have been reported in many of the diseases including male infertility [44]. Cell division in the testis may be the reason for the accumulation of tissue specific mosaicism at higher level in spermatozoa. PCR amplification of mtDNA has shown a higher incidence of mtDNA deletion in asthenozoospermic patients as compared with unaffected individuals. Men with idiopathic infertility have low levels of antioxidant protection and an increased number of mitochondrial

sequence variations [45]. Moreover, it is believed that mtDNA mutation may impair electron transport chain resulting in enhanced production of mitochondria ROS due to incomplete reduction of oxygen [46]. This excessive ROS production may induce the opening of membrane permeability transition pores and release of free radicals, Cytochrome C and other apoptogenic factors that ultimately lead to apoptosis [47]. Although mtDNA mutations have been identified in many studies, their role as a diagnostic marker in male infertility is still under debate. Nonetheless, male infertility due to mtDNA mutation can be successfully treated by ICSI, as paternal mtDNA mutations are not transmitted to the offspring.

CONCLUSION

Male factor infertility is an important cause of infertility in couples and hence, couldn't be ignored or underestimated. Especially in idiopathic male infertility cases where the exact cause is not known, in addition to the routine tests such as detailed and precise medical history, physical examination, routine semen analysis, genetic tests such as karyotype analysis, Y-chromosome microdeletions, AR gene polymorphism, *CFTR* mutation analysis, mtDNA mutation screening are also administered so as to obtain a correct diagnosis and to determine the best treatment strategies. Genetic counselling could also be offered in case when either the male or female partner is suspected to have a genetic abnormality and a higher risk of having genetic disease in the offspring. Novel molecular sperm diagnostic approaches are also emerging which utilizes the application of proteomics, transcriptomics and metabolomics to analyze sperm whole genome and provides a more comprehensive analysis of the biochemical basis of defective semen quality. The goal of all the diagnostic techniques used for the evaluation of infertile men is based on providing benefits to the patients which could be easily implemented from laboratory and the clinic as well. The available evidence suggests that OS is central to the etiology of male infertility but the exact effects of OS on oxidative DNA damage in spermatozoa have not yet been comprehensively investigated. Standard semen parameters are poor predictors of reproductive outcomes. Therefore, a need exists to develop other cost-effective laboratory techniques for the estimation of seminal levels of OS and oxidative DNA damage. Antioxidant scavenging systems have an important role in the inactivation of ROS and various antioxidant therapies and various combinations of antioxidants, such as vitamin C, vitamin E, selenium, zinc and glutathione, have long been used as treatments of male infertility. A large number of studies have already been conducted to determine the efficacy of antioxidant supplementation in the treatment of male infertility, in terms of improving both the rates of fertilization and pregnancy outcomes. But there are many studies which documented that indiscriminate and prolonged usage of antioxidants is rather harmful and associated with the generation of "Reductive Stress" impairing numerous redox sensitive metabolic processes. Therefore, the present therapeutic approaches for the treatment of male infertility includes development of simple lifestyle interventions such as yoga and meditation that have prolonged impact on the sperm genome and epigenome. Yoga and meditation also improves cardiovagal tone and increases perfusion to hippocampus, cingulated gyrus and cerebral cortex and reduced levels of cortisol which further aids in reduction of OS and its associated sperm DNA damage. In a previous study done in our lab, we have documented that men having children with retinoblastoma, the most common intraocular cancer might have an ongoing OS damaging the sperm DNA, and hence at an increased risk of having subsequent children with imprinting disorders and other health issues. Controlling or reducing sperm oxidative DNA damage through yoga and meditation hence might not only preserve their fertility but also reduce the risk of imprinting disorders such as retinoblastoma as OS is associated with aberrant methylation patterns. OS and oxidative DNA damage are associated with poor lifestyle habits and environmental exposures, therefore adoption of a healthy lifestyle, possibly including lifestyle-related interventions like yoga and meditation, might help in reducing the risk of oxidative DNA damage and improve sperm function and thereby, reduce the incidence of male factor infertility and childhood disease burden in the next generation.

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IMPORTANCE OF GENETIC STUDIES IN FEMALE FERTILITY

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INTRODUCTION

Infertility is a condition with psychological, economic, medical implications resulting in trauma, stress, particularly in a social set-up like ours, with a strong emphasis on child-bearing. According to the International Committee for Monitoring Assisted Reproductive Technology, World Health Organization (WHO), infertility is a disease of reproductive system defined by failure to achieve the clinical pregnancy after 12 months or more of regular unprotected sexual intercourse. It can also be defined as failure of couple to conceive after 12 months of regular intercourse without the use of contraception in women <35 years; and after 6 months of regular intercourse without the use of contraception in women ≥35 years. The term subfertility is used if there is a remaining chance for natural conception and child birth, for example cases with repeated abortions or cases with oligozoospermia.

Global incidence of infertility

There are no reliable figures for global incidence of infertility however as the WHO estimates 60-80 million couples worldwide suffer from infertility. The Worldwide prevalence of infertility ranges from 3% to 16%. It was reported that ~ 40% of infertility cases were related to men, ~40% to women and ~ 20% to both sexes. In addition, over 25% of infertility cases, no detectable cause can be traced after routine tests, which leaves the case as unexplained infertility.

Despite the prevalence of male and female infertility the research on genetic causes has expanded rapidly in last few years. This is attributed to the development of *in vitro* fertilising techniques. Development of ICSI has raised major concerns about safety for offspring's as it bypasses the mechanism of natural selection. Genetic tests are now available to explore the cause of the infertility and assess the risk of a given couple to transmit its genetic characteristics. This allows at-risk couples to take an informed decision when electing for a medically assisted reproduction. It also allows the professionals to offer a prenatal diagnosis when appropriate. Thus, the genetic work-up of the infertile couple has become good practice for an appropriate diagnosis, treatment and prognostic assessment.

Genetic counselling

Genetic counselling must always be a part of extensive evaluation of infertile couples. Genetic counselling focuses on extensive medical and family history evaluation and is required in order to offer appropriate genetic testing to the couple. Genetic counselling provides the couple with background of testing, possible consequence for the carriers and potentially affected family members. While taking history the existence of syndromal genetic disease or multifactorial features should be assessed. The disclosure of genetic testing results has to be done in person. Genetic counselling can be offered in normal results but is mandatory if there are significant findings.

Genetic Testing

There are various tests that are available and which can be offered either independently or in combination depending on the history [Table 1].

Causes of Female Infertility

Causes of female infertility include ovulatory disorders, chromosomal abnormalities, endometriosis, pelvic adhesions, tubal blockage and other tubal abnormalities, and hyperprolactinemia. There is growing evidence that genetic abnormalities are present in as many as 10% of infertile females. There is also evidence to suggest that chromosomal abnormalities and single gene mutations contribute to the cause of infertility in a proportion of couples seeking infertility therapy.

Table 1. Type of genetic tests

Sr. No	Type of genetic test	Detected by light microscopy	Resolution (DNA base pair)
1	Cytogenetics	Yes	≥ 5 mb
2.	Molecular cytogenetics	Yes Indirect diagnosis (Fluorescence microscopy)	≥ 1 kb
3.	Molecular	No	≥ 1 bp

Genetic causes of female infertility include ovulatory disorders such as Kallmann syndrome, fragile 'X' syndrome, as well as karyotype abnormalities, and primary ciliary dyskinesia. Infertility can also be noted as a minor manifestation in many other genetic conditions including galactosemia, mucopolysaccharidosis, Prader-Willi, cystic fibrosis, pseudohypoparathyroidism type 1a, progressive external ophthalmoplegia, autoimmune polyglandular syndrome type I, ovarian leukodystrophy, ataxia telangiectasia, Demirhan syndrome, and blepharophimosis-ptosis-epicanthus inversus syndrome.

In addition to the single gene defects noted earlier, additional rare genetic abnormalities have been identified in infertile women. These include mutations in the follicle-stimulating hormone (FSH), luteinizing-hormone (LH), and gonadotropin-releasing hormone (GnRH) receptors, and mutations in the androgen receptor causing androgen insensitivity syndrome.

Genetic causes of Infertility

Chromosomal abnormalities are of interest in fertility treatment because chromosome disorders occur in more than half of all first-trimester pregnancy losses. Many of these early miscarriages are due to the random occurrence of a chromosomal abnormality in the embryo. Peripheral blood karyotype is strongly recommended during diagnostic workup of cases with infertility, primary ovarian dysfunction or recurrent fetal loss prior to offering any ART procedures. Chromosomal abnormalities are classified as either sex chromosomal (numerical and structural) or autosomal abnormalities.

Sex chromosomal abnormalities

The phenotype of women affected by sex chromosomal aberrations is highly variable, in terms of external and internal genitalia and physical features. However, a feature shared by all these chromosome imbalances is primary ovarian dysfunction (hypergonadotropic) with primary or secondary amenorrhoea (including Premature Ovarian Failure) or oligomenorrhoea.

Turner syndrome is the most common chromosomal abnormality in infertile women but also a variety of structural autosomal aberrations are also found. Diagnosis of 45,X karyotype is often delayed or some are diagnosed when they present with primary ovarian failure or primary amenorrhea. These cases usually have normal intelligence. Another important aspect for prognosis is presence of genetic mosaicism [Figure 1]. The high incidence of mosaicism in Turner syndrome of 50% is quite remarkable. Thus, apart from 45,X cell lines, also cell lines with structural alterations of the X chromosome (39%), the Y chromosome (6%) [Figure 2] and numerical alterations (7%) can occur. In some mosaicisms, for example mos 45,X/ 46,XX, germ cell formation is functional, which allows for a normal or only slightly impaired fertility. If the chromosomopathy is detected early, the patient should be informed about the high risk of premature ovarian failure and thus be recommended to procreate early in life. Pregnant women with Turner syndrome or mosaicism have an empirical chance of 15% to have children with 45,X monosomy. A mosaicism mos 45,X/ 46,XY is associated with a high risk for gonadoblastoma, thus, gonadectomy should be considered. The 47,XXX karyotype has an incidence

of 1 in 1000.Two thirds of the carriers have a clinically normal phenotype. One third has learning difficulties and psychotic disorders. Premature ovarian failure with infertility has been described for carriers of this chromosomal abnormality. In women with XXX karyotype, the risk for the offspring to carry X chromosomal aneuploidy is < 1%.

Structural alterations involving the X chromosome increase the risk for premature ovarian failure and infertility. The loss (deletion) of the short arm of the X chromosome results in the typical clinical picture of Turner syndrome. The chromosomal findings can vary from a simple loss of the short arm (monosomy Xp) to its partial loss or the loss of the short arms due to the fusion of the long arms of the X chromosome at the centromere. The latter results in the karyotype of monosomy Xp combined with trisomy Xq (Isochromosom Xq).

Structural chromosomal abnormalities

The structural chromosomal abnormalities include translocations, deletions and duplications. The most common structural alterations are translocations, which are further subdivided into balanced (chromosomal exchange without loss or gain of genetic material). Balanced translocations involving two autosomes occur in 3.35% of all newborns. These are further distinguished into:

1. Robertsonian translocations, in which two acrocentric chromosomes fuse (incidence 1.35%).
2. Classical balanced translocations (incidence 2.0%). Balanced translocations, hampering the normal course of meiosis, are increasingly found in the patient population with infertility or subfertility. In balanced translocations, the chromosomes form translocation quadrivalents instead of bivalents, which can cause

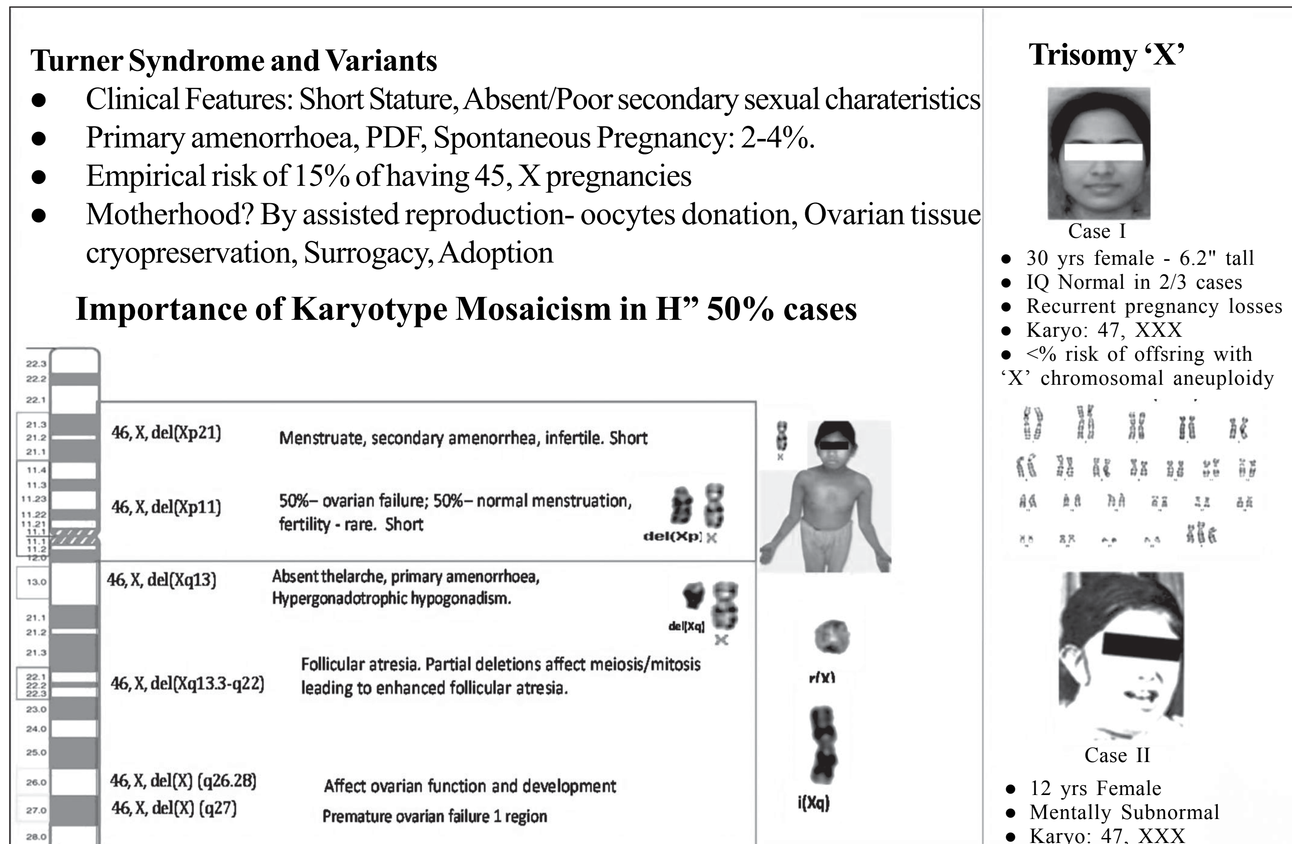

Figure 1. Turner syndrome and variants

chromosomal segragation problems and germ cell arrest. Amongst all infertility patients females are less affected bytranslocations than males. Translocations can very often lead to abortions due to chromosomal imbalances.

Molecular genetic testing

FMR1 gene (Fragile 'X' syndrome)

This is the most common cause of mental retardation in males and it is caused by the expansion of the CGG trinucleotide repeat in exon 1 of the FMR1 gene located in Xq27.3 [Figure 3]. Mutations in the FMR1 gene located in the long arm of the X chromosome (Xq27.3) are differentiated into full mutations, premutations and intermediate alleles. Full mutations in the male cause the fragile X syndrome (FXS) with mental retardation and other associated symptoms. In the female, the risk for learning difficulties or minor mental disability is increased. Premutations in the female correlate with infertility as well as with an increased risk for late onset progressive neurodegenerative diseases. Normal alleles have 5-49 repeats, intermediate alleles 50-58, premutations 59-200 and full mutations > 200. An intermediate allele has a low risk of expansion (~ 6.6%) and its expansion to a full mutation has not been described yet. A full mutation develops due to transmission of a premutation from the mother with the subsequent expansion of CGG repeats. A full mutation results in the hypermethylation of the surrounding DNA region with a more or less complete transcriptional gene silencing and thus a missing gene product. In contrast, premutations result in an overexpression of the gene. The prevalence of full mutation is rather low in women. It should be emphasized that carriers of premutations have an increased risk for male offspring with fragile X syndrome and an increased risk for female progeny with learning difficulties or slight mental impairment. Furthermore, premutation has been shown to be

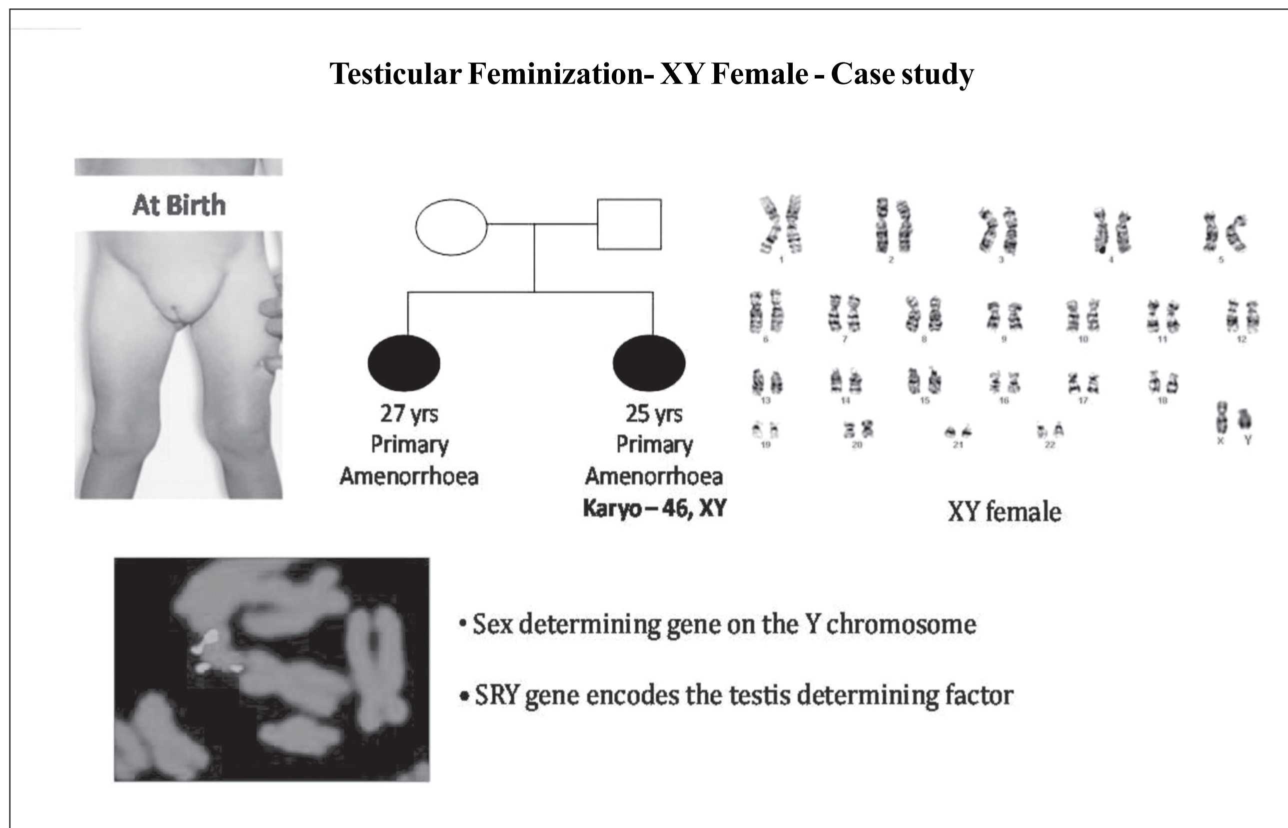

Figure 2.Testicular Feminization-XY female

associated with low response to ovarian stimulation during *in vitro* ART.

21-hydroxylase deficiency (CYP21A2 gene) – adrenogenital syndrome

21-hydroxylase deficiency by a gene mutation can be a monogenic cause of infertility and often results is polycystic ovary syndrome in women. Approximately 95% of all androgenital syndrome cases are caused by the autosomal recessive transmission of 21-hydroxylase deficiency caused by a mutation in the CYP21A2 gene located on chromosome 6. The disease is further classified into the classic, severe type (two severe mutations) and the nonclassic type – also known as late-onset androgenital syndrome.The latter comprises either two alleles with mild mutations or compound heterozygosity (two alleles with different mutations of the CYP21A2 gene) with one allele carrying a mild and the other a severe mutation. The prevalence of the classic type is 1:10000 to 1:15000. Late-onset androgenital syndrome with usually > 20% enzymatic activity has a prevalence of 1 in 1000. In the context of subfertility/infertility of the female, late onset androgenital syndrome is of importance, as the classic form can already be diagnosed in child or infancy stages (newborn screening). Apart from polycystic ovaries and menstrual disorders, secondary amenorrhea or oligomenorrhea, hirsutism and acne as well as increased DHEAS and 17-OH progesterone blood levels in the follicular phase of the cycle are typical symptoms of late-onset androgenital syndrome. Where there is reason to suspect late-onset androgenital syndrome, molecular diagnostics should be performed in order to exclude or confirm mutations in the CYP21A2 gene. If the patient is found to be homozygous or is a compound heterozygote, the partner should be tested.

CFTR gene

Mutations in the CFTR gene have not been unequivocally associated with reduced female infertility. However, women affected by cystic fibrosis are subfertile and have a higher risk of complicated pregnancy.

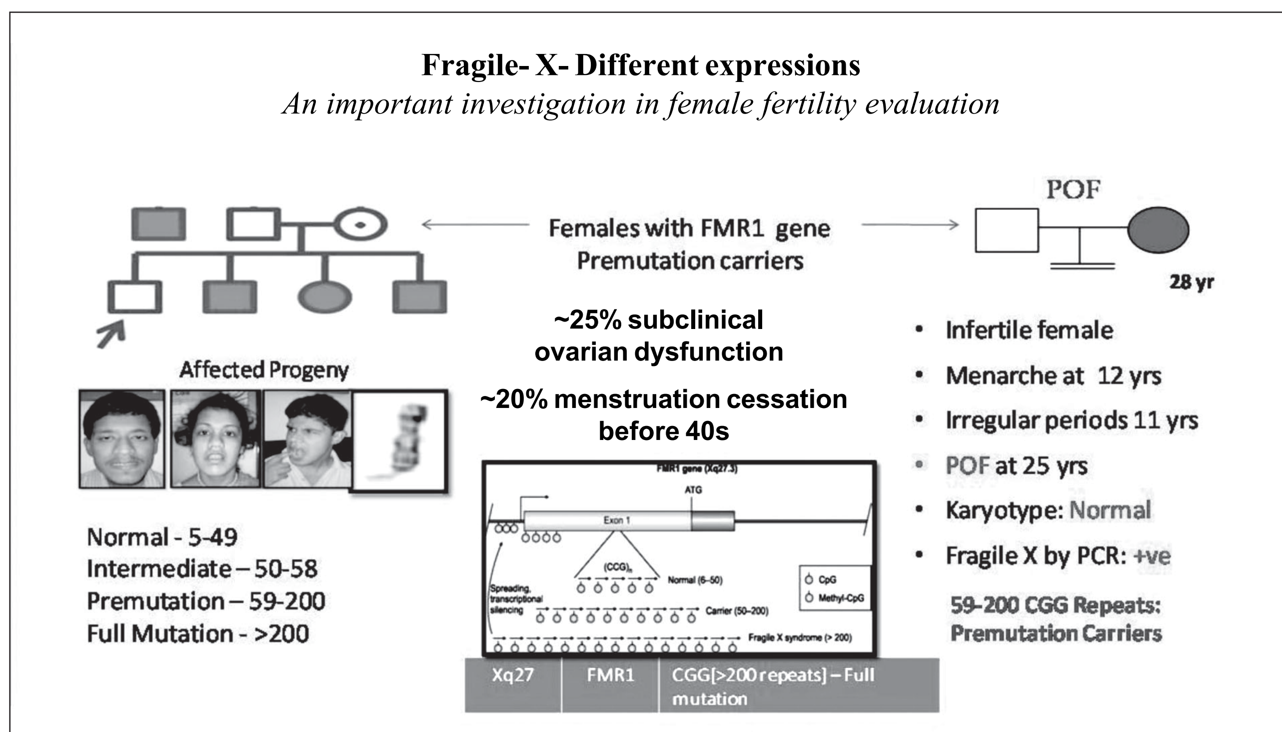

Figure 3. Fragile-X-Different expression

The prevalence of the CFTR mutation in the general population (1:25), and the higher probability of a CFTR mutation to occur in the male partners of ICSI couples, recommend this test in the female partners candidate to ART. Screening for CFTR mutations (including the 5T allele) is strongly recommended in infertile individuals with a diagnosis of bilateral or unilateral congenital absence of vas deferens (CBAVD or CUAVD).

Whenever the couple is planning a pregnancy by ART, this test should be performed in both the affected male and his partner.

KAL1 gene

Kallmann syndrome due to mutation in the KAL1 gene is exceedingly rare in women, resulting in primary amenorrhoea with hypergonadotropinism and anosmia. A higher incidence of uterine malformations has been also reported. Heterozygous females have no discernible abnormalities.

Array CGH

Array comparative genomic hybridization (a-CGH) analysis is also being researched on cohorts of women with premature ovarian failure for analysis of copy number variants (CNVs) over the genome. A number of potential candidate genes including *PTHB1* and *ADAMTS19* have been identified in this fashion, although larger follow-up studies are needed to confirm these findings.

CONCLUSION

Genetic testing must be offered to all couple's undergoing infertility treatment. This must be along with appropriate genetic counselling. Evaluation of the abortus material is important for psychological support of the couple and of particular importance in offering prognosis for the next pregnancy.

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ASSOCIATION OF IRS2 GENE GLY1057ASP POLYMORPHISM WITH POLYCYSTIC OVARIAN SYNDROME: A HOSPITAL BASED STUDY

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Introduction

Polycystic ovarian syndrome (PCOS) is one of the most common endocrine disorders affecting 5-10% women of reproductive age group and is the leading cause of female infertility [1]. PCOS represents the major cause of anovulatory infertility and is associated with distressing cutaneous manifestations of androgen excess, such as hirsutism, acne & acanthosis nigricans [2]. Moreover, obesity as well as insulin resistance co-occurs with the syndrome [3]. Obesity is seen in about 70% of patients, while insulin resistance is seen in 84-94% of patients. Also low levels of high density lipoproteins (HDL) are seen in about 55% of the patients. Hence, it is a life-long condition with adverse reproductive as well as metabolic implications in affected women [4]. The familial clustering of PCOS is well documented in several studies & this indicates genetic components of its pathogenesis. Approximately 20-40% of the first-degree female family members of patients with PCOS are also diagnosed with the syndrome. Moreover, associated co-morbidities, such as hyperandrogenemia, hyperinsulinemia and disturbed insulin secretion, also cluster within these families of patients with PCOS [5]. Finally, high heritability ($h^2 = 0.7$) in a Dutch twin study. PCOS seems to be more common amongst sisters of monozygotic twin pairs compared to dizygotic twins indicating a high degree of heritability in patients with PCOS [6]. Phenotypic variation that can be attributed is additive. The PCOS phenotype can be structured in three components: manifestation of anovulation, hyperandrogenism and the metabolic syndrome which includes hyperinsulinemia secondary to insulin resistance. Nonetheless, the mode of heritance of PCOS remains unclear, and both dominant and multigenic modes of transmission have been proposed.

Insulin resistance (IR) is now known to be intrinsic to this disorder, contributing in a major way to its pathogenesis. It is known that IR progresses towards the development of compensatory hyperinsulinemia, which drives hyperandrogenemia in these women. Several evidences suggest hyperinsulinemia to be the primary factor contributing to the ovarian hyperandrogenemia. Pharmacological infusion of insulin levels has been found to improve hyperinsulinemia as well as hyperandrogenemia and restore ovulation in the women with PCOS. However, reduction of androgen levels by bilateral oophorectomy or administration of GnRH agonist or/ and androgenic compounds were seen to have no effect on IR or hyperinsulinemia in the PCOS women. This would have been expected if hyperandrogenemia was the cause of hyperinsulinemia. Insulin can augment ovarian steroidogenesis directly, alone and/ or augment LH-mediated androgen production [7].

The candidate genes involved in insulin secretion and action are *IGF2, IRS1*, and *IRS2*. IRS proteins are critical for insulin mediated signal transduction in insulin target tissues. Two common SNPs in IRS particularly, Gly972Arg in IRS-1 and Gly1057Asp in IRS-2, have been shown to influence the susceptibility to IR in PCOS and T2DM. With review of literature we came to know that association studies with type 2 diabetes have shown that Gly1057Asp SNP of IRS-2 is prevalent in the Indian population while Gly972Arg SNP of IRS-1 is absent [8]. In contrast to the meta-analysis, association studies in Indian population have shown negative correlation between Gly972Arg IRS-1 and IR in PCOS [9]. In this light there is a possibility that IRS-2 might be involved with IR in PCOS. However, there are no studies exploring the association between

Gly1057Asp of IRS-2 SNP and IR in PCOS. Thus we aimed to study the role of Gly1057Asp IRS-2 (rs1805097) SNP in insulin resistance among polycystic ovarian syndrome patients.

Study design and patient recruitment: A case-control design was adopted for the study. A total of 30 PCOS and an equal number of women with regular menstrual cycle were enrolled from the Department of Obstetrics and Gynaecology of Sri Devaraj Urs Medical College during Aug 2015 to July 2016. The study was approved by the Institutional Ethics Committee. Informed consent was obtained from each participant before enrolment. All the participants were subjected to physical examination, serum glucose level, thyroid profile to rule out thyroid disorders, hormonal assay of FSH, LH, Prolactin was done. PCOS was diagnosed with oligo anovulation & ovarian PCOS morphology.

Sample collection & DNA isolation: 3 ml of peripheral venous blood was collected in EDTA tube under aseptic condition and stored at 4°C till further analysis. DNA was isolated from the peripheral blood lymphocytes by using salting out method. The quality and concentration of the DNA samples were assessed by spectrophotometry (Perkin Elmer Lambda 35 model).

Genotyping IRS-2 G1057D: Genomic DNA was amplified by polymerase chain reaction on Bio Rad C1000 Touch Thermal Cycler. The primer pairs used were 5'-AGC TCC CCC AAG TCT CCT AA- 3' and 5'-GGC CAC ACC AAA AGC CAT C-3'. The PCR product was analyzed on 2% agarose gel. The 292 base pair amplicon was subjected to restriction digestion with 10 units of *Hha I* (New England Biolabs, USA) and analyzed on 2% agarose gel with ethidium bromide staining. The Gly1057 allele produces three fragments measuring 196, 70 and 26 base pairs. Asp1057 allele produces two fragments measuring 266 and 26 base pairs. Heterozygous samples will produce four fragment measuring 266, 196, 70 and 26 base pairs. The 26 and 70 base pair fragments were not visible on the gel due to their small size. The 196 and 266 base pair fragment were therefore used as reference to decide the genotype [8]. 20% of the samples were repeated and the results were found to be 100% concordant. The agarose gel image showing RFLP patterns are shown in the Figure 1.

Statistical methods: Sample size was calculated using the Open Epi web tool considering 95% confidence interval and 80% power [www.OpenEpi.com, updated April 04, 2013, accessed May 23, 2016]. Statistical analysis was done using the Statistical Packages for Social Sciences software (SPSS, Windows version release 13, SPSS Inc., Chicago, Illinois, USA). Allele and genotype frequencies of the two groups were compared using relevant contingency tables. Difference between the groups was determined by using Fisher's exact test. P-values ≤ 0.05 were considered as statistically significant. All statistical tests were two-tailed. The study population was tested for conformity to Hardy-Weinberg equilibrium using a web program [10].

RESULTS

Clinical parameters of the study population are given in Table 1. The results of the genotype analysis are shown in Table 2. The allelic frequencies of the SNP among cases and controls are given in Table 2. Frequency of the common allele in homozygous condition (Gly/Gly) was found to be 61.7% in cases and 38.3% in controls. Heterozygous combination (Gly/Asp) was 36% among the cases and 31% in controls. It was found that the occurrence of homozygous Asp/Asp genotype among PCOS subjects is almost two times that of control group (20% and 11% respectively). Control group was tested for conformity to Hardy-Weinberg equilibrium by chi-square test and was found to be in Hardy-Weinberg equilibrium. During screening we found 54% of patients & 57% patients have multiple cysts at right & left ovary respectively. Further 15 of the patients have Hirsutism score ≥10 who are also having impaired glucose metabolism. It is interesting to know that only 2 patients have hirsutism score more than 10, impaired glucose metabolisers & homozygous Asp/Asp.

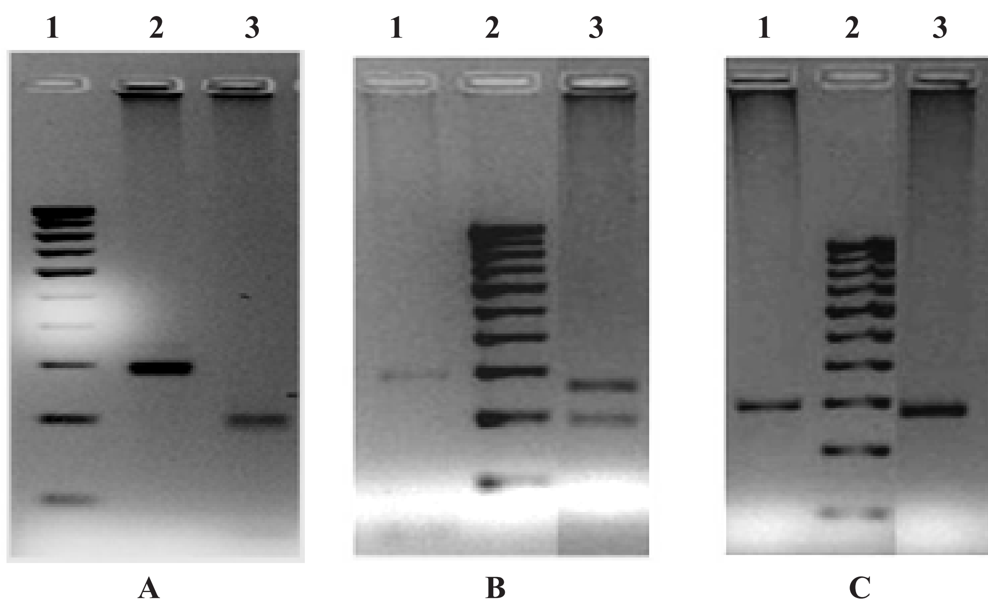

Figure 1: Representative agarose gel image showing RFLP pattern. A. RFLP pattern for Gly1057Gly genotype. Lane 1, 100 base pair ladder; lane 2, PCR amplicon (292 bp); lane 3, *Hha* I digestion product (196 bp). B. RFLP pattern for Gly1057Asp genotype. Lane 1, PCR amplicon (292 bp); lane 2, 100 bp ladder; lane 3, *Hha* I digestion product (196 + 266 bp). **C.** RFLP pattern for Asp1057Asp genotype. Lane 1, PCR amplicon (292 bp); lane 2, 100 bp ladder; lane 3, *Hha* I digestion product (266 bp).

Table 1: Clinical profile of PCOS patients

Parameter	N	Min.	Max.	Mean ± S.D.
Age	30	19	33	26.2±4
Pulse	30	68	88	78±5
SBP	30	116	134	124±6
DBP	30	70	88	80±4
Hirsutism score	30	02	24	11±5
Height	30	145	162	151.93±4.19
BMI	30	19.44	32.03	25.6±3.2
FBS	30	70	136	93.93±16.65
PPBS	30	122	167	138.87±12.52
FSH mIU/ml	30	3.90	11.20	6.39±1.92
LH mIU/ml	30	3.84	12.02	8.16±2.33

DISCUSSION

Meta-analyses involving association studies from various ethnicities indicate that Gly972Arg IRS-1 to be associated with IR in PCOS rather than Gly1057Asp. However, association studies in the Indian population have failed to show any correlation with IR in PCOS and Gly972Arg IRS-1. Furthermore, IR also occurs among T2DM patients. These IRS SNPs have been studied in the Indian T2DM patients who show IR. It is interesting to note that in case of T2DM IR, no association was found with Gly972Arg IRS-1 but a positive relationship was evident in the case of Gly1057Asp IRS-2. The results obtained from our study do not show any statistically significant difference in the distribution of Gly1057Asp SNP alleles between cases and controls. The results of our study appear to be in agreement with association studies reported from other populations [11]. However, it is not possible to confirm the conclusion at this stage as IR was not common in the study population. In the present study, all the patients were tested for fasting blood glucose level. We found that only two of the PCOS volunteers showed hyperglycemia. Thus, in the present group only 27% of the participants were potentially insulin resistant.

Table 2: Allele and genotype frequency of the study population

Allele/ Genotype	Cases (n = 30)	Controls (n = 30)	P - value
Gly	37(61.7%)	43(71.3%)	0.33
Asp	23(38.3%)	17(28.75)	
Gly/Gly	13(44%)	17(58%)	0.57
Gly/Asp	11(36%)	9(31%)	
Asp/Asp	6(20%)	4(11%)	

Calculation of insulin resistance also requires measurement of fasting insulin levels which is still in progress. It is interesting to note that the volunteers who were positive for hyperglycemia were homozygous for Asp1057 allele (Asp/Asp). Therefore it is difficult to rule out an association between IR in PCOS and IRS SNPs unless a larger study is performed taking only the subset of patients who are clinically confirmed to be suffering from hyperglycemia.

Till today, no gene polymorphism has emerged as universally accepted susceptibility gene polymorphism for PCOS. The conflicting results noticed in various studies might be attributed in part due to discrepancy in the diagnostic criteria, small sample size, failure to replicate results in independent studies, inadequate number of genes and their variants analyzed, presence of clinical heterogeneity among PCOS patients and ethnic variations between the populations. To overcome this, uniformity in diagnostic criteria of PCOS and sub-classification of cases according to clinical phenotypes like their IR status may provide further insights into the understanding of the molecular aspect of IR in PCOS.

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SCREENING FOR CONTIGUOUS GENE DELETIONS IN CHROMOSOME 15q15.3 AMONG INFERTILE MALES FROM CHENNAI - A PILOT STUDY

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INTRODUCTION

Infertility is defined as a couple's inability to conceive after one year of unprotected intercourse. It is estimated that globally, 60–80 million couples suffer from infertility every year, of which probably 15–20 million cases are in India alone [1]. Globally it affects 10% of couples, of which approximately 50% is attributed to a male factor [2]. Male fertility depends on successful spermatogenesis involving a number of genes. So, it is evident that deletions or mutations in genes controlling spermatogenesis would result in infertility [3]. It is estimated that a genomic defect contributes to nearly 50% of male factor infertility [4]. To this date ~400 genes have been implicated for male infertility [5]. The standard clinical evaluation for male infertility includes a complete medical history, physical examination, hormone level measurement and semen analysis. Male infertility manifests as chromosomal, mitochondrial, monogenic and multifactorial disorders of genetic origin. One of the most significant pathogenetic defects associated with male infertility are microdeletions commonly occurring in Yq11.23, at Azoospermia Factor (AZF) locus, which is further subdivided into AZFa, AZFb, and AZFc regions [6].

A number of syndromes often co-segregate with infertility and more than 70 such syndromes have been identified. In some syndromes, infertility becomes the primary problem and other features may be notable even well before the onset of puberty [7]. In several cases, infertility may be a part of one or more symptoms in unrelated physiologic systems, arising from a common genetic etiology. Deafness and Infertility Syndrome (DIS) is one such rare condition which is characterized by a contiguous gene deletion of the *CATSPER2* and *STRC* genes on chromosome 15q15.3 [8]. *CASTPER2* the cationic channel protein plays a vital role in hyperactivation of the sperm during fertilization. Mutations in this gene cause defects in motility and morphology of sperm and thus result in infertility [9]. The severity of hearing loss in DIS varies from mild to profound and may even be normal hearing. Owing to this variability in hearing loss, we attempted in this pilot study to screen the infertile males with apparently normal hearing for DIS contiguous gene deletion.

MATERIALS AND METHODS

A total of fifty infertile men attending Iswarya Women's Hospital & Fertility Centre, Adyar & Prashanth Fertility Research Centre, Nungambakkam in Chennai were recruited for this study. Information relating to family history, social habits and laboratory investigations were collected using a predesigned questionnaire. This study was approved by the Institutional Human Ethical Committee and informed consent was obtained from all the participating members. Genomic DNA was extracted from 10ml of blood sample by standard PCI method [10]. Effort was taken to ensure that none of the subjects complained of any hearing loss. Cytogenetic analyses were performed to exclude any numerical/ structural chromosomal abnormalities in the probands.

Multiplex PCR was also carried out using specific primers to screen for Y chromosome microdeletions (AZFa, AZFb and AZFc). Subsequently the probands were subjected to DIS contiguous gene deletion screening by multiplex PCR using STS markers D15S784 and REN37386 ; besides beta actin as a control primer [11].

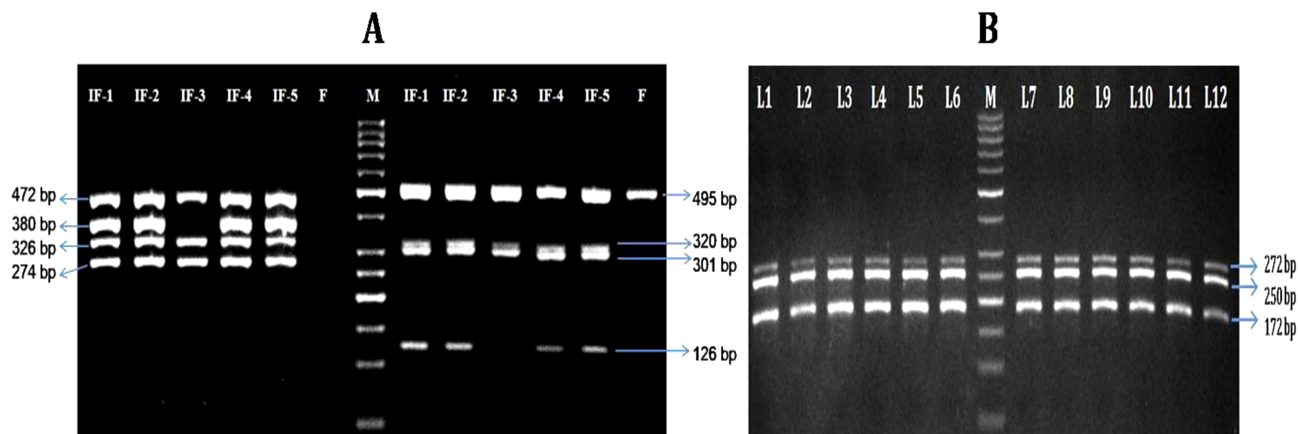

Figure 1: (A) Multiplex PCR products of STS markers on chromosome Y. Lanes IF-1 & 2 and IF-4 & 5 shows the amplification of markers, at AZFa, AZFb, AZFc and ZFY regions, that indicates the absence of Y chromosome microdeletion in four infertile subjects. Lane IF-3 did not show the amplification in markers sY254 and sY255 which indicates the presence of microdeletion at AZFc region. Lane F - female DNA. (B) Lanes L1 to L12 shows the amplification of the markers, D15S784 (172bp) and REN37366 (250bp) that indicates the absence of DIS deletion. The 272bp band represents beta actin gene amplification that was used as a control. Lane M - 50bp marker.

RESULT

Family history revealed that 11 out of 50 probands were consanguineously married. The mean age and duration of infertility of these Tamil speaking probands were 35.24 and 8.4 years respectively. Semen analysis showed azoospermia in 17, asthenozoospermia in 12, oligozoospermia in 5, teratozoospermia in 2, oligoasthenozoospermia in 7, oligoastheno-teratozoospermia in 5 and cryptozoospermia in 2 probands.

Screening for Y chromosome microdeletions showed deletion of two STS markers (sY254 and sY255) corresponding to AZFc locus in one proband with a frequency of 2% [Figure 1A]. None of the probands showed DIS contiguous gene deletion, thus excluding this as the etiologic factor for their infertility [Figure 1B].

DISCUSSION

To date, most of the male infertility studies related to Yq microdeletions are centered on the azoospermia factors (AZFa, AZFb, AZFc), which are considered to contain genes most important for spermatogenesis. It represents the most common structural chromosomal anomaly associated with non-obstructive azoospermia and severe oligozoospermia. Y chromosome microdeletions are reported to occur in 7% to 13% among infertile men worldwide [12-14] and in India it ranges from 3 to 29.34 % with an average frequency of 8.1% [15]. The removal of candidate genes from the AZF locus has variable clinical consequences. AZFc deletions are the most frequently observed in a majority of the Y chromosomal microdeletion screening studies. This may be due to the presence of a large number of repetitive sequences compared to another locus [16]. In this study we also observed only the deletion in AZFc region correlating with the previous studies.

This is the first attempt to screen for DIS (*CATSPER2* and *STRC*) deletion among a random cohort of infertile males with apparently normal hearing. Deafness and infertility syndrome is a rare contiguous gene deletion syndrome with a frequency of 1 in 40,000 individuals [8]. Males with this condition produce sperm that have decreased movement (motility), causing affected males to be infertile. An important gene associated with DIS is *CATSPER2* (Cation Channel Sperm Associated 2) which provides instructions for making protein

channels that regulate the entry of calcium cations into sperm cells through plasma membrane. This deletion was first identified in three French sibs with ~70-kb deletion in chromosome 15q15, which removed the entire *STRC* gene and the last 2 exons (225 bp) of the *CATSPER2* gene [9]. Various deletion sizes (90kb & 62kb) with additional co-morbidities like mental retardation, short stature; dysmorphic features and macrocephaly were reported [8,17]. Since its discovery, there are only 10 studies worldwide reporting this deletion mostly as sparse cases [8, 18, 19].

Jaiswal *et al.*, reported a case study with this deletion in two infertile brothers from North India [20]. The size of the deletion varies (193kb & 174kb) among the two brothers. Although they had DIS deletion none of the brothers showed any hearing loss phenotype other than primary infertility. Our attempt to screen for DIS deletion among a cohort of normal hearing south Indian infertile men did not yield any such deletion which may be a population specific variation. The etiology of male infertility may differ between ethnic populations. However, increasing the sample size may throw more light on its frequency. Another approach would be to verify the same deletion in a cohort where both deafness and male infertility is established. Further, more comprehensive approaches like targeted gene panel, whole exome sequencing and CNV assessment should be followed up to resolve their genetic etiology.

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DELINEATION OF A SMALL SUPERNUMERARY MARKER CHROMOSOME IN TURNER SYNDROME

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INTRODUCTION

Turner syndrome (TS) is considered one of the most common aneuploidies, occurring in 1 of 2,500 female live births. It is caused either by the complete or partial loss of one of the X chromosomes in all or a portion of cells, resulting in different karyotypes. Recent studies show that 50-60 % of TS patients have monosomy X (45,X), whereas mosaicism for 45,X has been reported in 20-30% of cases. Based on more recent studies, structural abnormalities of the Y chromosome account for ~ 6% of TS cases [1].

Small supernumerary marker chromosomes (sSMC) are structurally abnormal chromosomes that cannot be identified or characterized unambiguously by banding cytogenetics alone. They are equal in size to or smaller than a chromosome 20 of the same metaphase spread. sSMC are very rare chromosomal abnormality seen in Turner syndrome patients [2]. sSMC can present with different shapes (ring, centric minute and inverted duplication shape), and consist in the majority of the cases of pericentric chromosomal material. Besides, sSMC can be derived from any part of the human chromosomes and form neocentrics. If they derived from the chromosomal ends, in most cases they lead to partial tetrasomies [2]. The precise characterization of these marker chromosomes in Turner syndrome patients are essential because the presence of Y chromosome material in Turner syndrome patients are at higher risk of developing gonadal tumours. The main objective of the present study was to delineate a small supernumerary marker chromosome (sSMC) in a Turner syndrome patient.

MATERIALS AND METHODS

The present study was conducted at the Jubilee Centre for Medical Research, JMMC&RI, Thrissur, Kerala, India. Peripheral Blood sample was collected from the patient after getting informed written consent. Classical chromosomal analysis was performed after 72 hrs peripheral blood lymphocyte culture. Harvesting and GTG banding were performed as per the standard procedure. Fifty metaphases were karyotyped using Meta Systems Ikaros software (MetaSystems, Altlussheim, Germany). Karyotypes were described according to International System for Human Cytogenetic Nomenclature (ISCN 2016) [3]. Fluorescence *in situ* hybridization (FISH) was performed using Kreatech centromeric probes for chromosomes X and Y as per the standard procedure.

Genomic DNA was isolated from peripheral blood samples using KIT method. *SRY* (sex-determining region on the Y) and *TSPY* (testis specific-protein, Y-linked) were amplified by polymerase chain reaction using the primer pair; SRYF (5'-CATG AA CG CATTCA TCGTGT GG TC-3'), SRYR (5'-CTGCGGGAA GCAAACTGC AA TTGTT-3'), and TSPYF (5'-GATGACATA ATGGCGGAG-3'), and TSPYR (5'-CGATAGG CCTCCAC TTCATA-3'). The PCR product was analyzed on 2% agarose gel.

RESULTS AND DISCUSSION

G-banded chromosome analysis of 72hrs culture peripheral blood cells showed a mosaic Turner syndrome pattern. Sixty three percentage of cells showed monosomy X [45,X]. Remaining cells had monosomy X and a marker chromosome [45,X/46,X,+mar] [Figure 1]. The FISH result indicated that the marker chromosome was derived from chromosome Y. In PCR analysis we observed an absence and presence of SRY and TSPY gene sequences respectively.

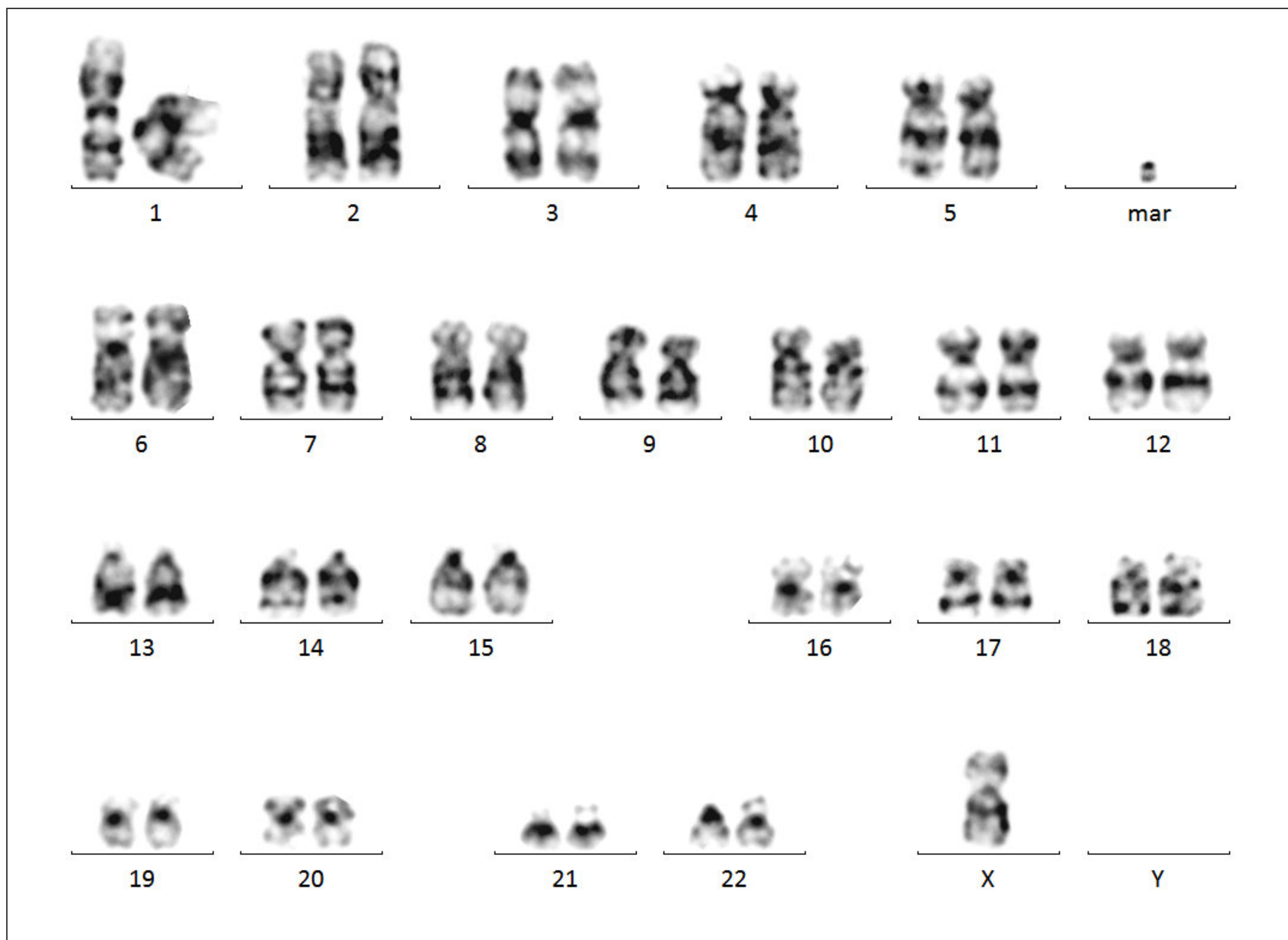

Figure 1.G-banded karyotype showing 46,X,+mar

Turner syndrome is regarded as one of the most common chromosomal abnormalities, with 45,X monosomy considered as the main reason behind 50-60% of patients. Other chromosomal abnormalities seen in Turner syndrome patients includes mosaicism of the X chromosome, isochromosomes of the X, Xp or Xq deletion, ring X structures, and structural abnormalities of the Y chromosome.

Small supernumerary marker chromosomes can be observed in a numerically normal 'basic karyotype', but also in numerically abnormal one like in a 'Turner-syndrome karyotype' (=sSMCT). At present 528 such cases with an sSMCT are reported. The majority of sSMCT(X) form ring-chromosomes, while most sSMCT(Y) are inverted duplicated/isodicentric ones. In our study by using conventional and molecular cytogenetic analysis, we identified a marker chromosome derived from Y chromosome in a Turner syndrome patient. The patient karyotype showed a mosaic pattern. When a mos 45,X/46,X,der(Y) or 45,X/46,XY present in a Turner syndrome patients its charact erization is important to counsel the patient concerning a possibility of gonadoblastoma and a preventive removal of gonadal tissue [4]. The presence of a gene on the Y chromosome, the gonadoblastoma-Y (GBY), predisposes XY females with dysgenetic gonads to develop gonadal tumors. Studies suggested that presence of TSPY gene which located on Ypter-p11.2 could cause gonadoblastoma in Turner

syndrome patiens [5]. In PCR analysis we identified the presence of TSPY gene in our patient. Since the presence of TSPY gene in our patient might increase the risk of developing gonadal tumors we recommended gonadectomy.

CONCLUSION

The origin of sSMC of Turner syndrome was from Y chromosome. The molecular analysis of the sSMC can provide useful information for genetic counselling and treatment of the Turner syndrome patients with a marker chromosome.

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SYMPOSIUM ON

PEDIATRIC GENETICS

HANDLES OF DYSMORPHOLOGY

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INTRODUCTION

Definitions

Dysmorphology is a discipline of clinical genetics which deals with the study of abnormal patterns of human growth and with the recognition and study of congenital human structural anomalies and patterns of birth defects. Congenital malformations/ birth defects can be sub-classified as major or minor anomalies. Major anomalies, which interfere with the normal functioning of an individual and pose a significant health problem or risk to life are present in 2-3% and minor anomalies which are usually only of cosmetic significance are present in around 15% of live births.

Congenital anomalies are classified, on the basis of the developmental stage in which the insult occurred, the process that caused the change and the end result, into:

Malformation: Primary intrinsic developmental defect usually caused by genetic/ environmental/ multi-factorial causes (recurrence risk varies accordingly) which occur during the period of organogenesis which is up to 8 weeks post fertilization for most organs. E.g. neural tube defect, ventricular septal defect, polydactyly etc.

Deformation: Distortion of a normally developed structure caused by mechanical forces usually in the latter half of gestation and most often involving musculo-skeletal tissues. E.g.club foot, torticollis, plagiocephaly etc.

Disruption: Breakdown of an intrinsically normally developing/ developed tissue due to some disruptive event such as a mechanical, vascular or infectious insult. E.g. amniotic band sequence.

Dysplasia: Abnormal cellular organization within a tissue, almost always of genetic cause. E.g. skeletal dysplasias.

A syndrome is a recognized composite pattern of 2 or more anomalies with a common specific aetiology. E.g. Turner syndrome, fetal phenytoin syndrome etc.

An association is a non-random occurrence of 2 or more anomalies that occur together more frequently than expected by chance alone, but without a known specific aetiology. E.g. VACTERL (vertebral defects, anal atresia or stenosis, cardiac defects, tracheo-esophageal fistula, radial defects and renal anomalies, limb defects) association.

A sequence is a pattern of anomalies resulting from a single primary anomaly or factor E.g. Potter sequence (Primary anomaly - bilateral renal aplasia/ dysplasia → decreased fetal urine production → severe oligohydramnios → compressive effects → flattened facies with flattened nose, deformed ears, pulmonary hypoplasia & positional limb defects).

Clinical Evaluation of a Dysmorphic Child

Clinical evaluation of a dysmorphic individual should include a thorough pre-, peri- and post-natal history, developmental history, detailed family history with minimum three-generation pedigree, thorough head to toe examination, anthropometric measurements and their comparison with standard tables/ graphs of age and gender norms and examination of both parents and other available family members for similar/ related features. Physical features not found as normal/ familial traits and present in specific conditions are of more diagnostic help and are called 'good handles' for diagnosis.

A genetic aetiology should be suspected in any individual with the following:

- Congenital anomalies: at least 1 major/ > 2 minor anomalies.
- Growth deficit (short stature/ failure to thrive)
- Developmental delay, intellectual disability or developmental regression

· Failure to develop secondary sexual characteristics
· Abnormal genitalia
· Appears 'different'/ 'unusual'

History: A detailed history covering the following aspects should be obtained:

- **Prenatal history:**
– Teratogenic exposures especially in the first trimester of pregnancy: infections/ medications/ drugs of abuse/ maternal illness/ radiation exposure
– Prenatal complications and antenatal ultrasonographic findings
- **Perinatal history:**
– Presentation/ mode/ complications of delivery
– Gestational age and condition (Apgar score) at birth
– Birth weight, birth length and head circumference, body proportions
- **Neonatal course:**
– Feeding and activity
– Any adverse events/ complications
- **Post neonatal:**
– Physical growth
– Developmental milestones
– Neurological symptoms especially seizures / visual or hearing deficits/ behavioural phenotype
– Other systemic symptoms
- **Family history:**
– At least three generation family history / pedigree
– History of recurrent pregnancy losses/ infertility
– Specific information/ medical records of other affected family members
– Consanguinity in parents
– Ethnic background

Physical examination: A thorough clinical examination must be done taking the following aspects into consideration:

· **General principles:**
– Thorough head to toe examination to be done.
– Measurements to be taken and compared with standard tables/ graphs of age and gender norms.
– Both parents and other available family members to be examined for similar or related features.
– Clinical photographs to be taken with informed consent of individual/ parent/ guardian: for records, syndrome search, referral and study of evolution of the phenotype.

- **Anthropometric measurements:**
– Height/ length, weight, head circumference
- **Assessment of proportionality & symmetry:**
– Upper segment/ lower segment ratio
– Arm span
– Individual limb segment measurements (in specific cases)
- **Head to toe assessment:** (for exact description of each feature refer to Am J Med Genet A 2009 Jan; 149A (1) & Aase JM Diagnostic Dysmorphology textbook).
– Each body part to be examined carefully from head to feet to look for anomalies
– Cranium – size; fontanelles; sutures; shape and symmetry
– Scalp hair - colour and texture; distribution; hair whorl patterns; position of anterior and posterior hairline
– Face
o overall impression of facial appearance: gestalt e.g. Down syndrome facies, coarse facies, myopathic facies
o overall shape/ symmetry/ size of face: triangular/ broad/ round
o face to be divided into sections: forehead, midface and oral region
o face to be viewed from front and from side
o lateral profile better for: depth or height of structures such as nasal bridge, position of mandible relative to maxilla and midface development
– Facial measurements:
o interpupillary distance, inner canthal distance, outer canthal distance, interalar distance, philtral length, upper lip thickness, lower lip thickness, intercommisural distance
o Measurements to be compared to age and sex norms (< or > 2SD => abnormal)
– Forehead - Size: small/ broad/ tall; Shape: sloping frontal bossing/ bitemporal narrowing/ metopic prominence; Supraorbital ridges: prominent/ underdeveloped

– Maxilla/ midface –
o Cheek bone: prominent/ underdeveloped/ fullness
o Malar region: prominence/ flattening
o Midface: prominence/ retrusion
o Nasolabial folds: prominent/ underdeveloped
– Mandible - size & shape: micrognathia/ retrognathia/ prominence
– Eyes- eyebrows; palpebral fissure length (short long); palpebral fissure slant (up/down); epicanthic folds; eye spacing (use a rough guide of 1:1:1 for ratio of left palpebral fissure length: inner canthal distance: right palpebral fissure length); palpebral fissure shape; iris colour; pupil shape; cornea/ sclera/ lens; globe position (assessed from lateral view: protuberant vs deep set globes)
– Nose – nasal root; nasal bridge: depressed prominent/ broad; nasal tip: broad/ flattened; columella (the vertical ridge separating the nostrils): wide/ overhanging; nostrils: patency and position (anteverted); alae nasi
– Mouth and perioral region - mouth size and shape; upper and lower lip shape and thickness; gum thickness; philtrum definition and length; jaw position (prognathia/micrognathia); palate shape
– Oral cavity - teeth/ frenulum/ tongue size and morphology
– Ears
o Ear position
o Ear rotation (normally 15 degrees posterior to the vertical plane of the head): anteriorly/ posteriorly rotated
o Ear shape and structure
o Accessory structures: pits/ skin tags
– Skeleton-
o Neck: length/ shape (webbed)
o Shape of thoracic cage
o Sternum: length & shape (pectus carinatum excavatum)
o Spine: length/ straight/curved
o Limbs: length/shape/ symmetry
– Joints – contractures; range of joint movement: laxity/ restriction; soft tissue webbing across joints (pterygium)
– Skin –
o Texture: smooth/ coarse/ dry/ ichthyotic
o Pigmentation: hypo/ hyperpigmentation; patchy / generalized
o Naevi/ lentigines
o Redundancy/ laxity
o Patchy pigmentation may indicate mosaicism
– Hands and Feet
o Overall shape and size of hand and foot
o Digit number
o Digit shape (e.g. cleinodactyly) and length
o Webbing between digits
o Palmar, plantar and digit creases
o Nail morphology
– Genitalia and Anus
o Phallus size and morphology
o Development, rugosity & pigmentation of scrotum
o Size and position of testes
o Development of labia
o Position of anus relative to genitalia and patency of anus

- **Systemic Examination:** cardiovascular/ per

abdomen/ neurological/ respiratory

- Physical features not found as normal or familial traits and which are present in only a few conditions or are pathognomonic of specific disorders are of more diagnostic help. These are said to be ‘good handles’ for diagnosis e.g. white forelock of hair which is a good diagnostic clue for Waardenburg syndrome.

Radiographs

The following radiographic assessment helps in the diagnostic evaluation:

- X ray wrist + hand (anteroposterior (AP) view) in cases with short stature: for bone age assessment
- Genetic skeletal survey for suspected skeletal dysplasias/ disproportionate short stature:

– AP & lateral views of skull
– AP & lateral views of spine (cervical to sacrum)
– AP view of pelvis with bilateral hip joints
– AP view of one hand and one foot
– AP view of one upper limb (shoulder to elbow; elbow to wrist)
– AP view of one leg (knee to ankle)

Imaging studies

The following imaging modalities may be used in the evaluation:

- Neuroimaging:
  - MRI brain: in presence of neurological deficits/ seizures/ microcephaly or macrocephaly
  - CT Scan brain: for suspected TORCH infections/ cranial contour abnormalities/ craniosynostosis (3D CT)
- USG abdomen/ 2D Echo: to look for visceral malformations

Analysis

- All clinical and lab findings must be analysed together in order to get a diagnosis; all features must fit into the diagnosis as far as possible
- If the condition cannot be diagnosed based on previous experience or existing knowledge, one should take the help of resources such as dysmorphology databases (e.g. LDDB - London Dysmorphology Database and POSSUM – Pictures of Standard Syndromes and Undiagnosed Malformations), online resources (OMIM – Online Mendelian Inheritance in Man) and dysmorphology textbooks.

Genetic Testing

The following genetic tests can help in confirming the aetiology in affected cases:

- **Karyotyping**: to be done in cases with:
  - congenital malformations
  - prenatal onset growth retardation
  - disorder of sexual development
  - developmental delay
  - history of multiple miscarriages in the family
- **Fluorescence *in situ* hybridization (FISH)/ Multiplex ligation - dependent probe amplification (MLPA):** when the phenotype suggestive of a specific microdeletion syndrome e.g. Di George syndrome (22q microdeletion)/ Angelman syndrome (15q microdeletion)/ Williams syndrome (7q microdeletion)
- **Metabolic testing:**

Relevant biochemical investigations should be done if a metabolic etiology is suspected. Metabolic disorders with dysmorphism include:

  - Mucopolysaccharidoses, oligosaccharidoses, mucolipidosis, GM1 gangliosidosis
  - Peroxisomal disorders
  - Disorders of cholesterol metabolism (e.g. Smith Lemli Opitz syndrome)
- **Single gene mutation analysis:** DNA-based molecular genetic tests to be done when a specific monogenic disorder is suspected.
- **Cytogenetic microarray (CMA) study:**
  - o Can be done in any case with multiple malformations with or without associated intellectual disability and without any other identified genetic/ non-genetic cause
  - o CMA scans the entire genome for copy number variations (microdeletions/ microduplications)

Intervention

- Appropriate medical/ surgical management wherever feasible: eg. surgical correction of cardiac defect, correction of hearing deficit etc.
- Genetic counseling
- Prenatal diagnosis wherever feasible

Genetic Counseling

- Deformations/ disruptions have low risk of recurrence
- *De novo* chromosomal abnormalities and microdeletions have risk of recurrence of <1%
- In single gene disorders, risk of recurrence will vary according to the mode of inheritance: autosomal dominant (50% in sibs and offspring if inherited and nil in sibs if de novo)/ autosomal recessive (25% in sibs)/ X-linked (50% in male sibs)

Prenatal Diagnosis

- Targeted mutation analysis/ chromosomal analysis/ metabolic testing in fetal tissue depending upon diagnosis of proband: Chorionic villus sample/ amniotic fluid/ pre implantation genetic diagnosis
- Fetal anomaly scan to look for the same/ associated malformations
- 3D/ 4D USG for better visualisation of the facial profile/ external dysmorphisms
- Fetal echocardiogram for detecting fetal cardiac anomalies

- Limitations of scan based prenatal diagnosis:
  - o cannot detect certain malformations like imperforate anus
  - o cannot determine intellectual status
  - o cannot pick up some features e.g. microcephaly/ lissencephaly until late gestation

Follow up

- To assess growth & development
- To study course of the disease
- To monitor for known/ anticipated associated complications
- To offer newly available diagnostic tests
- To offer newly available therapeutic options
- Sometimes phenotype evolves with age and reassessment at a later age in an undiagnosed case might make diagnosis clear
- To discuss reproductive risks.

RESOURCES FOR REFERENCE

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POTENTIAL USE OF NEXT GENERATION SEQUENCING (NGS) IN THE DIAGNOSIS OF MONOGENIC DISORDERS

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INTRODUCTION

Mendelian diseases, also known as monogenic disorders are caused by mutation in single gene. Monogenic disorders are considered to be rare individually but collectively occur at a rate of 40 to 82 per 1000 live births [1]. Sanger sequencing been a first effective tool in molecular diagnosis of monogenic disorders to confirm a suspected diagnosis and allow more accurate genetic counseling. However, Sanger sequencing can only analyze one DNA segment at a time and is thus laborious and time consuming. Recent advances over the past decades have allowed high throughput sequencing called next generation sequencing (NGS). This allows for simultaneous interrogation of multiple genes through one single reaction and has been proven to be a cost effective and efficient tool in investigating patients with monogenic disorders [2-3].

The clinical utility of NGS varies for different disorders in clinical setup. In the majority of cases, the finding does not alter the clinical management/ Treatment of patient, but it does allow an end to an expensive and stressful diagnostic odyssey [4]. Identification of the molecular etiology, facilitates genetic counseling and allows accurate risk prediction in successive pregnancies [5]. We are presenting the clinical usefulness of NGS application in the setting of a genetic clinic in a tertiary hospital

MATERIALS AND METHODS

The study is a retrospective data analysis conducted at Genetic Clinic, SAT Hospital & Genetic Lab, Child Development Centre, Government Medical College Campus, Trivandrum. The period of study was two and a half years from January 2015 to July 2017. A total of 69 cases with varied clinical presentation like Acardi-Goutieres syndrome, Cornelia de Lange, Goltz Syndrome, Long QT Syndrome, Osteogenesis Imperfecta etc. were subjected to NGS technique and the data were used for clinical diagnosis and genetic counseling.

The clinical geneticist performed detailed phenotyping of the affected individual(s) which would include evaluations from other subspecialist and/ or performing biochemical and/ or radiological tests as well as detailed pedigree was analyzed. The pre-test genetic counseling was done in all cases where sensitivity and limitations of the specific NGS test was explained. Peripheral blood samples (3 ml) were collected in EDTA vaccutainer after getting the written informed consent. The NGS testing was outsourced in standard laboratory in India. Indication for testing and the clinical utility of the test was analyzed. Sanger validation of the candidate variant was performed/ recommended for proband as well as family members to rule out false positives/ to confirm their significance. Prenatal testing was also offered for a few cases.

RESULT AND DISCUSSION

In this study, 69 cases with varied clinical indications were referred for NGS testing to detect rare variants in patients with a phenotype suspected to be due to a Monogenic Disorder. The variants are broadly classified as Pathogenic, Likely Pathogenic and Variant of Uncertain Significance (VUS). Pathogenic variants are defined as those variants that adversely alter protein function and have either been reported previously in other affected individuals and it confirms the diagnosis of suspected disease. In case of likely pathogenic variants, the scientific evidence is currently insufficient to support the findings. Here

additional evidence is expected to confirm its pathogenicity. VUS are variants which are difficult to classify it as either pathogenic or non-pathogenic based on current available scientific evidence. Interpretation of VUS in such instances requires segregation analysis (by analyzing the variant in other family members) presence of the variant in affected but not in unaffected family members adds further evidence to the possible causal relationship of a given variant [6].

Of the 69 cases analyzed in this study with NGS results, 64 cases were the affected patients (probands) and 5 cases were couple with history of previous child with phenotypically a monogenic disorder. The variations reported in 64 cases: pathogenic in 28 cases (44%), likely pathogenic in 6 cases (10%) and variant of unknown significance (VUS) in 17 cases (26%). The result was non-contributory in 13 cases. Sanger sequencing of the variant identified in the proband was done in all cases to avoid false positive testing in NGS method. The case where parental testing is required also was done to delineate the pathogenicity of the variant in NGS data.

Case Reports: The three interesting rare case reports were discussed to highlight the utility of NGS, in cases where there is an unusual phenotype.

Case 1: A three year old boy born of a consanguineous marriage, presented with growth retardation, triangular face, microcephaly, long fingers, contractures and distal athrogryposis, where phenotypically a syndromic short stature like Seckel syndrome was suspected. Cytogenetic testing revealed a normal karyotype. In view of syndromic short stature and consanguinity NGS based multigene testing was done (Clinical Exome) to identify the affected gene so that prenatal diagnosis can be offered in next pregnancy. The test result showed homozygous missense variation in exon 2 of the *ORC6* gene suggestive of Meier-Gorlin syndrome-3. The characteristic feature of this syndrome is absent patella, which was established in this child.

Case 2: An eight year old boy born of a non-consanguineous marriage, presented with clinical features of short stature, mild facial dysmorphism, congenital ichthyosis, hepatomegaly and delayed boneage. His younger sibling has episodes of seizures and ichthyosis and there is a family history of ichthyosis and seizures. Based on pedigree analysis and clinical phenotype he was suspected to be affected with any form of X-linked ichthyosis.He has been evaluated for pathogenic gene variations and was detected with homozygous nonsense variation in Exon1 of the *ABHD5* gene diagnostic of Chanarin-Dorfman syndrome. This is a rare form of nonbullous congenital ichthyosiform erythroderma. One of the characteristic features of this condition was lipid droplets in granulocytes which were demonstrated by peripheral smear examination.

Case 3: A 20 yr old boy born of a non-consanguineous marriage was tested for Marfan Syndrome in view of the clinical phenotype and family history (His father and elder sister also had some features of Marfan syndrome). He has been evaluated for pathogenic variation in *FBN1* gene where no variant was observed. However, secondary findings revealed a heterozygous variation in exon 9 of *SMAD3* gene. Pathogenic variations in the *SMAD3* gene have been showed to be associated with Loeys Dietz syndrome, which manifests as skeletal and vascular abnormalities similar to Marfan syndrome.

These three cases illustrate the importance of NGS techniques in the diagnosis of rare phenotypes.NGS also helps in the molecular diagnosis of conditions where a number of genes are involved in the same phenotype. Classical example of this situation is limb-girdle muscular dystrophy where a large number of genes are involved in the phenotype. Sanger sequencing of individual genes one by one, will be a laborious and expensive technique. NGS methodology will be a robust technique in this situation.

The NGS also plays a vital role in carrier screening of parents where the proband is having a genetic disorder, where the mutation was not proven. In 5 cases where the genetic diagnosis of the proband was not confirmed, parental screening has been performed by NGS platform. The utility of Carrier screening by NGS platform was 4 cases out of 5 cases (80%) [Table 1].

Table 1: Showing NGS utility to detect carrier status of parents

NO	Carrier Screening of Parents by NGS Platform
1.	Indication: History of previous two fetus , terminated at 24th& 28th weeks of gestation period respectively, were found to have corpus callosum agenesis, deformed cerebellum, cerebellar vermis absent, enlarged cisterna magna and hydrocephalus.
	Result: Heterozygous variation in *CCDC88C* gene was identified in both couple, which is associated with congenital hydrocephalus.
2.	Indication: Their first child diagnosed to have homogenous renal enlargement during fetal life, suggestive of ARPKD expired at 3 months of age.
	Result:Heterozygous variation in *NPHP3* gene was identified in both couple , which is associated with Meckel syndrome type 7.
3.	Indication: Previous baby with hemophilia. The child was diagnosed with hemophilia A at 6 months of age. He had intracerebral bleed and died at 1 year of age. No mutation analysis was done in the child.
	Result: Inversion mutation was not present in the mother. Mother harbors a heterozygous pathogenic variant in Exon 24 of the *F8* gene. Later prenatal diagnosis also done in next pregnancy.
4.	Indication: Previous Child with microcephaly, hypotonia& seizures. A case of neonatal encephalopathy died in the newborn period. Child was found to have increased Valine & Leucine by TMS.?MSUD
	Result: Both the parents were detected with heterozygous variant, in Intron 12 of the *HIBCH* gene which can lead to Leigh encephalopathy in the affected child. In this case also prenatal diagnosis was offered.

We were also able to provide prenatal diagnosis in four cases which includes Congenital adrenal hyperplasia, Merosin deficient congenital muscular dystrophy type 1, Goltz syndrome and Duchenne muscular dystrophy.

CONCLUSION

NGS is a robust technology where multiple genes can be sequenced parallel which will reduce the turnaround time. However this is not the single test which will give solution to all genetic disorders. The selection of patients for the testing is the most important criteria for better cost effectiveness. Similarly these expensive testing should accompany pre test and post test genetic counseling. The usefulness and limitations of the test should be informed to the caregivers. Overall clinical utility of NGS was helpful in confirmation of diagnosis and genetic counseling in 37 cases out of 69 cases (54%).

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DOWN SYNDROME WITH AUTISM SPECTRUM DISORDERS: A SUBJECT OF CONCERN

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INTRODUCTION

Down syndrome (DS) is the most common genetic cause for intellectual disability. The trisomy of chromosome 21 results in Down syndrome and it occurs in 1 of every 732 live births [1]. Its frequency is 1:350 and 1:1000 in woman with 35 years and less than 30 years of age. The cause of DS is multifactorial including genetic, environmental, age dependent and age-independent risk factors. In children with DS, the diagnosis of autism is usually missed since they are considered to be sociable. More than 90% of DS cases occur due to errors of nondysjunction, 4% due to parental or *de novo* translocation and 1% cases result due to mosaicism [2].

Autism is a complex neurodevelopmental disorder characterized by impairments in social interaction and communication, repetitive and stereotypic behaviours [3]. It is four times more common in males as compared to females, usually diagnosed by age of 3 years. It is not associated with known racial, ethnic or social boundaries. It is often linked with social and pragmatic deficits. Hence, they should be analysed for language and social adaptive skills. A mental delay is observed in children with thyroid hormone discrepancy.

In recent years, prevalence of dual diagnosis i.e.Down syndrome with autism or autistic spectrum disorders (ASD) is found to be increasing. The frequency of ASD in Down syndrome has been reported to vary from16-19% [4]. In Down syndrome cases with associated conditions like autism, speech and language milestones are also found to be delayed. DS centers on one chromosome while autism is associated to a number of genes on different chromosomes. More recent research indicates social impairments and related behavioral challenges in individuals with DS, although there are limited studies on it. Our aim was to evaluate cytogenetic findings, thyroid profiles, psychological, speech and language parameters in 65 individuals showing Down syndrome with and without autism spectrum disorders (DSA).

MATERIALS & METHODS

We have examined 40 individuals with pure DS and compared them with 25 cases showing DS with comorbid condition of autism spectrum disorders. The patients attending the Institute of Communicative and Cognitive Neurosciences, Shoranur, were enrolled in the study. Conventional cytogenetic analysis, assessment of thyroid function, psychological, speech and language evaluations were performed in all cases. The genetic study was performed using conventional cytogenetic technique. Routinely, Giemsa (GTG) banding technique was carried out, in each case, 25-50 metaphases were examined. Nearly 50 to 100 metaphases were scored in cases of mosaicism.

The Vineland Social Maturity Scale (VSMS) was used in the evaluation of social competence [5].The VSMS included 117 items in eight subscales, it evaluates personal and social skills. Raw scores were converted to an age equivalent score i.e. social age and social quotient (SQ). The VSMS measured different levels of social and adaptive functioning ranging from below average to profound level of retardation.

The Statistical analysis was performed to find whether the two groups i.e. DS and DSA differed in chronological age and maternal age of conception. We have also tried to compare the scores of VSMS (Social Quotient) in two groups using ANOVA.

RESULTS

The age of the patients ranged from 0.1-14 years. The male and female ratio was 1.4:1 and 1.1:1 in DS and DSA respectively. In both groups 86% of mothers were less than 35 years of age. The results of cytogenetic analysis is shown in Table 1, none of them with pure DS had mosaicism as compared to 8% in DSA. Hypothyroidism was detected in 23% cases.

Table 1. Cytogenetic findings in children with Down syndrome with and without autism spectrum disorders

Sn.	Karyotype	DS without ASD	DS with ASD
1	Pure Trisomy	38 (95%)	21(84%)
2	Translocations:	2(5%)	2(8%)
	➢t (14;21)	1(2.5%)	-
	➢t (21;21)	-	1(4%)
	➢t (15;21)	1(2.5%)	1(4%)
3	Mosaicism	-	2(8%)

The expressive language age was delayed than receptive language age in both groups. The mean maternal age at the time of conception of the DS children is shown in Table 2. The social quotient in DSA as compared to pure DS was 52.5 ± 17.9 vs 67.62 ± 22.6 (*P*=0.012). In the current work, the ratio of young mothers to older mothers was much higher (86% vs 14%), which is comparable with 91.6% vs 8.4% in another survey [6]. In contrast to this, 54% vs 46% noticed in another study [7].

DISCUSSION

More recent research indicates social impairments and related behavioral challenges in individuals with DS, although, there are limited studies on it. Approximately 16-19% of individuals with DS were found to show ASD and about 8-9% have autism [4], as compared to 1% ASD in the typical population [8]. However, there is some variability in those rates due to difficulties of making a dual diagnosis. Due to impairments from intellectual disability in DS, the diagnosis of ASD is difficult. In individuals with DS and ASD, Down syndrome can be confirmed by genetic testing, in contrast, there are presently no genetic diagnostic tests for ASD, and its diagnosis is made strictly based on behavioral study.

Studies show that there was a considerable delay in the diagnosis of autism in DS as compared to children with autism but no DS. The cohort with DSA showed poor social and adaptive functioning as compared to pure DS as indicated by poor social quotient in DSA group. In concordance with another study [9], the comparison between DS and DSA showed that those with ASD were found to have significantly more impaired language abilities. The frequency of mosaicism was also more in cohort with DSA. A number of chromosomes and genetic

Table 2: Comparison in individuals showing Down syndrome with and without autism spectrum disorders

Parameters	DS without ASD	DS with ASD	F value	P value
	Mean (SD)			
Chronological age (years)	4.26 ± 3.4	4.58 ± 2.9	0.15	0.698
Maternal age (years)	26.7 ± 5.74	29.24 ± 5.3	3.19	0.079
Maternal age of trisomy 21	26.7 ± 5.8	30.5 ± 5.39	4.09	0.048
Maternal age of translocation	26 ± 5.4	24 ± 4.24	0.16	0.728
Maternal age of mosaicism	0	29 ± 2.8	-	-
Social Quotient	67.6 ± 22.6	52.5 ± 17.9	6.78	0.012

locations have been associated with ASD, and none of the single gene is solely responsible for its occurrence. Therfore, it can be said that ASD is caused by complex interactions between several genes rather than a single genetic mutation which results in variation of different biological levels [10,11,12]. Because all individuals with the classic presentation of DS have trisomy 21, it is possible that those with co-occurring DS and ASD may have additional genetic variants which can act as modifiers of the phenotype, leading to the development of ASD [13]. Besides the chromosomal changes, epigenetic modifications also play an important role in the development of differential manifestation of phenotypic features in DS. More research should focus on the underlying etiology in case of DS with ASD. Such findings can aid in proper management strategies.

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A RARE CASE OF TWO DISTINCT AUTOSOMAL DOMINANT DISORDERS IN A NEWBORN - FAMILIAL MATERNAL DI-GEORGE SYNDROME ALONG WITH PATERNAL NEUROFIBROMATOSIS

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INTRODUCTION

Di-George syndrome also known as 22q11.2 deletion syndrome is caused by a microdeletion near the middle of chromosome 22 at a location designated q11.2, and has autosomal dominant mode of inheritance. It occurs in approximately 1 in 4000 births. It is also known as Velo-Cardio-Facial Syndrome (VCFS), conotruncal syndrome and CATCH 22. The features of this syndrome vary widely, even among affected members of the same family. Individuals with Di-George syndrome are suspectible to infections due to poor T cell production and function and often have altered facial characteristics, abnormal gland development or defects in organs such as the heart. It is a lifelong condition that mostly affects infants and children [1].

In this case, a newborn female baby was referred to Lifeline Genetics and Research Centre, Unit of Fetal Medicine, Kerala, India in the month of January 2017 for evaluation of dysmorphism such as broad flat nasal bridge, bilateral low set ears, prominent forehead with wrinkles, puffy eyelids, right bifid thumb. Based on the pedigree and evaluation of the mother, maternal uncle and maternal grandmother a probable diagnosis of Di-George syndrome was made.

Neurofibromatosis type 1 (NF1), also referred to as Recklinghausen's disease, affects about 1 in 3500 individuals and presents with a variety of characteristic abnormalities like multiple cutaneous lesions and tumors of the peripheral and central nervous system. *NF-I* gene present on chromosome 17q codes for a huge protein neurofibromin, a GTPase activating protein (GAP) that modulates signalling through Ras pathway [2].

Neurofibromatosis type 2 (NF2) is much more rare occurring in less than 1 in 25000 individuals [3]. NF 2 is mainly restricted to tumors of the central and peripheral nervous system. *NF-II* gene is present on chromosome 22q.

MATERIALS AND METHODS

The patient's details were recorded in a predesigned proforma after getting written informed consent and heparanized blood sample was collected.

1.Blood Karyotyping - Culturing and Harvesting

Blood cultures were set up by adding 0.5ml heparinised blood, 0.1ml Phytohaemagglutinin and 0.5ml Fetal Bovine Serum into 5ml RPMI media.The cultures were incubated at 37°C for 72hrs. At 69th hour of incubation, 30µl colchicine (0.15%) solution was added to the cultures to arrest cell division at metaphase stage. After 72 hours of incubation and centrifugation the cultures were subjected to hypotonic solution (0.56% KCl) treatment for four and half minutes. Again centrifuged, supernatant was discarded and the cells were fixed with chilled acetomethanol (1:3). The cultures were kept in freezer for overnight.

Slide Preparation and GTG Banding

For slide preparation three to four drops of cell suspension was dropped evenly on a clean, chilled wet slide and the slides were labelled, air dried and kept for proper ageing. Then the slides were treated with Trypsin (0.025%) solution for 10-20seconds and stained in Giemsa stain. The slides were screened under microscope (Olympus BX51) and well spreaded metaphases were analysed using cytovision software 2.7 (Applied imaging, USA). The

chromosomes were arranged according to their banding pattern.

2.FISH Test for 22q11.2 Microdeletion

The procedures for culturing and harvesting the cells from blood cultures were same as that of blood karyotyping. For slide preparation, three to four drops of cell suspension was dropped onto the centre of the marked area on a chilled wet slide.

Pre Hybridization Treatment

The slides were air dried and treated with 2X SSC for 30 minutes at 37° C and pepsin-HCl for 15 minutes at 37° C. The slides were given PBS (2 minutes) and formaldehyde (5 minutes) wash. Then the slides were dehydrated in a series of alcohol grades (70%, 85% and 100%) for 2 minutes each. The slides were air dried. Then probes TUPLE 1 and ARSA were applied to the slide, cover slip placed and sealed with rubber cement. The slides were then kept for denaturation and hybridization under moist condition in Thermobrite.

Post Hybridization Treatment

The cover slip of slides were removed in wash buffer II and incubated in wash buffer I (72°C) for 2 minutes and then in wash buffer II for 2minutes at room temperature. The slides were again dehydrated in a series of alcohol grades (70%, 85% and 100%) for 2 minutes each. Then air dried slides were counterstained with DAPI (4,6-diamidino-2-phenylindole).

For FISH analysis, locus specific DiGeorge Region probes were used to bind to chromosome 22. TUPLE 1 labelled with a red dye which binds to 22q11.2 region and ARSA labelled with green dye which binds to 22q13.3 region of chromosome 22 which is not deleted in 22q11.2 deletion syndrome was used. FISH study was carried out on both interphase and metaphase cells. The cells were analyzed using a fluorescent microscope.

3. Molecular Analysis

To check for mutations in *NF-I* gene, EDTA blood samples were sent to Strand Life Sciences Pvt. Ltd, Bangalore.

RESULT AND DISCUSSION

Most people with 22q11.2 deletion syndrome are missing a sequence of about 3 million DNA building blocks (base pairs) on one copy of chromosome 22 in each cell. This region contains 30 to 40 genes, many of which have not been well characterized. The loss of a particular gene on chromosome 22, *TBX1*, is probably responsible for many of the syndrome's characteristic signs such as heart defects, cleft palate, distinctive facial features, hearing loss, and low calcium levels.

The inheritance of 22q11.2 deletion syndrome is considered autosomal dominant because a deletion in one copy of chromosome 22 in each cell is sufficient to cause the condition. Most cases of 22q11.2 deletion syndrome are not inherited and are sporadic. If a 22q11.2 deletion is detected in a child, both parents should be offered the FISH test to determine if the child's deletion is inherited. In approximately 10% of families, like in the present study the deletion is inherited and an individual with a 22q11.2 deletion has a 50% chance with each pregnancy of passing it on to their offspring [4].

Here in this case the child's karyotype report was normal. FISH test using LSI probes specific for 22q11.2 microdeletion showed two green signals (ARSA signal for 22q13.3) and only one red signal (TUPLE 1 signal for 22q11.2) thus confirming a microdeletion in one of the two copies of chromosome 22. The same FISH test was done for mother, maternal uncle and maternal grandmother and the results confirmed that they are have microdeletion, thereby confirming Di-George syndrome. Hence the child has inherited CATCH 22 gene deletion from the maternal grandmother indicating as expected autosomal dominant mode of inheritance.

NF1 is caused by a spectrum of mutations that affect the *NF1* gene located at the 17q11.2 chromosome. Only 50% of the NF1 patients have a positive family history of the disease. The rest of the patients represent spontaneous mutations [5]. Mutations in *NF-I* gene result in a large number of nervous system tumors including neurofibromas,

plexiform neurofibroma, optic nerve gliomas, meningiomas. In addition to neurofibromas which appear as multiple, soft rubbery cutaneous tumors, other cutaneous manifestations include café-au-lait spots.

Along with the maternal side evaluation child's paternal side was also evaluated and found that father and paternal grandfather has symptoms of Neurofibromatosis and therefore gene testing was done for father to find out the mutation but paternal grandfather refused to do gene testing.

Next Generation Sequencing based mutation analysis showed heterozygous mutation (chr17:29533315C >T, c.1318C >T, p.Arg440Ter) in *NF-I* gene confirming that father is affected with Neurofibromatosis type 1. The identified heterozygous nonsense substitution (p.Arg440Ter) is predicted to cause premature termination of the protein. The truncated protein is predicted to have a length of 439 amino acids as opposed to the original length of 2818 amino acids. The resultant protein is likely to lack multiple functional domains of the protein which result in loss of function. Moreover, due to introduction of a premature stop codon, this aberrant transcript will likely be targeted by the nonsense mediated mRNA decay (NMD) mechanism.

The same gene testing was done for the child which showed the same mutation by Sanger Sequencing indicating hereditary Neurofibromatosis with an autosomal dominant mode of inheritance.

The present study showed that the child is having familial maternal Di-George syndrome along with paternal Neurofibromatosis. The child is having clinical features of Di-George syndrome presently and no obvious features of Neurofibromatosis. Since the mutation was present in the child a close follow up and review was advised. The couple was given detailed genetic counselling. Mode of inheritance and co-existence of two genetic disorders was explained. They were advised prenatal diagnosis in all future pregnancies. The child was also referred to Paediatric Neurologist. Hence the present study shows co-existence of two separate genetic disorders in one patient.

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CHROMOSOMAL ABERRATIONS AND *IRF6* POLYMORPHISM IN OROFACIAL CLEFTS

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INTRODUCTION

Orofacial cleft (OFC) is one of the most common congenital anomalies with significant medical, psychological, social, and economic ramifications. The main categories are isolated cleft palate (CP) and cleft lip with or without cleft palate (CL/P).The incidence of CL/P worldwide is 1 in 700 live births and it is nearly 1 in 500 in India [1, 2]. Its incidence varies according to race, gender and cleft type. Cleft lip or palate, is a common congenital defect recognized at the time of birth. It results from failure of fusion of maxillary processes or palatal shelves. Effects on speech, hearing, appearance, and psychology can lead to long lasting adverse outcomes for health and social integration. Complex molecular signalling pathways regulate cellular processes essential for lip and palate morphogenesis. During the 4th and 12th week of embryogenesis, the formation of the frontonasal prominence, the paired maxillary processes, and the paired mandibular processes, which surround the primitive oral cavity and merging of the medial nasal processes with one another and with the maxillary processes on each side leads to formation of the upper lip and the primary palate. Any disturbance by teratogens during this critical period can lead to failure of the closure mechanism. Genetic and environment factors dysregulate these pathways causing the malformations.

The cleft can be either syndromic, where the patient has more than one malformation associated with the cleft or non-syndromic, if there is only a single malformation [3]. More than 400 syndromes have been studied where CL/P is a feature. Non-syndromic (isolated) cleft lip with or without cleft palate (CL/P) is the most common OFC [4]. Chromosomal anomalies are important hints for genes that involve in clefting of the lip and palate. Chromosomal deletions and duplications were surveyed to see phenotypes that are linked to partial aneuploidies. The regions 1q25, 3p21, 4p15, 4q32 and 10p15 were found to be associated with clefts. The 4p15 region within which lies the homeobox MSX1 gene is found to be the site of deletion which leads to Wolf-Hirschhorn syndrome, associated with OFCs [5].

More than 20 Non-syndromic Cleft lip and Palate candidate genes have shown 2–6% of the total number of individuals with Non-syndromic Cleft lip and Palate having polymorphisms in these genes MSX1, JAG2, FOXE1, SATB2, GLI2, RYK1, LHX8, and many others [6,7]. However, a large number of individuals about 94–98% with Non-syndromic Cleft lip and Palate are not carriers of mutations in any of the candidate genes. Many candidate genes are analysed to screen specific polymorphic variants and mutations/ polymorphisms that elevate the risk of Non-syndromic Cleft lip and palate. The gene IRF6 identified in Van der Woude syndrome is confirmed in multiple other populations around the globe such as Thailand, Taiwan, Singapore, Korea, Italy, Belgium, US, South America including Norway. This gene is also responsible for about 12–18% of Nonsyndromic Cleft Lip/ Palate.

MATERIALS AND METHODS

The study was performed in Jubilee Mission Medical College and Research Institute, Thrissur. Cleft lip and Palate patients were recruited from the Department of Plastic Surgery, Burns, Charles Pinto Centre for Cleft Lip, Palate and Craniofacial Anomalies, JMMC& RI. A total of 50 patients between the age group: 1- 35 years were selected. Detailed information about the family and medical history of the patients were recorded in a standard Proforma. The study was conducted from January to June 2017. The informed written consent was obtained from the all subjects and the Ethical Clearance was obtained from the Institutional Ethical Committee of JMMC & RI for this study.

The patients' peripheral blood (2ml) was collected aseptically in EDTA vacutainers and (2ml) in heparin vacutainers. Conventional cytogenetic analysis was performed to investigate the frequency of chromosomal abnormalities and to confirm the heterochromatin variations in chromosomes, C-banding was done. GTG banding (G-band by trypsin and Giemsa) and C-banding (Centromeres banding) were performed according to the standard procedure. Atleast 20 well spreaded and good banded metaphase spreads from each case were captured and analyzed using cytogenetic software (Metasystem Ikaros, Germany) and karyotyped as per the international system for human cytogenetic nomenclature (ISCN) guidelines (ISCN, 2016).

For the analysis of IRF6 polymorphism, PCR for exons 3, 4 and 7 were performed. Agarose gel electrophoresis was done to confirm the PCR product amplification.

RESULTS

Cytogenetic analysis of 50 cleft lip and palate patients revealed 8 cases with abnormal karyotype. GTG banding and C banding technique shows heterochromatin variants of 1st and 9th chromosomes, duplication [46,XY,dup(3)], inversion [46,XY,inv(9)(p11q13)][Figure 1], presence of an extra large satellite [46,XX,22ps+], translocation with a heterochromatin variant [46,XY,1qh+,t(7;14)(q21;q23)].

IRF6 Exon Analysis

We have selected 32 patients with cleft lip with/or without cleft palate to understand the contribution of IRF6 gene in the pathogenesis of orofacial clefts. The blood samples were subjected for molecular studies. Three exons were selected to screen for the possible variants present in the study population. Exon 3 and 4 showed no variation and three of the individuals from the study group showed a single nucleotide variation in the exon 7 of IRF6 gene. The polymorphism found in the study do not show a strong association in the present scenario as the sample size is small. The study has designed to be a pilot investigation among the cleft lip and/or palate patient population. Exon 7 of IRF6 gene showed a variation in the reverse strand at the 209790735 G>A position. In the consensus coding sequence of IRF6 the variation was found to be at 820 G>A transition which happen to change the amino acid code GCT (Valine) to ACT (Isoleucine).The polymorphic allele distribution among the patient population revealed a homozygous A/A (having a clinical feature of Cleft lip and Complete Cleft of Primary & Secondary Palate), two heterozygous A/G (having clinical features of Unilateral cleft of left lip with 1° Palate and Complete Cleft Lip & Palate) and other 29 individuals with homozygous G/G.

DISCUSSION

This study was aimed to understand the involvement of chromosomal instability in the individuals affected with cleft lip and palate. The study was also aimed to understand the association of the IRF6 Gene variants in individuals affected with orofacial clefts. It is known that in multifactorial genetic diseases which are of CLP type, the biggest share of genetic impact comes to several genes, but along with them morphogenesis could be controlled by many more genes. Few studies show chromosomal involvement in cleft lip and palate which are not confirmed like one study which shows eight cases of trisomy 13, five of trisomy 18, one unbalanced translocation between chromosomes 7 and 8, and one deletion 4p- in cleft lip and palate patients [9]. At the same time, there are studies which shows negative

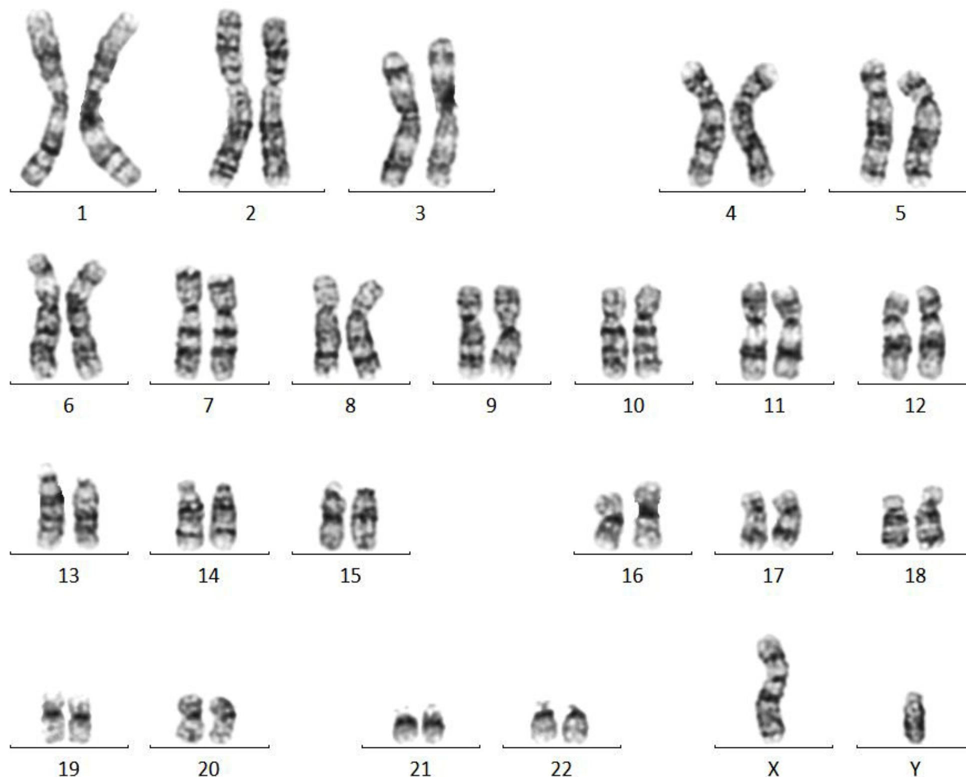

Figure 1. G banded karyotype showing heterochromatic variation in chromosome 9 [46,XY,inv(9)(p11q13)]

results in relation with chromosomal morphology and cleft lip and palate [10]. About 25% of neonates with CL-P show associated malformations, syndromes or aneuploidy [11]. Many more conceptuses (up to 80%) than infants with CL-P have associated trisomies and multiple, potentially lethal malformations. These foetuses either die in utero or within the neonatal period, or are aborted and therefore are never seen as CL-P patients. Therefore, foetuses with sonographically identified CL-P represent quite a different group of patients from neonates affected by the malformation [12].

The earlier studies indicated that the chromosome 1, 2, 4, 6, 17, 19, X are closely related with NSCL/P abnormality [13]. The results of the present study with the help of GTG banding and C banding technique shows heterochromatin variants of 1st and 9th chromosome. Using the G-banding method (or even the specific C-banding method), heterochromatin variants are quite easily recognized undermicroscope; thus, they became a major point of interest for early cytogeneticists [14].

The translocation (46,XY,1qh+,t(7;14)(q21;q23)) and the duplication in the chromosome 3 has not been reported in the literature previously. The heterochromatin variant of the chromosome q12 of both 9th and 1st chromosome is observed in individuals affected with cleft lip and palate and it indicated that Chromosome 9 presents the highest degree of morphological variations among the non-acrocentric human chromosomes research [15].

SNP analysis and DNA sequence variation studies have proved that IRF6 gene is a hot spot or risk loci for orofacial clefting [16]. According to kondo *et al*. interferon regulatory factor 6 is located at 1q32 to 1q41 and they are highly active during the palatal formation and fusion [17].

This study can be considred as a pilot investigation to screen the hot spots of IRF6 gene in the individual affected with orofacial clefts. Sequencing analysis of exons 3 and 4 was devoid of genetic variants. Exon 7 of IRF6 gene showed a variation in the reverse strand at the 209790735 G>A position. In the consensus coding sequence of IRF6 the variation was found to be at 820G>A transition which happen to change the amino acid code GCT (Valine) to ACT (Isoleucine).

The polymorphic allele distribution among the patient population revealed a homozygous A/A (having a clinical feature of Cleft lip and Complete Cleft of Primary & Secondary Palate), two heterozygous A/G (having clinical features of Unilateral cleft of left lip with 1° Palate and Complete Cleft Lip & Palate) and other 29 individuals with homozygous G/G. Homozygous A/A and heterozygous A/G in different populations are strongly associated with NSCLP and this is considered as one of the risk allele to develop orofacial clefts. In 2004 after zucherro *et al.* reviewed that 12% of the orofacial clefts are developed from IRF6 genetic variation at 1q32 [17].

In the present study, IRF6 hot spots and genetic variations are not seemingly contributing to the development of oral clefting in the studied group. A higher number of affected individuals should be studied and screened for a variety of genetic loci to elucidate the pathogenic origin of the condition.

CONCLUSION

We identified chromosomal abnormalities in 8 cases out of 50 patients. Even if the result obtained from 90% of the study population seems to be normal, this study must be continued with a higher number of affected individuals and healthy controls to understand the genetic distribution and etiology of the condition.

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GENETIC AND OTHER ETIOLOGICAL ASSOCIATIONS IN CHILDREN WITH PDP (PERVASIVE DEVELOPMENTAL DISORDERS)

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INTRODUCTION

Autism is a neurodevelopmental disorder recognized in children between the age of one and three with characteristic features such as social and communication deficits, and ritualistic repetitive behavior. It affects recognition of emotions, sharing foci of interest with other people, meaning and uses of language, forming attachments, empathy and whole lot of other competencies that allow a child to become a family member and a social being. If carefully watched, mother or primary caretaker can also observe the child's general disinterest in the human face and the lack of response to stimuli of emotional nature. Autism has been linked to various factors such as maternal, congenital, perinatal and environmental. Factors thought to affect the child include maternal age, infections, exanthema, placenta praevia and autoimmune disease. Perinatal factors include poor apgar, resuscitation and intervention during delivery and birth trauma. Postnatal factors include respiratory illness, gastroenteritis, viral infections, convulsions, head trauma, living in nuclear families and vaccination (especially measles, mumps and rubella). The disorder also seems to be due to variations in the brain [1]. Existing literature also says the same thing, that it is most probably genetic but mode of transmission is ill understood [2,3].

The disorder seems to be due to variations in the size of the brain. Structural radio imaging studies have been inconclusive and have yielded variable results. Brain size has been reported to have increased by cranial imaging techniques and necropsy. Occipitofrontal head circumference is the measurement most widely accepted and these children are supposed to be macrocephalic. Autism has been studied in twins and in multiple incidence autism families (families with more than one member with clinically diagnosed autism), and it has been found that in general, autism or autistic traits are more common in certain families. It has been noticed that parents of autistic children may exhibit poor executive functions. This could be as a result of shared genes or shared environment. It is likely that it is due to genetic factors. A hunt has been on for candidate genes on each chromosome. Chromosomes 7, 13, 15 & 22 have been the foci of interact [1]. Also as the disorder has been noticed more commonly in males, it was postulated that the gene may be present in the Y chromosome. Fragile sites on the X-chromosome have also seen linked with autism. All the above studies were pointing to different factors. There is a possibility that there are different kinds of disorders with common symptoms. Some workers have quoted the presence of two types of autism – a high – functioning type and a low functioning type - both of which may arise from separate genetic causes.

Much as there are controversies regarding the etiological or genetic basis of autism, it must be emphasized that timely detection and early intervention may mitigate the emotional, social and cognitive deficits of those disabled and improve the outcome. The main objectives of the present study were 1) To study the genetics correlates and other possible etiological factors in children with PDD 2) To ascertain whether non-autistic family members exhibit behavioral characteristics in the autism spectrum.

MATERIALS & METHODS

Two hunred children were selected from the child psychiatry OPD of NIMHANS, Bangalore.Their physical features were noted. Families were interviewed using a questionnaire. Photographs were taken after prior informed consent. Blood samples

were taken for Karyotyping. Tables and graphs were drawn after subjecting the data to statistical analysis.

RESULTS AND DISCUSSION

Possible Etiological Associations

Based on history: Majority of the patients were from high (46%) or medium income groups (48 %). Those from low income groups were less (6%). One of the reasons for this could be that the people from the higher strata may have had higher awareness. That allowed them to take medical advice and therefore come to NIMHANS.

Residence: Maximum number of patients hailed from Karnataka. The rest of them were from Kerala, other parts of South India and West Bengal. The larger number of patients being from these areas may be due to easy access to NIMHANS and increased awareness among patients.

Consanguinity: Most of the patients (78%) were non-consanguineous individuals and others (22%) were found to be consanguineous individuals.

Age & education of father

83 % - High age group (35 years or more)
17% - Medium age group
52 % - Fathers were postgraduates
38 % - Fathers were graduates
10% - Low education level (under graduates)

Age & education of mother

52 % - Mother was highly educated and age above 28 years.
8 % - Medium education (22-28 years)
10% - Low education (below 20 years)

Health of mother: Most mothers were healthy (77%). Only 23 % of mothers had health problems (such as hypertension, hyperthyroidism, diabetes, autoimmune diseases and psychiatric problems).

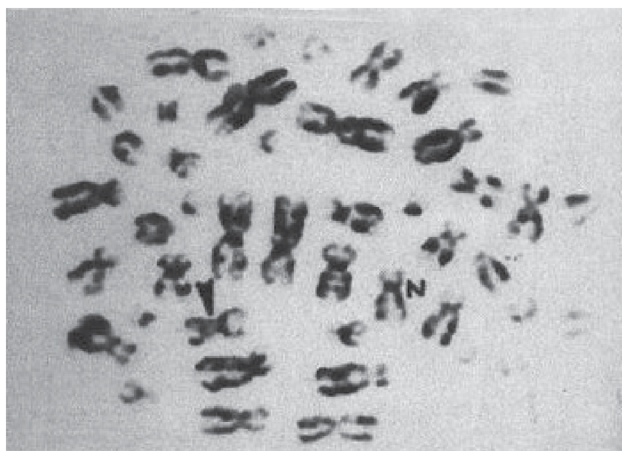
Type of family: 66 % of these children are from nuclear families & 34 % from joint families.

Drugs taken by mother: 52 % of mothers did not take any drug. 48% of mothers had taken drugs like pain killers, antihypertensive medication or hypoglycemic agents.

Labour duration	Type of delivery
55 % - Normal duration of labor 28 % - Long period of labor 17 %- Short duration of labor	36 % - Caesarean section 64 % - Normal delivery So no significant etiological factors – head trauma.

Apgar score	Fetal distress	Maturity	Exanthems
Medium - 49 % Good - 8 % Very good - 31%	Present in 19 % cases Absent in 81 % cases	Full term - 77% Pre-term - 20 % Post mature - 3 %	No Exanthems - 82 % (antenatal) Some rashes - 18 % Some of these Rubella - 6%

Foetal movements	Vaccination	Reaction to vaccination	Karyotyping
Normal - 50% Increased - 22 % Decreased - 28 %	100 % of the patients were vaccinated so there was no possibility of comparison.	30% - No infection 52%- Mild respiratory infection. 18%- Severe respiratory infection. The infections were treated with course of antibiotics.	98 %- No abnormalities 2%-Chromosomal abnormalities Inversion 9 – inherited from mother – male child [Figure 1&2]. Translocation t (7,14) – female child – documented rubella syndrome.



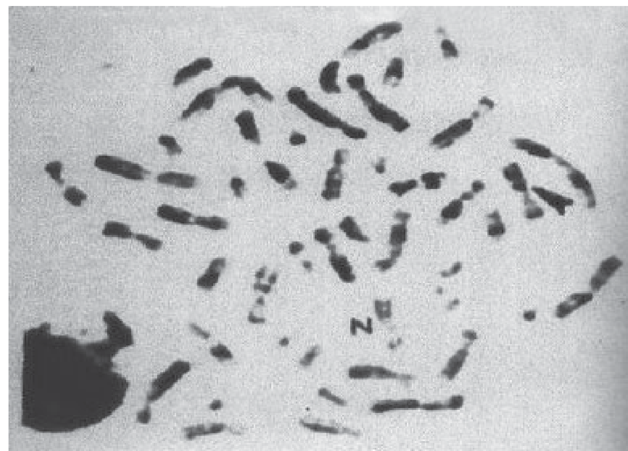


Figure 1& 2. G- Banded metaphases showing inv(9)

Metaphase of case No 42 showing 46,XY, inv(9) (p13q21). Normal chromosome 9 is shown by the letter 'N' and the inverted chromosome is shown by an arrow

Metaphase of mother of case No.42 showing the same inversion 46,XY, inv(9) (p13q21).

Family history

17 cases	- No first degree relative affected.
17 cases	- Only 1 first degree relative affected.
15 cases	- 2 first degrees relatives affected.
1 case	- More than 2 first degree relatives affected.
26 cases	- 0 to 5 2nd degree relatives affected.
14 cases	- 6 to 10 2nd degree relatives affected.
11 to 15 cases	- 9- 2nd degree relatives affected.
1 case	- More than 16 members affected.

Structural imaging: No consistent findings. (1,7,21,26) 8 cases found to be abnormal

Head circumference	Most cases normal
Shape of forehead	42 % cases – abnormal shape of forehead
Chest abnormalities	62 % - Abnormal 38 % - Normal
Skeletal abnormalities	Scoliosis- 16 cases
Neuro muscular co-ordination	90%- Good co-ordination 10 % - Slightly less co-ordination

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GENETIC SOFT MARKERS IN FETAL NEUROSONOGRAM

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INTRODUCTION

In India birth defects prevalence is 70 /1000 live births that account for 7% neonatal and 20% infant mortality [1]. Every year 17 lakh children are born with various birth defects in India. In the next pregnancy, mothers of these children are considered as high risk cases and would require special guidance for early diagnosis in antenatal period to rule out recurrence of similar condition. In modern days, every pregnancy is precious and need best evaluation methods for successful outcome. The direct genetic diagnosis is not possible due to several factors and may not be necessary in all cases. As an indirect, non invasive and affordable method the genetic ultrasound made a major breakthrough in providing advanced care in modern obstetric practice by identifying the fetus at risk of genetic abnormalities in all antenatal cases i.e. in both high risk and low risk pregnancies.

Assessment of functional status of fetal brain still remained unresolved issue and is very vital for couple who are presenting with history of previous child affected with neuro development failure due to an inherited neurological diseases and other factors. Besides inherited genetic diseases other causative factors like intrauterine fetal infections, maternal alcohol consumption, and exposure to various types of environmental pollution, radiations and hypoxia in prenatal stage are main responsible causes for neurodevelopment failure in fetus. In many cases, cause remains unexplained and recurrence is also very common. Certain morphological deviations called soft markers in fetal brain in prenatal stage gives clue about functional status of brain after birth [2]. These soft markers are known as Neurogenetic soft markers, are assessed in all fetuses in antenatal period between 18 to 20 weeks of gestation. Significant number of fetuses with chromosomal and Single Gene defects may present with these soft markers, giving an indication for further genetic evaluation and diagnosis at chromosomal and molecular level [Table 1 & 2].

Five Neurogenetic soft markers evaluated in routine antenatal ultrasound scanning for fetal imaging are:

1) Mild Ventriculomegaly

Ventriculomegaly is defined as a measurement >10 mm across the atria of the posterior horn of lateral ventricles at any period of the gestation [Figure 1]. Mild 10 to 12 mm, moderate 13 to 15 mm, and severe ≥16 mm. Most commonly, the term "ventriculomegaly" is used when the ventricles are mildly enlarged, and "hydrocephalus" is used when they measure >15 mm. It is the most commonly detected Ultrasonographic abnormality of the central nervous system and is clinically important because it can be caused by a variety of disorders which may result in motor and cognitive impairment in child.

Ventriculomegaly is associated with chromosomal and single gene defects, in utero infections, anatomical defects like Chiari malformations, neural tube defects, Dandy Walker malformations, agenesis of the corpus callosum, or destruction, vascular anomalies, or an obstruction within the ventricular system and intracranial haemorrhage and autoimmune disorders. The likelihood ratio with this soft maker is 9/ for the risk of karyotype abnormality.

2) Cavum septum pellucidum

The septum pellucidum is a thin vertical membrane that connects the corpus callosum to the columns of the fornix and separates the lateral ventricles.

Table 1. Chromosomal abnormalities associated with Neurodevelopment failure

Down Syndrome	Trisomy 21 (47 chromosomes)
Edward Syndrome	Trisomy 18 (extra copy of 18th chromosome)
Patau Syndrome	Trisomy 13 (extra copy of 13th chromosome)
Trisomy 16 Syndrome	Trisomy 16 (extra copy of 16th chromosome)
Turner syndrome	Monosomy X (absence of one copy of X chromosome)
Klinefelter's Syndrome	XXY (extra copy of X chromosome)
Triple XXX Syndrome	XXX (extra copy of X chromosome)
XYY Syndrome	XYY (extra copy of Y chromosome)
Jocobsen syndrome	Terminal 11q deletion
Cri-du-chat syndrome	Chromosome 5p deletion

Table 2. Single gene defects associated with Inherited Neurological diseases

Inheritance Pattern	Disease	Gene
Autosomal recessive	Alcardi-Goutieres syndrome	TREX1
	Infantile diffuse degeneration of cerebral grey matter with hepatic cirrhosis	POLG
	Ataxia telangiectasia	ATM
	Canavan disease	ASPA
	Citrullineamia	ASS1
	Cohen syndrome	VPS13B
	Fatal infantile cardioencephalomyopathy due to cytochrome C oxidase deficiency	SCO2
	Friedreich ataxia	FXN
	Gangliosidosis	GLB1
	Gaucher disease type 2 and type 3	GBA
	Glycine encephalopathy	GLDC
	Leigh syndrome	SURF1
	Leukoencephalopathy with vanishing white matter	EIF2B2
	Metachromatic leukodystropy	ARSA
	Neuronal ceroidlipofuscinosis 2	TPP1
	Phenylketonuria	PAH
	Sandhoff disease	HEXB
	Smith-Lemli-Optiz Syndrome	DHCR7
	Spinal muscular atropy2	SMN1
	Succinic semialdehyde dehydrogenase deficiency	ALDH5A1
	Tay-Sacs disease	HEXA
	Zellweger syndrome	PEX1
Autosomal dominant	Alzheimer disease	APP
	Amyloidosis hereditary neuropathic disorder	TTR
	Charcot-Marie-Tooth disease axonal type 2E, Demyelinating type 1A and type 1B	NEFL,PMP22,MPZ

	Fascioscapulohumeral muscular dystrophy 1A Familiar retinoblastoma Hypokalaemic periodic paralysis Huntington disease Myotonic dystrophy 1 Neurofibromatosis type 1 and type Nonsyndromic holoprosencephaly Spinocerebellar ataxia type 1,type2, type6 and type7 Tuberous sclerosis 1	FRG1 RB1 CACNA1S HTT DMPK NF1,NF2 SHH ATXN1,ATXN2, CANA1A, ATXN7 TS1
X-Linked	Adernoleukodystropy Becker muscular dystropy Charcot-Marie-Tooth disease Duchenne muscular dystrophy Emery-Dreifuss muscular dystrophy Fragile X Syndrome Hydrocephalus Lesch-Nyhan syndrome Pelizaeus-Merzbacher-like disease Rett syndrome Spinal and bulbar muscular atropy	ABCD1 DMD GJB1 DMD EMD FMR1 L1CAM HPRT1 PLP1 MECP2 AR
Mitochondrial	Mitochondrial encephalomyopathy, Lactic acidosis and stroke-like episodes	MTND1P1, MTND5P1 MT-TH, MT-TL1 MT-TV
Prion disorder	Gerstmann-straussler-sheinker syndrome	GSS

The septum has a right and left leaf, which are part of the medial hemispheric border [Figure 2]. The cavum septum pellucidum is reliably visible prenatally by ultrasonography and its size can be accurately quantified. In normal fetuses, the cavum septum pellucidum should always be visualized between 18 and 37 weeks. Failure to detect the cavum septum pellucidum within this time interval or increased size of the cavum may indicate abnormal cerebral development and requires further investigation. The width of the cavum septi pellucidi ranged from 2.0 to 10.0 mm with a mean of 5.50 ± 1.48 mm. Absent CSP is a very important CNS malformation marker and it triggers extended fetal morpho-genetic evaluation. Almost half of the cases associated genetic disorders and 39% associated structural malformations like optic nerve hypoplasia, holoprosencephaly, septo-optic dysplasia, abnormalities of the corpus callosum, and Chiari II malformation, midline defects, schizencephaly.

An enlarged cavum septi pellucidi or cavum vergae warrants consideration of genetic counseling and testing. However, long-term follow-up is recommended in the apparently normal neonates and infants, as they may develop abnormal psychological behavior lately

3) Mega Cisterna Magna

An enlarged cisternal magna is defined as an anterio posterior diameter >10 mm. The cisterna magna is measured on a transaxial view of the fetal head angled 15 degrees caudal to the canthomeatal line [Figure 3]. The anterio posterior diameter is taken between the inferio-posterior surface of the vemis of the cerebellum to the inner surface of the cranium. The measurement

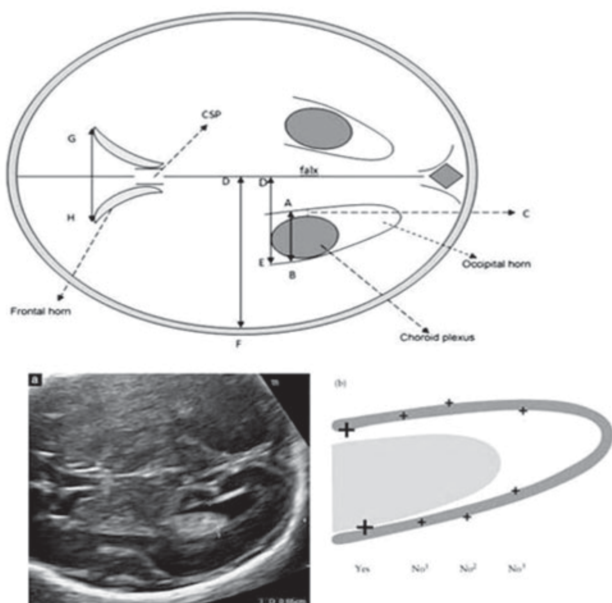


Figure 1. Lateral ventricles in fetal brain

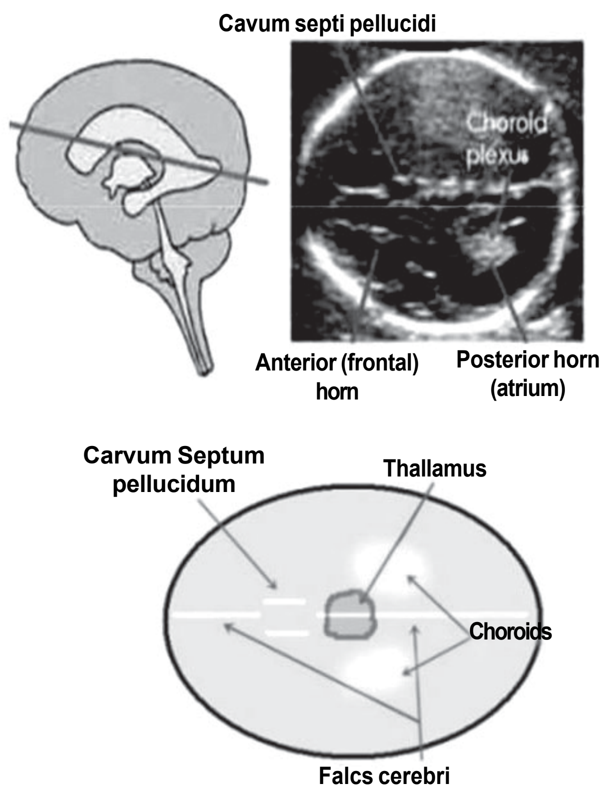


Figure 2. Carvum Septum pellucidum in fetal brain

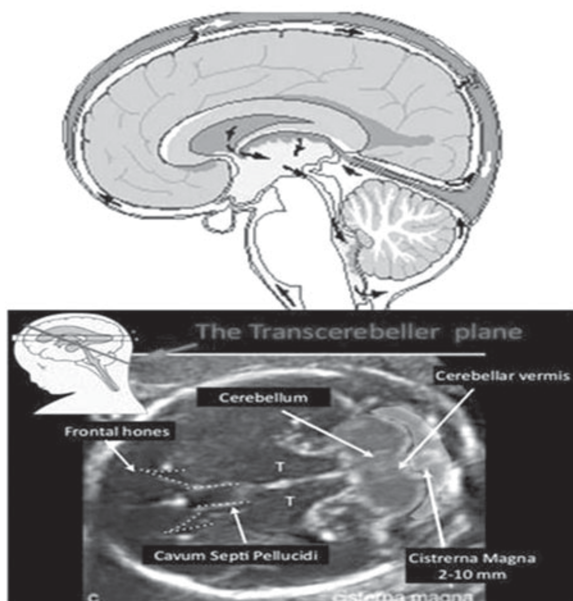


Figure 3. Cisterna Magna in fetal brain

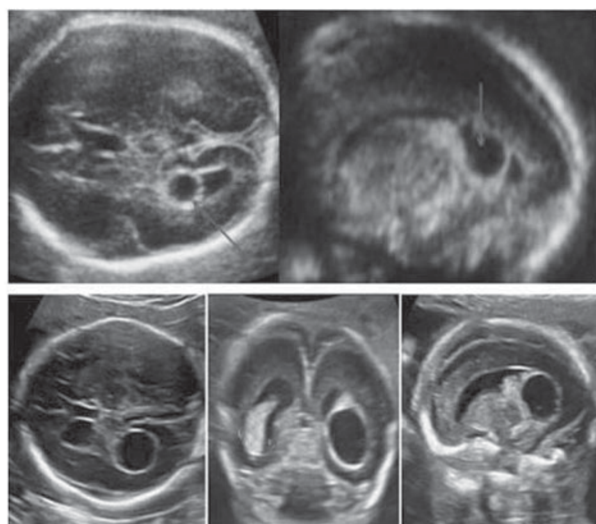


Figure 4. Choroid Plexus Cysts in fetal brain

will be falsely exaggerated by a steep scan angle through the posterior fossa or dolichocephaly. A mega cisterna magna can be associated with infarction, inflammation and infections particularly cytomegalovirus and chromosomal abnormalities-especially trisomy 18, the likelihood ratio for chromosomal abnormalities is 1.9 with this marker. Anatomic defects like arachnoid cyst, Dandy Walker malformation, and Dandy Walker variant and syndromic oro-facial–digital syndrome, Meckel-Gruber syndrome, and DiGeorge syndrome abnormalities are associated with this marker. Mega cisterna magna needs to be distinguished from other causes of an enlarged retro-cerebellar CSF space, arachnoid cyst, epidermoid cyst, cerebellar atrophy & cerebellar hypoplasia.

A detailed fetal examination should be performed, looking for other anomalies, growth restriction, or abnormalities in amniotic fluid volume. An isolated enlarged cistern magna may not be an indication for fetal karyotyping. But with an enlarged cisterna magna, review and follow-up ultrasound scans are essential. Fetal karyotyping should be offered, if an enlarged cisterna magna is association with other abnormal findings.

4) Choroid Plexus Cysts

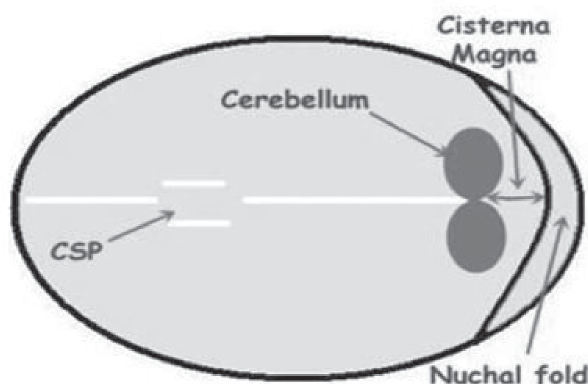
These are found in about 1–2% of pregnancies and are usually of no pathological significance. These are sonographically discrete, small cysts more than 3 mm found in the choroid plexus within the lateral cerebral ventricles of the developing fetus at 14 to 24 weeks of gestation. Imaging of choroid plexus is performed in the transverse plane of fetal head at the same level that the lateral cerebral ventricle is evaluated [Figure 4].The choroid plexus should be inspected bilaterally for the presence of cysts. The size of CPCs is not of clinical relevance.

Evaluation of the choroid plexus near field ventricle is more difficult owing to imaging artefact, when other defects are present, there is a high risk of chromosomal defects, usually trisomy 18 but occasionally trisomy 21. For isolated choroid plexus cysts, the risk for trisomy 18 and trisomy 21 is 1.5-times the background risk. Counseling and possible invasive testing might be necessary if maternal age is 35 years or older or the maternal serum screen is positive for either trisomy 18 or 21 [4].

5) Thickened Nuchal Fold

The nuchal fold is the skin thickness in the posterior aspect of the fetal neck [Figure 5]. Its measurement is obtained in a transverse section of the fetal head at the level of cavum septum pellucidum and thalami, angled posteriorly to include the cerebellum. The measurement is taken from the outer edge of the occiput bone to the outer skin limit directly in the midline.

The significant measurements are ≥ 6 mm at 18 to 24 weeks and ≥ 5 mm at 16 to 18 weeks . A thick nuchal fold at ≥ 6 mm increases the risk of Down syndrome by approximately17-fold [4].



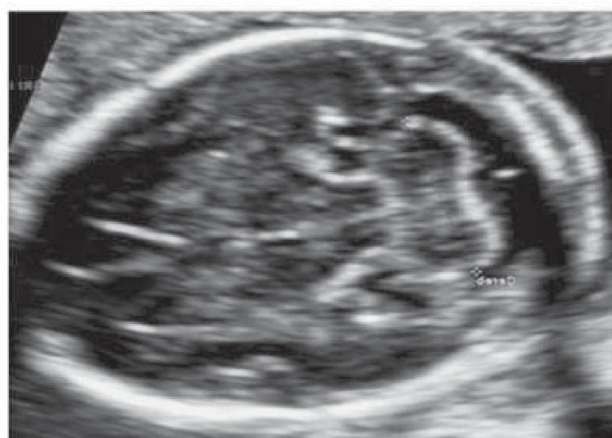


Figure 5. Nuchal fold thickness of fetal brain

It could also be associated with single gene abnormalities such as Noonan syndrome, multiple pterygium syndrome, skeletal dysplasias, congenital cardiac defects and all the conditions which may cause hydrops fetalis.

Thickened nuchal fold significantly increases the risk of fetal aneuploidy. Expert review along with karyotyping and fetal echocardiography should be recommended [5].

Conclusion

These genetic soft markers are of paramount importance in predicting neurodevelopmental status of infant after birth and also increased risk of IUGR, IUD and Stillbirth etc. In all high pregnancies with previous child with neurodevelopmental delay, these neurosonographic soft markers evaluation must be performed routinely. In low risk pregnancies screening for these soft makers helps in identifying sub group of fetuses at risk and that requires increased surveillance and genetic evaluation.

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SYMPOSIUM ON

GENETICS OF METABOLIC DISORDERS

BIOCHEMICAL BASIS OF METABOLIC DISORDERS

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INTRODUCTION

Inborn errors of metabolism (IEM) result from the deficiency of enzymes in metabolic pathways. Most IEM are inherited as autosomal recessive disorders. More than 500 different IEMs are known.

Deficiency of an enzyme in a pathway can produce the following effects –

1 Accumulation of the substrate of that enzyme
2 The pathway may be directed to an alternate pathway, leading to accumulation of abnormal metabolites
3 Biochemically important products of the main pathway are reduced

These basic metabolic alterations are responsible for the diverse clinical manifestations.

There are some disorders which are asymptomatic (essential pentosuria, alkaptonuria etc). But most of the IEM can lead to mental retardation and even death if untreated. The effects of toxic substances and their by-products increase with time if the offending diet is not restricted or suitable mechanisms for removing the accumulating toxins are not taken. Therefore, it is not surprising that some disorders may be mild at onset, but deteriorate with time. Many metabolic disorders however have an acute onset of clinical symptoms.

Common clinical features are –

1 Neurological - Psychomotor delay, mental retardation, seizures, dystonia, ataxia, lethargy, coma, encephalitis, speech delay, hyperactivity etc
2 Non-neurological - Failure to thrive, organomegaly, vomiting, skin rashes, metabolic acidosis, hyperammonemia, hypoglycemia (low blood sugar), lactic acidosis and ketonuria.

Most IEMs can be treated, with special diets, avoidance of particular food stuff and other specific measures. Treatment has to be started very early in life, and has to be continued for a lifetime. An early accurate diagnosis is mandatory.

This review discusses some important IEMs seen in the Indian population with a focus on those disorders which can be diagnosed easily.

Classification of IEM -

IEM can be classified into -

1. Amino acid disorders
2. Organic acidurias
3. Urea cycle disorders
4. Carbohydrate disorders
5. Mitochondrial fatty acid disorders
6. Mitochondrial disorders
7. Peroxisomal disorders
8. Lysosomal storage disorders
9. Purine and Pyrimidine disorders
10. Porphyrias
11. Metal metabolism disorders.

Another classification of IEM is

1. Disorders of intermediary metabolism,
2. Disorders of biosynthesis and breakdown of complex molecules and
3. Neurotransmitters defects and related diseases.

Disorders of intermediary metabolism include amino acid disorders, carbohydrate disorders, fatty acid and mitochondrial energy metabolism defects. They can be diagnosed by estimation of lactate, ammonia, plasma amino acids, urine organic acids and acyl carnitine profile. Clinical signs and symptoms appear very early.

The second group has slow progression and less chance of acute metabolic decompensation which is very common in the first group. Diagnosis requires specialized investigations. These diseases include lysosomal storage disorders, peroxisomal disorders, disorders of purines, pyrimidines, isoprenoid and sterol pathways, bile acid and heme, lipoproteins and congenital diseases of glycosylation (CDG).

The third group includes disorders of glycine and serine, pterin and biogenic amines and gamma amino butyric acid (GABA).

Amino acid disorders

There are many amino acidurias which are encountered in clinical practice. Diagnosis can be made by quantification of amino acid levels in blood, urine and in some cases, by CSF.

Hyperphenylalaninemias

These are disorders of phenylalanine metabolism, due to deficiency of the enzyme phenylalanine hydroxylase (PAH). Normal level of phenylalanine in blood is 47-74 μmol/L. Hyperphenylalaninemia is defined as a value above 120 μmol/L. The gene for PAH is located in 12q22-24.1.

Hyperphenylalaninemia can be broadly classified into-
1. PKU (Phenylketonuria)
2. Non-PKU hyperphenylalaninemia.

The overall incidence of hyperphenylalaninemia is estimated to be 1 per 1 million births, but there is considerable difference in the prevalence across different populations. It is considered to be a common cause of mental retardation in some populations. Clinical features include mental retardation and impaired cognitive function.

PKU is characterized by very high levels of phenylalanine (above 1000 μmol/L). Lesser elevation of phenylalanine is seen in non-PKU type. The pathogenic mechanism of hyperphenylalaninemia is due to the accumulation of phenyl ketones, comprising phenyl pyruvate, phenyl acetate, phenyl lactate, phenyl ethyl amine and phenyl acetyl glutamine (Figure 1). Diagnosis can be made by urine screening tests (ferric chloride and Guthrie tests) and by blood phenylalanine level quantification. PKU diagnostic kits are also available. Here, a drop of heel-prick blood on dried filter paper is taken; this has the ease of transport to distant laboratories and stability on storage. Treatment includes low phenylalanine diet, enzyme supplementation and gene therapy.

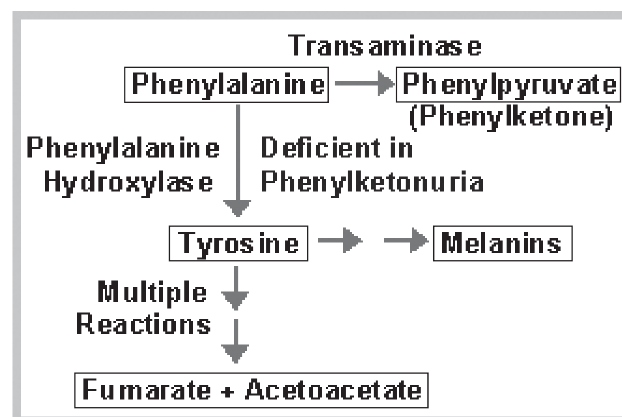

Figure 1. Metabolic pathway for phenylalanine showing abnormality in PKU

Alkaptonuria

Alkaptonuria is a rare, hereditary disease where homogentisic acid, an intermediate product in the metabolism of phenylalanine and tyrosine is excreted. Metabolic defect is characterized by the triad of homogentisic aciduria, ochronosis and severe crippling arthritis (in adults). Deficient enzyme is homogentisic acid oxidase. If urine containing homogentisic acid is allowed to stand for sometime, it gradually turns black; this is due to polyphenol polymers. They get deposited in connective tissues leading to ochronosis. Benedict's test gives a muddy brown color (Figure 2).

Maple syrup urine disease (MSUD)

MSUD (branched chain ketoaciduria) is due to deficiency of branched chain alpha ketoacid dehydrogenase (BCKAD complex). This leads to accumulation of branched chain amino acids, viz. leucine, valine and isoleucine, and the corresponding branched chain alpha keto acids (Figure 3).

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Diagnosis can be made by –
1. Dinitro phenyl hydrazine (DNPH) test in urine
2. Distinctive odour of maple syrup or burnt sugar to urine
3. Elevated branched chain amino acids in blood
4. Elevated L – alloisoleucine

Treatment includes –
1. Restriction of branched chain amino acids (achieved by protein restriction and substituted artificial food)
2. Treatment of infections and ketoacidosis
3. Treatment of acute episodes – Rapid removal of toxins, nutritional support, minimize catabolic stress/promote anabolism and peritoneal dialysis/ hemodialysis.
4. Liver transplantation
5. Somatic gene therapy

Figure 2: Diaper staining in an infant with alkaptonuria

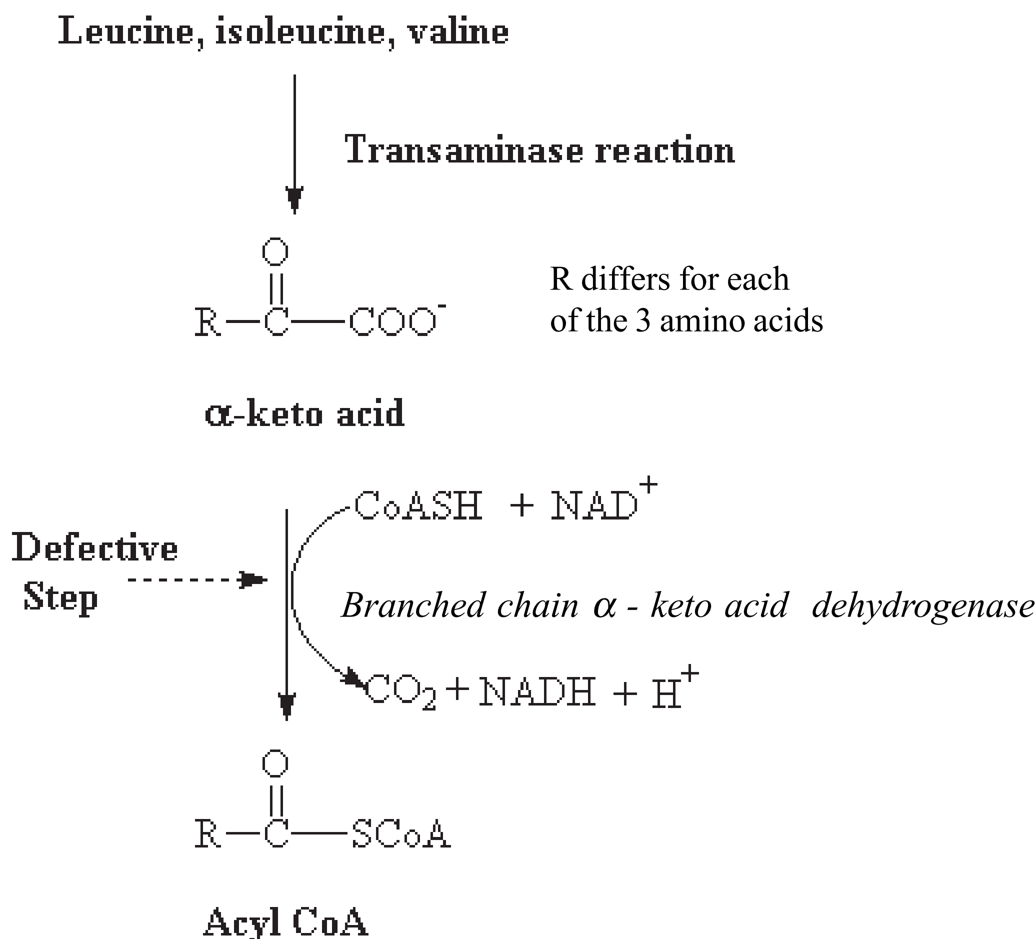

Figure 3: Metabolic pathway for branched chain amino acid (showing defective step in maple syrup urine disease)

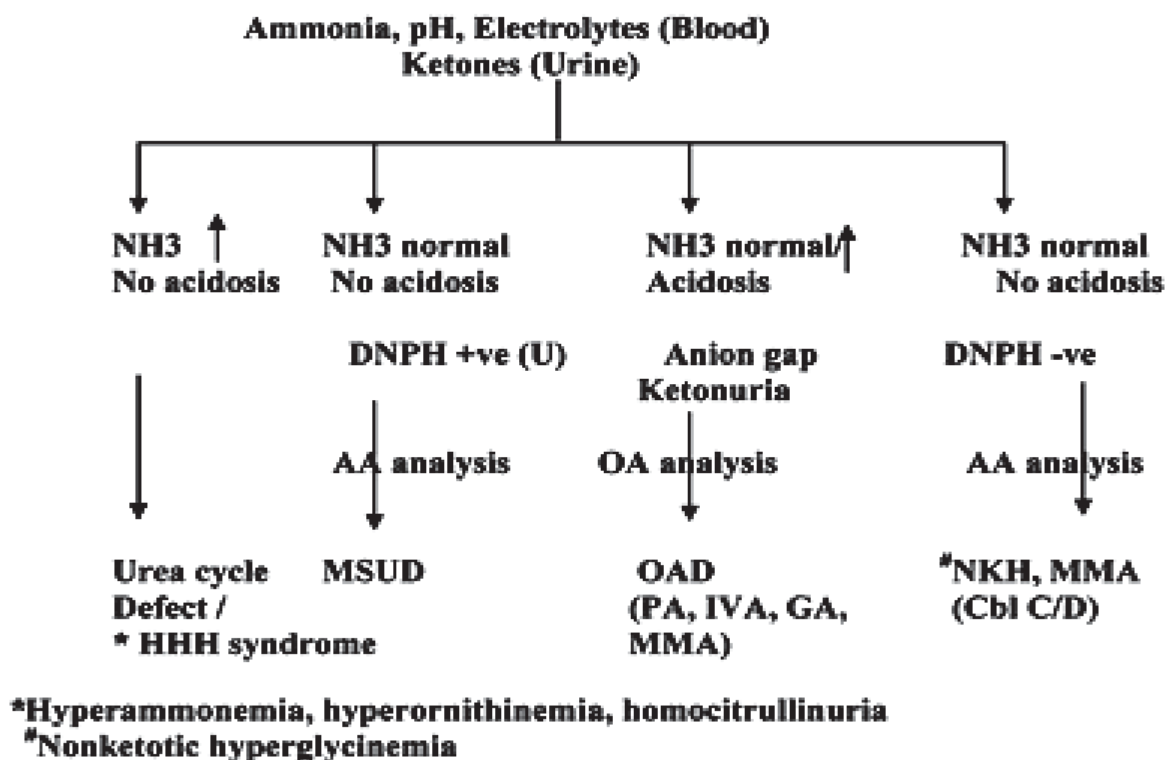

Figure 4: Flow chart for the diagnosis of organic acidurias

Majority of untreated patients die within the early months of life due to recurrent metabolic crisis and neurological deterioration. Patients on whom treatment is initiated after 10 days of life rarely achieve normal intellect.

Urea cycle disorders

Urea cycle disorders comprise a group of 5 disorders; carbamoyl phosphate synthetase deficiency (CPSD), ornithine transcarbamylase deficiency (OTCD), arginosuccinic acid synthetase deficiency (ASD), arginosuccinase deficiency (ALD) and arginase deficiency. The first 2-3 are known as proximal urea cycle disorders and the remaining as distal urea cycle disorders (Figure 4).

The first 4 generally present with hyperammonemia and metabolic alkalosis in the neonatal period, which is the hallmark of urea cycle disorders. Hyperammonemia is neither as severe nor as common in arginase deficiency.

Depending on time or presentation, urea cycle disorders are classified into –

1. Neonatal-onset group, and
2. Later-onset group.

Infant is normal for 24 hours, between 24 and 72 hrs infant develops signs and symptoms of hyperammonemia, viz. lethargy, difficulty in feeding, vomiting, hypothermia and hyperventilation. History of consanguineous marriage and neonatal sib death is often present.

Symptoms prior to 24 hrs is indicative of transient hyperammonemia of the newborn (THAN), in a pre-term infant and inborn errors of metabolism, like pyruvate dehydrogenase deficiency, glutaric acidemia Type II in a term baby. If symptoms appear after 24 hrs, it may be either organic aciduria, where ketoacidosis (metabolic acidosis) will be present or urea cycle disorder, where there is no ketoacidosis. Plasma citrulline analysis along with urinary orotate will help in the differential diagnosis of urea cycle

disorders. Plasma citrulline absent with low orotate indicates CPSD, and high orotate indicates OTCD. Plasma citrulline 100-300 µmol/L is indicative of ALD and citrulline >1000 µmol/L is diagnostic of ASD.

Treatment for urea cycle disorders include –

1. Low protein, high calorie diet
2. Sodium benzoate
3. Ondansetron hydrochloride
4. Sodium phenyl acetate
5. 10% arginine hydrochloride
6. Mannitol
7. Corticosteroids are contra-indicated.

About 3-4 days may be needed for an acute attack of hyperammonemia to subside.

Non-ketotic hyperglycinemia (NKH)

NKH is an inborn error of glycine degradation. Large amounts of glycine accumulate in all body tissues, including blood and brain; leading to intractable seizures, characteristic of NKH. Diagnosis is by estimating CSF/plasma glycine ratio. A ratio >0.08 is diagnostic. Normal CSF/Plasma ratio is 0.012-0.040. There is no effective treatment for NKH.

Propionic aciduria (PA)

Propionyl CoA carboxylase (PCC) is deficient in this condition. PCC deficiency leads to propionic acidemia, and elevated 3 hydroxy propionate, methyl citrate, tiglyl glycine, and unusual ketone bodies in urine. Severe metabolic ketoacidosis in neonatal period is seen. Alkali therapy and protein restriction are needed.

Methylmalonic aciduria (MMA)

Inherited deficiency of the mutase enzyme or abnormalities in cobalamin can result in methyl malonic aciduria. Neonatal or infantile metabolic ketoacidosis are the hallmarks. Features include failure to thrive, developmental retardation, megaloblastic anemia and macrocytosis. Therapy includes protein restriction, pharmacological doses of hydroxocobalamin and betaine supplementation. Prenatal diagnosis is possible by enzyme assays on chorionic villus biopsy or cultured amniotic cells and chemical determinations on amniotic fluid or maternal urine.

Methylmalonic aciduria and propionic aciduria are organic acidurias and are very commonly encountered in our population. Treatment with multi-vitamin supplementation, including betaine, cobalamin has also been reported to be useful. Other common organic acidurias include isovaleric acidurias (IVA) and glutaric acidurias (GA).

Other common disorders

Congenital hypothyroidism and congenital adrenal hyperplasia (estimated by 17 alpha-Hydroxy progesterone) are diagnosed by newborn screening ELISA kits, and are commonly seen in all parts of India. Galactosemia, glucose 6 phosphate dehydrogenase (G6PD) deficiency, homocystinuria and thalassemia are also common. Simple tests can be used to diagnose these conditions as well.

SUMMARY

Diagnosis of IEM is important because most IEM can be treated and the child can lead a near-normal life. Others have the importance that prenatal diagnosis can be given in future pregnancies. India is a country with more than 28 million births every year and we are faced with about 30, 000 patients with metabolic disorders every year. Hence though individually rare, our burden of IEM is high. There are special food supplements now available in the market for treatment of IEM; for example phenylalanine deficient supplements in PKU, branched chain amino acid deficient supplements in MSUD.

Most developed countries have screening programs for detecting IEM. In India, many people now understand the importance of early diagnosis. Tandem mass spectrometry and molecular diagnosis by mutation detection have become popular in the West for the diagnosis of metabolic disorders. In India also, some centers do offer similar facilities. What we are lacking at the moment is a nation-wide screening program. A proper screening program will be able to identify a child with IEM in the initial stages itself.

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UNCOVERING NOVEL DISEASE GENES IN MENDELIAN DISORDERS BY EXOME SEQUENCING

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INTRODUCTION

There have been various means of gene identification methods so far. These techniques have mostly been successful in identifying Mendelian disorders. Earlier gross genetic changes like large translocation as evidenced by changes in karyotyping increased our knowledge of genetic basis of pathogenesis of disease. Although karyotyping is easy to perform it has poor resolution for detection of most genetic disorders. Classical linkage analysis by use of microsatellite markers despite being a laborious method was useful for identification of novel disease genes. Homozygosity mapping enables gene identification in recessive diseases in consanguineous families. Next generation sequencing methods (NGS) have made it possible to identify disease genes by whole exome or whole genome sequencing.

In routine clinical diagnostics for inherited disorders, conventional Sanger sequencing is the gold standard. But when the disease associated genes are numerous in number, for e.g. in retinitis pigmentosa mutations are present in more than 120 genes, it is practically not feasible to perform Sanger sequencing. Exome sequencing might be more practically useful especially but incomplete representation and coverage of exons may lead to missing clinically relevant mutations which doesn't make it a substitute for Sanger sequencing. A process to streamline the findings by exome sequencing supported by Sanger sequencing is probably the best approach find novel disease genes.

Whole exome sequencing is a test to identify disease causing variants in approximately 95% of the protein coding sequence of all current clinically – offered disease genes. It has been reported that 85% of the known disease-causing variants occur within 1% of the genome which contains the exons and splice junctions [1]. Sequencing and analyzing only this portion of the genome works as an efficient and powerful clinical diagnostic tool. The advantages of this method over routine genetic testing and even targeted sequencing is the ability to provide molecular diagnosis to maximum number of patients in a short period of time especially since only a fraction of patients get a molecular diagnosis due to the genetic heterogeneity and inefficiency of the conventional technique [2]. The overall cost of undergoing traditional diagnostic assessments and the time taken may very well cost many folds more than exome sequencing [3].

MATERIALS AND METHODS

During the first evaluation of an infant or child a careful history is taken of pregnancy and delivery, as well as a medical and dysmorphological clinical/ neurological history. A three-generation family history is taken to investigate consanguinity. The collection and genomic analysis of the DNA were approved by the institutional review board and written informed consent was obtained. Blood samples were collected and genomic DNA extracted using alkaline lysis and ethanol precipitation (GentraPuregene; Qiagen Corp., Valencia, CA USA).

Sequencing was performed on the Illumina HiSeq 2000 using 100 bp paired-end read sequencing protocol (Axeq Technologies Asia, Macrogen Inc., Seoul, South Korea).

The three main steps in any NGS technique are 1) Template generation or Library preparation 2) Sequencing and 3) Data analysis

Library preparation: First step is to prepare the library of DNA fragments which is dependent on multiple factors like purity, integrity and quantity of the original DNA [4]. Adapter Ligation involves artificial DNA linkers which are unique to each sequencing platform being attached to the 3' and 5' ends of the DNA fragments. Sometimes short synthetic sequences are added to identify a sample's fragments to enable pooling of samples at subsequent steps, a process referred to as indexing or barcoding [5].

Immobilization: DNA fragment population is attached to solid surfaces or beads to allow downstream sequencing reaction to operate as millions of microreactions in parallel in spatially distinct areas [6].

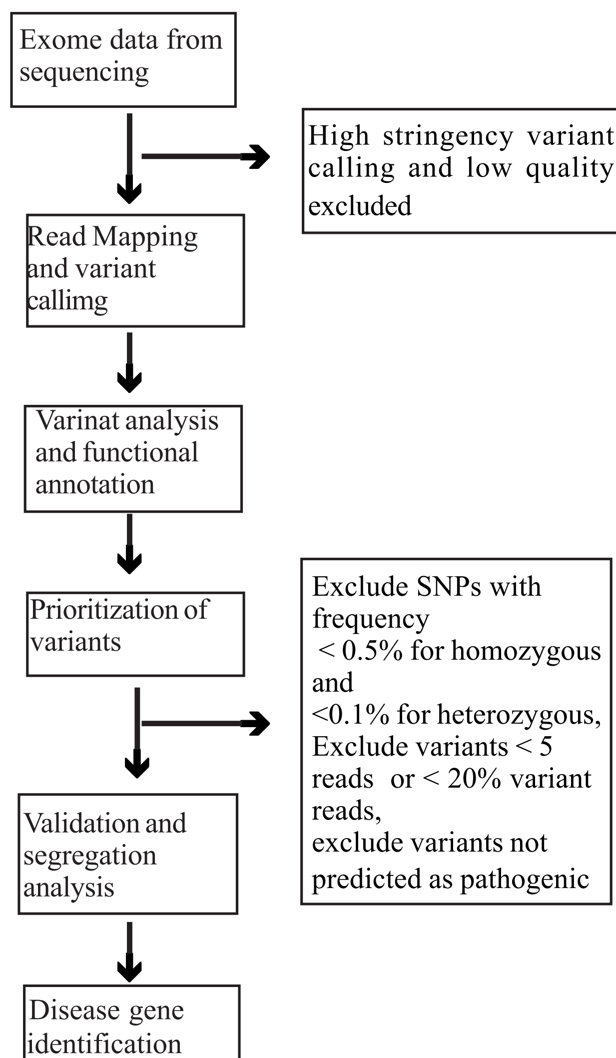

Figure 1. Data Analysis work flow

Amplification: The method involves solid phase amplification- initial priming and extension of single strand template followed by amplification of the template with the adjacent primers to form bridges and make clusters.

Sequencing: It basically involves a series of chemical reactions that are carried out which lead to addition of bases either by synthesis or ligation, washing of reagents, and signal detection and repetition of this cyclical process continues in parallel to sequence all DNA templates.

Data analysis: It is crucial feature of any NGS project and it will depend on the goal and type of project. Alignment is the process of assigning or mapping each read to a corresponding position in a reference sequence. Reads were aligned to the reference human genome (hg19) using the Burrows–Wheeler alignment tool using default parameters [7]. Reads with a mapping quality score ≤10 were discarded using SAM tools and the Picard Mark Duplicates tool was used to identify and discard read duplicates [8, 9]. Resequencing or Variant calling refers to the search for variants in a sample as compared to the reference genome. SNPs and indels were identified using the Genome Analysis Toolkit (GATK) version 1.6-9,17Variantswere annotated using Annovar [10]. Known polymorphisms were identified using dbSNP132, dbSNP135, 1000 genomes and NHLBI exome project and minor allele frequencies were recorded from 1000 genomes and ESP5400. Minor allele frequencies were recorded manually from dbSNP when required. After variants are identified, they are filtered and annotated to find clinically significant variation. We used the missense prediction programs, PolyPhen-2, SIFT and MutTaster [11]. Results from these three different tools were combined to decide whether a specific variant is benign or pathogenic. Variant confirmation of the remaining variants is done by PCR with Sanger sequencing.

DISCUSSION

Using this approach [Figure 1], validation led to a total of 8 variants in the proband which were followed by Sanger sequencing and segregation analysis.

The strength of our approach lies in the parallel analysis of all disease genes in our patients. Systematic variant prioritization reduced the initially high number of indels and SNPs to a number which can be sequenced and validated. After the variants have been trimmed, efforts are made to find possible functional associations between the gene and the disease from molecular expression in relevant tissues and published animal model studies. Novel genes need to be validated in animal models as proof of pathogenicity which is ongoing.

Our NGS approach resulted in narrowing down molecular diagnosis in patients;the patient is not taken on a diagnostic odyssey and low detection rates by other methods. Similar approaches in conditions like intellectual disability and retinitis pigmentosa have resulted in a diagnostic yield of 53% and 36% respectively [11,12].

Limitations: The accuracy of most current platforms is decreased in homopolymer stretches, GC and AT-rich regions. Platform to platform variability results in discordance in 19.9% SNVs and 73.5% indels for the same individual [13]. Variant types that are not well detected are trinucleotide repeats, copy-number variants, large indels, structural variants, aneuploidy and epigenetic alterations. Interpretative uncertainty can arise in cases where the pathogenicity of the variant cannot be established for sure. Thus, patients need to be explained the outcome with proper pre-test and post -test counseling. Regularly reanalyzing negative cases based on recent literature must be done [14]. The procedure due to sequencing costs remains expensive.

The diagnosis of unidentifiable Mendelian disorders is important because -

1. Identification of the mutation establishes the molecular diagnosis, which is useful for improved clinical management.
2. It allows for family counseling, risk prediction and prognostic information.
3. It will allow for the design of a detailed panel of genes for targeted gene sequencing for clinical investigation of patients.
4. Detailed genotype-phenotype correlations can be made for tailored approaches to treatment of the patients.

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CONTRIBUTION OF GNPTAB AND GNPTG MUTATIONS TO PERSISTENT STUTTERING

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INTRODUCTION

Stuttering is a complex speech disorder which impairs the fluency of speech that develops strong emotional impact in person who stutter (PWS). Speech is disrupted by repetitions, prolongations or abnormal stoppages called "blocks" accompanied by facial and body movements that develop in an effort to speak fluently. This disorder typically is seen among 5% of children among them 80% recover spontaneously leaving behind 1% of the population to be persistent [1]. It affects more males than females [2]. The current landscape of scientific research into stuttering has been fueled in large measure by technological advances that allow for increasingly reliable and precise study of genes, neuroanatomy, neurophysiology, and motor coordination.

Though the underlying cause for stuttering remains speculative, it has a strong evidence of genetic basis. Twin and adoption studies show high heritability of trait. Monozygotic twins have high concordance but always less than one indicating the role of environmental factors. Adoption studies were limited in stuttering with statistical limitations [3]. Familial studies to identify the mode of inheritance revealed familial clustering with high incidence in family than general population. Initial linkage studies were only suggestive although significant linkage was achieved in conditional models [4,5,6].

A single genome wide association study (GWAS) by Kraft 2010, did not reveal any significant association indicating the genetic heterogeneity involved. However, significant linkage was found at chromosomes 12, 3, 16, 2, 14, 15 & 10 in subsequent studies [7,8,9,10,11]. These studies led to the identification of genes involved in the lysosomal targeting pathway, signifying deficits in intracellular trafficking identified in many neurological disorders.

The gene GNPTAB suggested to have a causative role in stuttering was identified in a consanguineous family from Pakistan, showing linkage to chromosome 12. This gene encodes for N-acetylglucosamine-1-phosphotransferase. A non- synonymous variant c.G3598A in this gene was observed, that is present at a highly conserved site leading to substitution of glutamic acid to lysine at 1200(p.Glu1200Lys, c.G3598A). Subsequent analysis of this gene identified three other mutations (p.Ser321Gly, c.961A>G; p.Ala455Ser, c.1363G>T; p.Phe624Leu, c.1875C>G).

Additional studies that were extended to functionally related genes called GNPTG that encodes the gamma subunit of N-acetylglucosamine-1-phosphotransferase and NAGPA that encodes for N-acetylglucosamine-1-phosphodiester alpha N-acetylglucoaminidase, also referred to as uncovering enzyme (UCE). GNPTG and NAGPA genes are present in the same chromosome 16 lying close to each other harbored mutations in PWS (People Who Stutter). All the three genes are involved in a two step biochemical pathway that adds mannose 6 phosphate signal to the hydrolases targeted to lysosomes.

GNPTAB/G is already implicated in mucolipidosis types II and III characterized by skeletal, cardiac, and ocular disorders in addition to deficits in speech particularly in expressive speech domain [12]. The causative role of these genes in stuttering is supported by the fact that these patients also have deficits in speech. The mutations in stuttering were missense

mutations rather than protein truncation or deletions, which are typically observed in mucolipidosis and presumably have more effect on protein function [13].

Another gene AP4E1 in chromosome 15 that encodes adaptor protein complex 4, epsilon 1 subunit was found to co segregate in a Camaroon family during whole exome analysis. This gene was found to be interacting physically with NAGPA and hence have a close relationship with lysosome targeting machinery [14]. All the four genes cumulatively accounts for 20% of unrelated cases of persistent stuttering and they point to a single pathway that leads to deficits in intracellular trafficking. Conversely, these evidences also suggest the need to unravel genetic and mutation landscape of stuttering among different ethnicities. However, the role of these four genes implicative in stuttering is unclear [3].

Thus the present study, aims to identify the causative role of GNPTAB and GNPTG among Indian PWS that helps to provide important targets for intervention.

METHODOLOGY

Recruitment of probands: Stuttering probands were ascertained from various potential avenues like schools, speech therapy clinics and self-help groups. Phenotyping involves a detailed fluency evaluation for characterization of speech of the children. Stuttering Severity Instrument (SSI-3; Riley, 1994), a standardized evaluation tool was used to assess all the participants. SSI-3 that includes characterization of stuttering in terms a) Frequency b) Duration and c) Physical concomitants. Stuttering severity index scores were calculated for all three domains. Overall scores on SSI-3 was used to classify the degree of stuttering in an individual as mild (0-15), moderate (16-23), severe (24-45). Rate of speech, speech naturalness and nature of breathing was also assessed.

Once the probands are identified, home visits were made to fill detailed and structured schedule that included demographic and medically relevant informations like gender, age at onset, nature of onset, handedness, peri&post natal life of proband, family history, consanguinity, birth order, sibship size etc. Detailed pedigrees including the first (parents and siblings), second (grandparents) and third degree relatives (first cousins, aunts and uncles) was constructed during the extensive interview of the proband and / or the informant to consider the status in each relative individually.

Sample collection: Blood samples were collected from the individuals after obtaining written informed consent. 10 ml of blood was collected by venipuncture method in EDTA coated vacutainer.

DNA Isolation by Phenol-Chloroform-Extraction Method: Genomic DNA was isolated from the peripheral lymphocytes (WBC) using Phenol Chloroform Isoamyl alcohol (PCI) procedure. The isolated DNA was purified and dissolved and quantified using the Nanodrop (Thermo- Scientific).

Mutation analysis: The mutation specific exons implicated in genes GNPTAB and GNPTG [Table 1]were alone genotyped among 60 individuals with stuttering. The DNA was amplified by hot start PCR using the respective primers [Table 1] and purified using FAVOURGEN kit. The purified PCR products were sequenced using ABI 3730XL sequencing at Bangalore. The sequencing results were then analysed using FinchTV and NCBI-BLAST programs. On identification of the variant, bidirectional sequencing was done to confirm.This study was approved by the Institute of Human Ethical Committee, University of Madras.

Bionformatic analysis: Both the Native protein and mutated protein was subjected to pair wise alignment using the Geneious Pro version 6.1.2. The pair wise alignment was carried out by MAFFT alignment. Default parameters were set to assess and predict the effect of SNP identified in this study.

RESULTS

To assess the causative role of GNPTAB/G genes in stuttering we analyzed 60 stuttering probands from Tamil Nadu. Our data comprised of 56 males and 4 females with a M/F ratio of 14:1. The age distributions of the probands were 4-32 with mean age of 17.56. Among them 33 were evaluated to be severe, 16 moderate and 11 mild PWS. There was higher incidence of familial stuttering (65%) when compared to sporadic occurrence (35%). On analyzing the pedigree 30% (18) of them born to consanguineous parents. Sequencing of the selected exons with

Table 1: Primer sequences of implicated mutations in stuttering

GNPTAB Exon No	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing Temperature	Product size (bp)	Mutations implicated
9	TGCTGTCTCTTTGAATTTTGG	AGGAAGGGAAGGCAATGAAG	60	521	p.Ser321Gly
11	TCAACGCAGCAGGATCTAAA	AGGTTTGCACCACCACACTT	61	591	p.Ala455Ser
13_1	CAAGGACGACATGCAAATTC	GCGTCTTTTGGAAGGAGTGA	61	649	p.Phe624Leu
13_2	TACAGCCCAGAAGGGTTACG	AATCAGAGATGGGGGCTTTT	62	597	
13_3	TGCAGAGGTTGACTTTTCCTG	TCACACTTGGGCTGTTTCCT	62	597	
19	TCATTCCCCCAGAGAATCAT	AGCTTGGGCAACAAGAACAA	60	460	p.Glu1200Lys
GNPTG	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing Temperature	Product size (bp)	Mutations implicated
1	AGGCCCTCAAACCCTGAC	TCCTCCACCACCTTCATCTT	62	497	p.Leu5_ Arg7dup
2	GTTGCTCCTCGGGCTCTC	AAGGCTGACAAACCAATGCT	62	435	p.Ala25Glu
9 & 10	CAGGACCTGGCCGATGAG	AGTTTCTGCCAAAACACCAG	62	500	p.Leu230Val

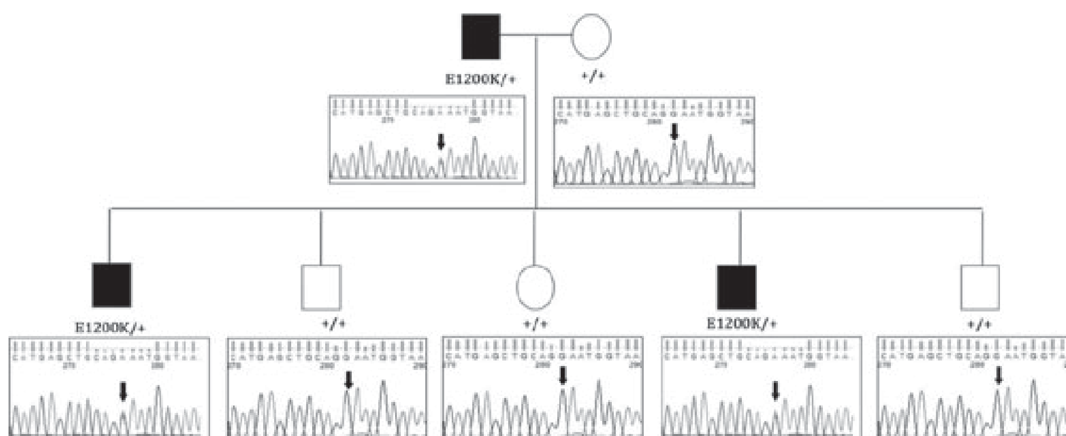

Figure 1. Partial chromatogram of the Glu1200Lys mutation segregating in a stuttering family

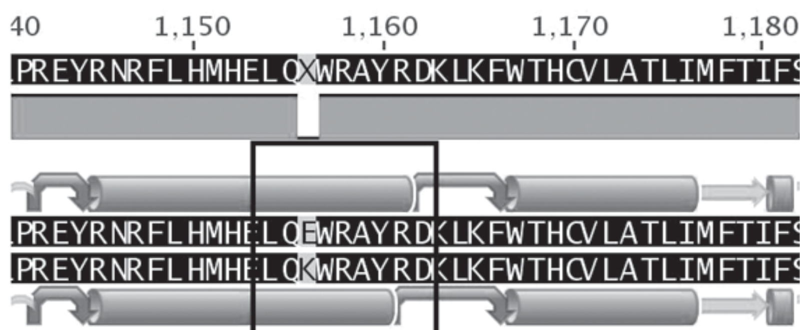

Figure 2. The alignment of secondary structure identified the loss of helix and addition of turn near the mutated region.

previously reported mutations in GNPTAB/G genes in 60 unrelated individuals with persistent stuttering showed the presence of a heterozygous missense pathogenic mutation (Glu1200Lys) in exon 19 of GNPTAB gene in one proband. On expansion of his family we found that the proband's father and elder

Table 2. List of variations found in GNTAB and GNPTG genes

GENE/ Exon	rs number	Codon change	Amino acid change	No of proband	AF in ExAC database	SIFT	Polyphen
GNPTAB							
Exon 19	rs137853825	c.3598G>A	Glu1200Lys	1	0.003552	Deleterious	Possibly damaging
Exon 13_1	rs10778148	c.1932A>G	Thr644Thr	51	0.5885	Synonymous	
GNPTG							
Exon 1	rs554707396	-4 C>T	5'UTR	1	0.00142	May be promoter region	
Exon 9	rs532275192	c.702T>C	Pro234Pro	1	0.00046	Synonymous	
Exon 10	rs377647926	c.813G>A	Thr271Thr	18	0.00002471	Synonymous	
Exon 10	rs759796840	c.802A>C	Ile268Leu	1	0.00000825	deleterious	benign

brother were also having stuttering. The mutation analysis of seven participating members in this family showed that the affected members carried the mutation in heterozygous condition, while the normal speaking members showed absence of this mutation [Figure1].

Bioinformatic analysis: The secondary structure prediction shows a change in the alpha helix due to the mutant E1200K (Figure 2).

Similarly, among these probands, a deleterious mutation viz., Ile268Leu in one other proband was found in exon 10 of GNPTG gene (Figure 3). Pathogenicity predictions tools like SIFT and PolyPhen-2 predicts this variant to be deletorious (0.04) and benign (0.168) respectively. None of the other mutations reported were observed in our study.

DISCUSSION

Two mutations were identified in this study namely Glu1200Lys and Ile268Leu. The mutation Glu1200Lys has been previously reported and study by Fedyna *et al* [15] establish this as founder mutation specific to Asian population. Incidence of this mutation in our study (1.7%) among south Indian ethnicity PWS for the first time favours the hypothesis of the founder effect.The segregation analysis in the family members shows that this mutation is seen in heterozygous state in the affected members and its absence in the unaffected members. This infact suggest that there could be second pathogenic mutation in this gene acting *in trans* or other additive genetic factors that may play a causative role in this family.

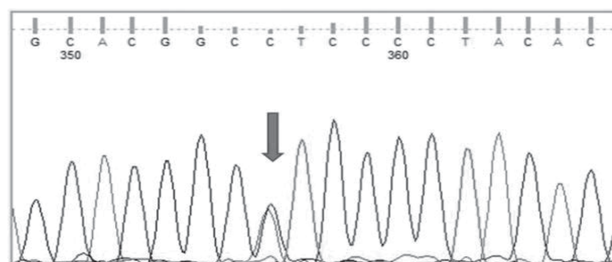

Figure 3. Partial chromatogram of Ile268Leu mutation in heterozygous condition in stuttering proband

However, considering the occurrence of this variant to be pathogenic among PWS is likely less due to its high minor allele frequency in the south Asian ancestry (0.002) as observed from from the ExAC database. Further, predictions based on the protein structure damage suggest that the mutant position is highly conserved (consurf = 8), and the mutation causes disruption in the helical segment that may perhaps be crucial for interaction with other subunits or proteins in lysosomal targeting pathway and its involvement in stuttering. Screening for the remaining exons may clarify the causative role of this gene in stuttering. Also in 2016, 3- to 8-day old mice pups engineered to carry two copies of the Glu1200Lys mutation were observed to have significantly longer pauses in their spontaneous vocalizations than littermates not carrying the mutations, consistent with some features of human stuttering [16].

Commenting on the presence Ile268Leu mutation in our stuttering proband would be premature until we find links such as common pathways that may be

involved in speech processing in both the phenotypes. This mutation has been reported very recently in a single individual (rs759796840) and is not validated till date. So far this has been reported only in mucolipidosis (MLIII).

ACKNOWLEDGEMENT

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PREVALENCE OF T2DM, HYPERTENSION AND HYPERLIPIDEMIA ASSOCIATION STUDY WITH SNP AMONG DIFFERENT POPULATIONS OF PALAKKAD MUNICIPALITY, KERALA

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INTRODUCTION

The worldwide occurrence of diabetes in 2000 was approximately 2.8% and is estimated to grow to 4.4% by 2030 [1]. The epidemic of diabetes will continue to rise as there is growing prevalence of obesity in children, which predisposes to diabetes [2]. There is a strong linear relationship between high blood pressure (BP) levels and the risk of CVD.Co-existent CVD risk factors like dyslipidaemia, raised blood sugar levels and higher body weight are common among prehypertensives. Ray *et al* in their study found a high prevalence of prehypertension (79.8%), lipid abnormalities (about 67%) and overweight/obesity (29.9%) among Indian military subjects [3]. A population based screening survey from Kerala reported that only 11.4 per cent of study participants had BP in the normal range and all others had either hypertension or prehypertension [4]. Ray *et al.* noticed high cholesterol and triglyceride levels among 21.9 and 14.1 per cent respectively, though 92.1 per cent of the cohort reported moderate to heavy physical activity [3]. Prevalence of MS (a state of central obesity, high blood pressure, insulin resistance and dyslipidaemia) has reached epidemic proportions in India in recent years. Reported prevalence of MS in some regions of the country ranges from 23.2 to 41.1 per cent [5]. Hypertensive subjects have a 2.5 times greater risk of developing diabetes within 5 years than normotensives matched for age, sex, and race. Hypertension and diabetes significantly share common pathways such as obesity, inflammation, oxidative stress, insulin resistance, and mental stress. Obese persons have a significantly higher risk of hypertension and type 2 diabetes [6]. Obesity is largely determined by genes; approximately 50% to 90% of the variation in weight is the result of genetic predisposition according to twin studies [7,8]. Adrenergic-receptor beta 2 (ADRB2) and beta 3 (ADRB3) are obesity genes that play a key role in the regulation of energy balance by increasing lipolysis and thermogenesis [9]. The Glu27 allele in ADRB2 and the Arg64 allele in ADRB3 are associated with abdominal obesity and early onset of T2DM in many ethnic groups. Peroxisome proliferator-activated receptor gamma (PPARG) is required for adipocyte differentiation. Pro12Ala mutation decreases PPARG activity and resistance to T2DM [9]. SNPs can occur in any position of the genome and the ones occurring in the coding and regulatory regions are likely to have effects on the function of a gene [10,11]. Studies also show that about half of the SNP mutations occurring in the coding regions are missense while the rest are silent [12]. Since missense mutations are known to be one the main causes for major genetic disorders, many of these are the single causative factors for rare single gene inherited disorders. It is also expected that some more frequent missense mutations arising from SNPs in the coding regions will be associated with common genetic disorders [13].

The present work is a pilot project to find out the prevalence of T2DM, Hypertension and Hyperlipidemia among the patients visiting the Mercy College health Centre for a period of 3 years and SNP's associated with obesity induced T2DM using bioinformatics tools.

MATERIALS AND METHODS

This is a cross-sectional retrospective study of patients which included all adult T2DM patients who were registered in the Mercy health centre, inside Mercy College Campus belongs to Palakkad

Municipality from 2014-2017. A total number of 14,539 patient's records were randomly selected from the patient medical records section. Patients of T2DM of age group ranged from 25 years to 75 years of either sex were included in the study. Data obtained from the patient records included: Age, sex, height, weight, blood pressure value, duration of DM, type of treatment for Hypertension (HTN) and hyperlipedidemia. The HbA1c value, triglycerides (TGL), total cholesterol (TCL), LDL, and high-density lipoprotein (HDL) were considered as primary outcome measure. The whole study period was divided into three years 2014-15, 2015-16 and 2016-17. The patients were categorised into Diabetic (T2DM), Hypertensive & Diabetic and Diabetic, hypertensive & hyperlipidemia. SNPs for the T2DM and obesity were collected from dbSNP (NCBI). SNPs involved in functional proteins from whole genome sequencing data were taken for functional analysis (http://www.ncbi.nlm.nih.gov/snp).

RESULTS

Among the 1785 patients visited the clinic (297/ month) in 2014 60% belongs to type 2 Diabetic category, 30% suffered from Hypertension and Diabetic mellitus and the rest 10% have Diabetes, hypertension and hyperlipidaemia. The number of patients enhanced to 4661and 4936 in 2015 and 2016 respectively. In 2017, till July a total of 3157(451/ Month) patients visited the clinic of which 271 belongs to type 2 Diabetic category (60%), 135 (30%) belongs to Hypertensive and Diabetic and the rest 90(10%) suffer from Diabetes, hypertension and hyperlipidaemia [Figure 1&2]. Out of the total patients 67% belong to obese comes under 25-50yrs age group and 33% 50-75yrs [Figure 3].

A total of 84 SNPs were retrieved from dbSNP. Limits were activated to obtain SNPs "Homosapiens' 'Misssense'. 8SNPs were found in T2DM and obesity combined gene of humans [Table 1]. The table 1 explains the SNP result from dbSNP. One missense mutation was noticed in the Adrenergic-receptor beta 2 (ADRB2) and beta 3 (ADRB3) genes and where as six missense mutation was reported in gamma (PPARG) gene. Towards 2017, 45% of the patients were using oral hypoglycaemic drugs. We observed an increase in the use of lipid-lowering drug treatments (from 22% to 34%) and antihypertensive drug treatments (from 57% to 69%) and substantial decreases in mean HbA1c [Table 2]. The percentage of hypertensive patients (SBP ≥50mmHg or DBP ≥85 mmHg) increased from 2014 to 2017.

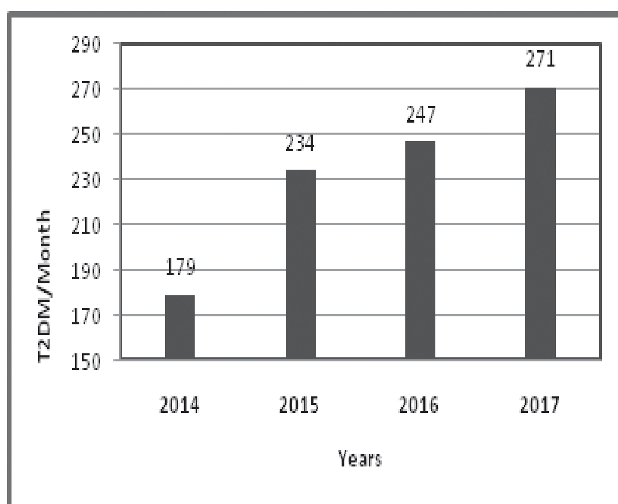

Figure 1. Incidence of Diabetic patients visited /Month at Mercy College Health centre during a period from 2014-2017

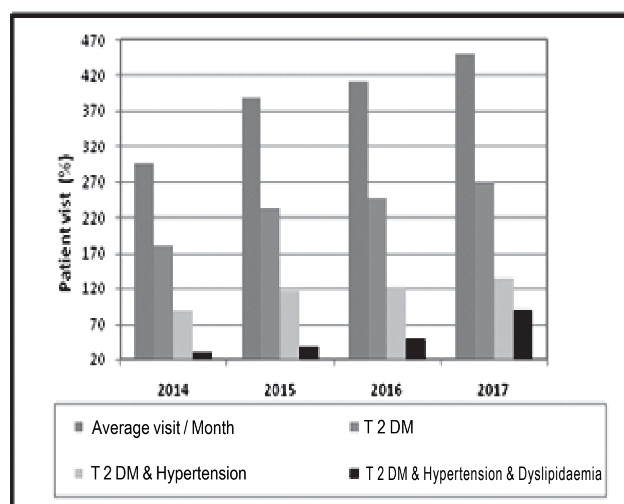

Figure 2.Percentage of patient visits/Months in T2DM, T2DM& Hypertensive and T2DM, Hypertensive &Dislipidimia category from 2014-2017 at Mercy College Health centre.

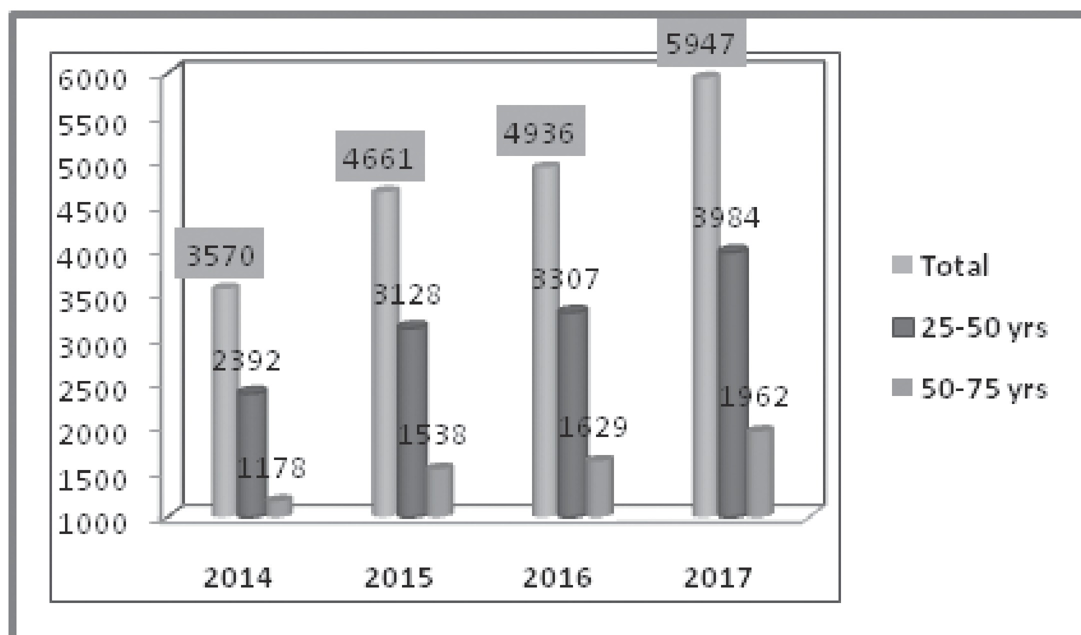

Figure 3. Patient distribution according to age-groups

Table 1. SNP association between T2DM and obesity

	SNP ID	Gene ID	Chromosome position	Allele change	Function	SNP to mRNA	Position	Residue change
1	rs1799999	PPP1R3A (5506)	7.113878379	GAC ⇒ TAC	missense	Fwd	2744	D[Asp] ⇒ Y [Tyr]
2	rs1801282	PPARG (5468)	3.12351626	CCA ⇒ GCA	missense	Fwd	125	P[Pro] ⇒ A [Ala]
3	rs1805192	PPARG (5468)	3.12379739	CCC ⇒ GCC	missense	Fwd	110	P[Pro] ⇒ A [Ala]
4	rs121909244	PPARG (5468)	3.12434111	CCG ⇒ CTG	missense	Fwd	1573	P[Pro] ⇒ L [Leu]
5	rs4994	ADRB3 (155)	8.37966280	TGG ⇒ CGG	missense	Fwd	384	W[Trp] ⇒ R [Arg]
6	rs1042714	ADRB2 (154)	5:148826910	CAA ⇒ GAA	missense	Fwd	318	Q[Gln] ⇒ E [Glu]
7	rs1800571	PPARG (5468)	3:12381349	CCA ⇒ CAA	missense	Fwd	330	P[Pro] ⇒ Q [Gln]
8	rs7732671	PPARGC1B (133522)	5:149832680	GCT ⇒ CCT	missense	Fwd	581	A [Ala] ⇒ P [Pro]

Table 2. Characteristics of type 2 diabetes patients

No	*Patient characteristics*	Years			
		2014	2015	2016	2017
1.	Body mass index (kg/m^2)	29.0 ± 4.1	29.4 ± 4.0	29.5 ± 3.8	30.1 ± 3.0
2.	HbA1c (% units)	7.5 ± 1.2	7.4 ± 1.2	7.3 ± 1.3	7.0 ± 1.2
3.	TC/HDL ratio	3.9 ± 1.1	4.1 ± 1.1	4.4 ± 1.2	4.5 ± 1.2
4.	Systolic blood pressure (mmHg)	145 ± 21	146 ± 20	150 ± 23	155 ± 23
5.	Diastolic blood pressure (mmHg)	80 ± 10	82 ± 10	84 ± 11	90± 11
6.	Number of glucose-lowering drugs				
	None	20%	18%	17%	12%
	1 oral/ Insulin	39%	41%	43%	45%
7.	Lipid-lowering drugs	22%	27%	30%	34%
8.	Antihypertensive drugs	57%	63%	66%	69%

DISCUSSION

Diabetes is a growing epidemic in both the developing and developed world and more so in the developing countries. Diabetes is known to be associated with hypertension. The presence of one increases the risk of having the other. As obesity is a common factor in the etiology of hypertension and diabetes, we would expect that hypertension, diabetes, and obesity not only share common pathophysiologic pathways but also common susceptibility genes. Most patients with type 2 diabetes are insulin resistant and about half of those with essential hypertension are insulin resistant. Prevalence of overweight and obesity are increasing in India in recent years even though under-nutrition continues to be an important public health issue even in the 21st century [5]. Heredity has recently been shown to influence fatness, regional fat distribution and response to over feeding. Genetics also plays a big role in developing obesity. Recent studies have concluded that about 25 to 40 percent of BMI is heritable [14]. A child with an obese parent, brother or sister is more likely to become obese [15]. The result of several studies suggests that the very fact of a women being obese during pregnancy may predispose her children to obesity [16]. Overweight and obese individuals are at an increased risk of hypertension, hypercholesterolemia and type 2 diabetes [17]. Studies on SNP analysis of T2DM and obese genes show that about half of the SNP mutations occurring in the coding regions are missense while the rest are silent [12]. Since missense mutations are known to be one the main causes for major genetic disorders, many of these are the single causative factors for rare single gene inherited disorders. It is also expected that some more frequent missense mutations arising from SNPs in the coding regions will be associated with common genetic disorders [13]. In this large observational study, we observed an overall increased use of antihypertensive and lipid-lowering drugs, and better control of risk factors between 2014 and 2017. For lipid-lowering treatment, improvements were mainly due to an increased proportion of type 2 diabetes patients who were initiated on drug treatment, whereas for hypertension improvements especially concerned the intensification of treatment in patients already on antihypertensive therapy.

The adrenergic receptors, ADRB2 and ADRB3, have an important role in lipolysis and thermogenesis, and the polymorphism of these genes causes

differences in energy expenditure. Alleles of both Glu27 in ADBR2 and Arg64 in ADRB3 are associated with obesity and/or T2DM in humans. PPARG activates differentiation of precursor cells of adipocytes to the small-sized adipocytes, which secrete factors that prevent diabetes mellitus. SNP analysis of ADRB2, ADRB3, and PPARG genes after DNA sequencing of the of the population under study has to be performed to confirm actual genetic reason for the high incidence of T2DM, Hypertension and Hyperlipidemia among different populations of Palakkad Municipality, Kerala.

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STUDY OF DRD2 *TAQ1A* POLYMORPHISM IN DEVELOPMENTAL STUTTERING

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INTRODUCTION

Language is one of the vital proficiencies mastered by our human brain, though learning to speak has the appearance of an ordinary phase in child development. Neural networks are established overtime as an individual learns and experiences things. Language and communication skills are therefore acquired after birth [1]. The infant brain is intricately poised to "crack the speech code" in a mode that the adult brain cannot. Complex mechanism of language construction is transformed into speech. Naturally when there is a disruption in speech fluency it becomes a burden and social stress [2].

There are developmental impairments in which various speech and language abilities are disproportionately disturbed compared with other cognitive skills. Study of impairments in speech, such as stuttering can be very valuable [3]. Developmental stuttering is a disorder of speech fluency characterized by involuntary repetitions, prolongations, and silent blocks, especially in the initial parts of utterances. It is also it is accompanied by emotions such as fear and anxiety, and behaviours such as avoidance and struggle. Typically it starts between 2-6 years of age [3,4].

Developmental stuttering interferes directly in interpersonal and professional relations, negatively impacting the quality of life [5]. Approximately 1% of the children population are estimated to be affected with stuttering [6].Though stuttering is poorly understood genetic and neurobiological approaches are giving new cues to the treatment of the disorder [3]. Stuttering runs in families confirming the genetic cause of the disorder. In monozygotic twins, the concordance rate is 70% and in dizygotic twins it is 30% and siblings of the same sex is about 18% [7,8].

Hyperdopaminergic activity at the basal ganglia has been evidenced as a cause for stuttering. Children generally showed increased number of dopamine receptors (type D_2) in basal ganglia. Single administration experiments with haloperidol, led to the hypothesis that D_2 receptor antagonists may be important in the treatment of developmental stuttering [9-12].The cause of dopaminergic hyperactivity is hypothesized to be due to an increase in either the post synaptic binding potential or the size of the reasonable presynaptic dopamine vesicular pool (in which the postsynaptic receptors may be down regulated).

Polymorphisms of three different dopaminergic genes, dopamine D_2 receptor (DRD2), dopamine â-hydroxylase (DBH), and dopamine transporter (DAT1) was found associated with stuttering [13]. Literature studies claims that a possible relationship exists between dopamine D2 receptor density in the striatum and DRD2 polymorphism at the TaqA1 locus [14]. The D2 dopamine receptor gene (DRD2) located at 11q22-q23 contains a *Taq* 1 restriction fragment length polymorphism, creating two alleles, A1 and A2- a 6.6 kb Taq1 fragment in the A1 allele and two fragments of 2.9kb and 3.7kb in the A2 allele [15-17].

Typical pattern of onset ofstuttering around age 2.5 to 3 years with a large percentage of recoveries, might be related to a natural phase of development of basal ganglia. Specifically it has been shown that children in general have a peak in the number of dopamine receptors type D2 in the basal ganglia at this time. Number of D2 receptors has also been reported to show correlation with cognitive performance [18].

The presence of one or two A1 alleles was found to be associated with reduced D2 receptor binding in all areas of striatum, significantly in ventral caudate and putamen. When the relationship between D2 expression and Taq1A genotype was examined within gender groups, this reduction was more marked in males than female particularly in putamen. When the density of D2 receptor binding was compared between these groups in different striatal areas, binding tended to be reduced in A1/A2 and A1/A1 cases when compared to A2 homozygotes. The results of the study indicate a statistically significant reduction between 30-40% in D2 receptor binding in the striatum of individuals of one A1 allele compared with those homozygous for the DRD2 allele [19].

The present study was aimed to investigate the genetic predisposition to stuttering due to DRD2 TaqA1 polymorphism.

MATERIALS & METHODS

Stuttering database was constructed after screening schools and visiting hospitals in Chennai using a standard schedule and Riley's stuttering scale (RSS). From this database 46 stutterers were randomly selected with variable severity; 5 -7 mL of blood was collected and genomic DNA was isolated by Phenol Chloroform Isoamyl protocol. This study was approved by the Institute of Human Ethical Committee, University of Madras. Genotyping for Taq1A polymorphism was performed by PCR using a Thermal cycler.

DNA (10-20 ng) was amplified in a 20 µl reaction mixture containing 0.2 mM of forward primer and 0.2 mM of reverse primer, 10xPCR buffer, 1.5mM $MgCl_2$, 200 mM dNTPs, and 1 unit of Taq DNA Polymerase (0.1µl) (Ampliqon). Amplification conditions were 95°C for 5 min, 95°C for 30 s, 58° for 30 s, 72°C for 45 s, Steps 2–4 were repeated by 35 cycles followed by 72°C for 3 min.

Genotypes of Taq1A was obtained using PCR-RFLP method with forward primer 5'CCT TCC TGA GTG TCA TCA AC 3' and reverse 5' ACG GCT CCT TGC CCT CTA G 3'. The PCR product was digested with Taq 1 restriction enzyme (Thermo Scientific) and the amplified products were checked in 2% gel containing ethidium bromide using gel electrophoresis. The bands were visualized and documented using Gel Documentation system (Vilber Lourmat).

Data analysis: The allele frequencies and their standard errors are computed by gene counting method. Chi square tests for the departure from the Hardy Weinberg expectations were performed. The genotype frequencies were compared with the ideal control population.

Estimation of gene frequencies: Let a, b, c denote the observed number of genotypes ++, -+ and — respectively.

P(+)=(2a+b)/2N Q(-)=(b+2c)/2N

The standard error is given by S.E (q)=Pq/2N

Testing for departure from Hardy Weinberg expectations by Chi-square test

$A=NP^{2;\ B}=2PqN;\ C=Nq^2$

$c_{cal}= (a-A)^2/A + (b-B)^2/B + (c-C)^2/C$

RESULTS & DISCUSSION

To assess the association of *DRD2*TaqA1 polymorphism with stuttering, 46 stuttering probands were genotyped. Among these probands 95.7% were males (n=44) and 4.3% were females (n=2) probands. The 46 PWS in our study were randomly selected irrespective of their severity status (mild =52.17%; moderate=8.69%; severe=19.56%).

In RFLP, the alleles are identified based on the presence or absence of cleavage site in the amplified product. The loss of restriction site produces a single fragment of 236bp resulting in a homozygote mutant (A1/A1).

Homozygote normal (A2/A2) having a restriction site produces two fragments of 124 bp and 112 bp, whereas a heterozygote (A1/A2) produces three fragments of 236 bp, 124 bp and 112 bp.In our study there were 17 homozygote normal (A2/A2), 26 heterozygotes (A1/A2) and 3 homozygote variant (A1/A1).

Table1. Genotype and allele frequencies of *Taq 1A* of DRD2 gene

Taq1A	Stuttering Probands (n=46)	Control (n=479)	*Chi square*	*P value*
Genotypes				
A2/A2 (CC)	17 (36.9)	147 ((30.7)	4.6	0.100
A1/A2 (CT)	26 (56.5)	240 (50)		
A1/A1 (TT)	3 (6.5)	92 (19.2)		
Allele				
A2 (C)	60 (65.2)	534 (55.7)	2.69	0.101
A1 (T)	32 (34.8)	424 (44.3)		

*The chi square and *p* values of genotype and allele frequency are indicated in the table. Both are in conformity with Hardy Weinberg equilibrium.

The allele frequency of *A1* and *A2* alleles were found to be 0.35 and 0.65(SE=0.002). The results of chi-square tests are in conformity with the Hardy Weinberg expectations (X^2=2.74; *p*=0.3). The allele frequencies in the combined caste population data (479 controls) from the same area were also reported to be in Hardy Weinberg equilibrium [20]. The genotype and the allele frequencies of DRD2 Taq1A in the two populations are given in the Table 1.

In a study by Comings *et al,* (1996) there was a significant correlation of A1 allele with variables of these probands (with Tourett's syndrome) that includes stuttering. It indicated greater association of *DRD2A1* allele with stuttering [14]. Since dopamine plays an important role in fine motor movements such as those involved in speech production our study aimed to assess the possibility of association of *Taq 1A* polymorphism with stuttering. However our study does not show any association for stuttering and DRD2 Taq1A polymorphism.

CONCLUSION

In summary our data lacks the association between Taq1A polymorphism and stuttering. It may due to small sample size of PWS which calls for further investigation on a larger sample scale and ethnic groups to confirm our findings.

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SYMPOSIUM ON

DIAGNOSTIC GENETICS

CORRELATION OF CYTOGENETIC AND RADIOLOGICAL EVALUATION OF FETAL CYSTIC HYGROMA TO DETERMINE THE RECURRENCE RISK– A SHORT STUDY

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INTRODUCTION

Cystic hygroma is a thin walled, multiseptate cystic structure that results from failure of lymphatic channels to communicate with the venous system. They typically develop as a part of lymphatic obstruction sequence, in which the lymph from head fails to drain into the jugular vein and accumulates in the jugular lymphatic sacs [1]. Most of the Cystic hygromas located in the posterior neck and head [Figure A] are frequently associated with chromosomal abnormalities with or without structural abnormalities, and most of the pregnancy outcomes are unfavourable [2].When an evidence of Cystic hygroma is seen in first trimester ultrasonography, the fetal karyotype and serial ultrasonograms to assess the growth of the mass and the development of hydrops are recommended in all cases [3] [Figure B]. Romero *et al*. cited 68% incidence of abnormal karyotypes in fetal Cystic hygromas, with the majority being 45,X or Turner syndrome, followed by Edward syndrome, Down syndrome and Patau syndrome. Non chromosomal disorders like Noonan syndrome, Multiple Pterygium syndrome and the Klippel Feil sequence are also associated with Cystic hygroma.

The patients with fetal cystic hygroma recruited in the present study were referred to Lifeline Genetics and Research Centre, Unit of Fetal Medicine, Kerala, India during the period of 2014- 2017. Once Cystic hygroma was diagnosed, the parents were counseled to explain the key points of management of the individual case on the basis of family history, the association of the malformation with chromosomopathies, heart disease and skeletal dysplasias. The parents were offered genetic investigation by chorionic villus sampling, amniocentesis or cordocentesis according to gestational age or postnatal evaluation by whole body X-ray and placental bits from products of conception (POC) based on the severity of Cystic hygroma [4].

MATERIALS AND METHODS

The present case study was divided into two groups: - Group I consisted of those patients who were willing for CVS/ Amniocentesis antenatally by prenatal diagnosis. Group II consisted of those who choose to do medical termination as the fetuses were unviable and opted for karyotyping from POC. The patients' details were recorded in a predesigned proforma after getting written informed consent. The study was approved by the Hospital ethics committee. Only after detailed ultrasonographic examination by a Fetal Medicine Specialist, the patients between 11-19 weeks were referred to the Genetics Department for genetic counseling. Clinical details and pedigree were entered into a predesigned proforma.

Prenatal Culturing – Group I: Here prenatal diagnostic tests include Chorionic villus sampling and Amniocentesis each of which is performed on specific gestational age.

Transabdominal Chorionic Villus Sampling: Twenty five to thirty milligram Chorionic villus sample were collected in Hank's Balanced Salt Solution (HBSS) under strict aseptic conditions. The villi were carefully separated and were enzyme macerated using trypsin and collagenase. Enzyme maceration helps tissue dissociation to cellular level, which was later used for cell culturing.

Transabdominal Amniocentesis: Under aseptic conditions a 22-guage disposable spinal needle with ultrasonographic monitoring is used for aspirating the amniotic fluid. Once the sample has been collected, it was labeled and transferred to the laboratory. Supernatant was removed after centrifugation and the remaining pellet was used for cell culturing.

Postnatal Culturing – Group II, Tissue Preparation – Product of Conception: Placental bits were collected from the product of conception in normal saline in sterile container after medical termination of pregnancy. The tissue was finely chopped and subjected to enzyme maceration. Cell culture protocol was same for Chorionic Villi and Product of Conception as described by Rooney and Czepulkowski (1992) with slight modifications. Cell culturing, harvesting, slide preparation and GTG banding have same protocol for all the three samples.

Cell Culturing: The pellet containing fetal cells was mixed with nine milliliter of amniomax cell culture media for long term culture. On the fifth and ninth day a media change was given after checking the cell colonies and growth.

Harvesting: Around tenth to eleventh day of culturing, after colony formation, cultures (CVS, POC and Amniotic fluid) were harvested to obtain metaphases. One milliliter of 0.15% colchicine solution (Sigma, USA) was added to each culture flask after checking the mitotic index to arrest the cell in metaphase stage. After three hours of colchicine addition, harvesting was done using 0.25% trypsin EDTA (Gibco). The detached cells were transferred to the same centrifuge tube, followed by hypotonic treatment (KCl-0.56%: Sodium citrate-1%, 4:1). Cells were fixed in acetomethanol (1:3).

Slide Preparation and GTG Banding: Two–three drops of cell suspension were evenly dropped onto chilled wet and clean slide and labeled correctly, air dried and kept it for proper ageing. Slides were treated with 0.025% trypsin solution for a particular time. Then the slides were dipped in Phosphate Buffer Solution (PBS) and stained with Giemsa stain. Twenty well spreaded G-banded metaphases were karyotyped using Olympus BX51 light microscope and cytovision software 2.7 (Applied imaging; USA). The chromosomes were analysed on the basis of their banding pattern and any abnormality was scored and reported [5].

RESULT AND DISCUSSION

Cystic hygroma patients selected in the present study were referred to Lifeline Genetics and Research Centre, Adoor, Kerala during the period of 2014-2017. Karyotyping was offered to all thirty five cases and the study was divided into two groups.

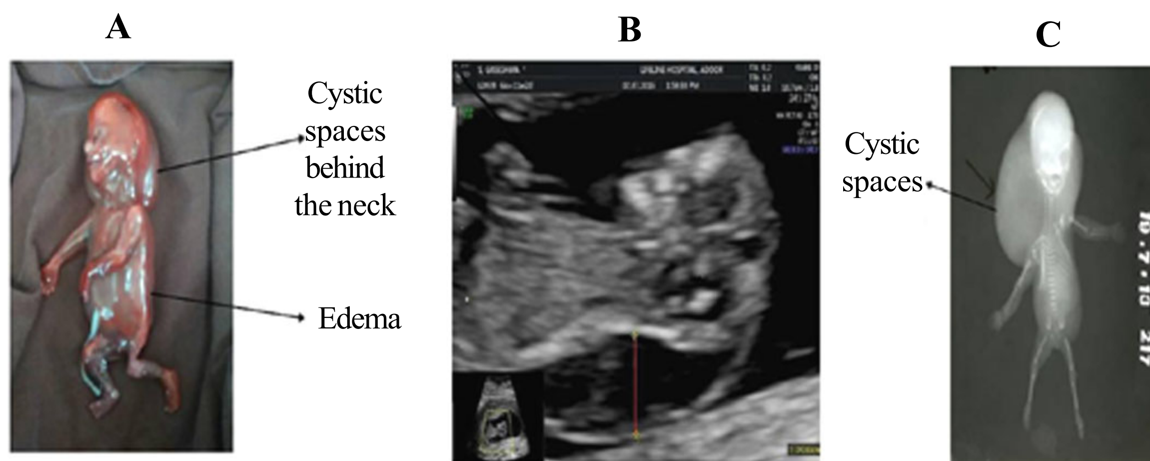

Figure A) Fetus with cystic hygroma after MTP B) Ultrasound image of a fetus with cystic hygroma C) X-ray image of fetus with cystic hygroma

In Group I (prenatal diagnosis), fetal karyotype was found to be normal in 48% (13/27) cases and the other 52% (14/27) of cases showed abnormal karyotype. The abnormal karyotype included Turner syndrome (Monosomy X), followed by Edward syndrome (Trisomy 18), Down syndrome (Trisomy 21), Patau syndrome (Trisomy 13) and other structural abnormalities (balanced/unbalanced translocations).

In Group II (Postnatal diagnosis) 50%cases were found to be abnormal (3- Edward syndrome, 1 - Turner syndrome),10% of cases showed normal karyotype and rest of the 40% POC samples either showed microbial contamination or failed to multiply in our laboratory conditions.

Chromosomal aneuploidies like Turner syndrome, Down syndrome (non disjunction Trisomy 21) and Edward syndrome (non disjunction Trisomy 18) are sporadic events and recurrence risk is <1% [6]. They are accidental *de novo* events and parents were advised prenatal diagnosis in all future pregnancies even though there is negligible recurrence risk. If one of the parents was found to be carrier of balanced translocation, the risk to offsprings may vary, it can be normal, can carry the translocation or can have duplications or deletions of genetic material. Therefore in comparison, Group I patients completely benefited from prenatal diagnosis, while Group II- postnatal diagnosis, some of the patients could not be benefited as there was no chromosome analysis to confirm abnormalities due to multiple reasons. Postnatal whole body X-ray studies helped in ruling out skeletal dysplasia [Figure C]. In the cases with normal karyotype, non chromosomal causes like Noonan syndrome, Multiple Pterygium, Apert syndrome and Achondroplasia etc. may be ruled out by X-ray [1]. Hence, this study has highlighted the association between cystic hygroma, ultrasonographic findings and the need for genetic evaluation of the fetus to predict the recurrence risk and future pregnancy management.

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CYTOGENETIC AND MOLECULAR STUDY ON RING CHROMOSOME 9 IN A CHILD WITH DEVELOPMENTAL DELAY

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INTRODUCTION

Ring chromosome usually result from two terminal breaks in both chromosome arms, followed by fusion of the broken ends or from the union of a broken chromosome end with the opposite telomere region, leading to the loss of genetic material. Ring chromosomes have been found in almost all human chromosomes. Among these ring chromosome 9 [r(9)] are rare cytogenetic findings in genetic disorders. Untill now only 30 postnatal ring 9 chromosome cases have been reported in the literature [1,2].

Ring chromosome formation on 9th chromosome may arise from sticky ends created by terminal deletion on both p and q arms and end to end fusion of palindromic telomere sequences [3]. Because of the difference in the extent of the deleted regions on both p and q arms in ring formation 9, there was a disparity in phenotypic features in reported cases and it is still considered as an inconclusive syndrome. The phenotypes of r(9) patients vary depending on the size and location of the deletions. The phenotype may be attributed to loss of variable portion of 9p and 9q lost in the formation of the ring, and genomic variations occurred due to instability of ring chromosomes [4]. The common features of ring chromosome 9 are developmental delay, facial dysmorphism, microcephaly, skeletal malformations, cardiac defects, genital abnormalities and infectious complications [1]. In this study we present conventional and molecular cytogenetic analysis in a child with dysmorphic features and developmental delay

MATERIALS AND METHODS

The present study was conducted at the Jubilee Centre for Medical Research, JMMC&RI, Thrissur, Kerala, India. Peripheral Blood samples were collected from the proband and her parents after getting informed written consent. Classical chromosomal analysis was performed after 72 hrs peripheral blood lymphocyte culture. G-Bands by Trypsin using Giemsa (GTG) banding was performed on metaphase chromosomes. Ninety five metaphases were karyotyped using MetaSystems Ikaros software (MetaSystems, Altlussheim, Germany). Karyotypes were described according to International System for Human Cytogenetic Nomenclature (ISCN 2016) [5].

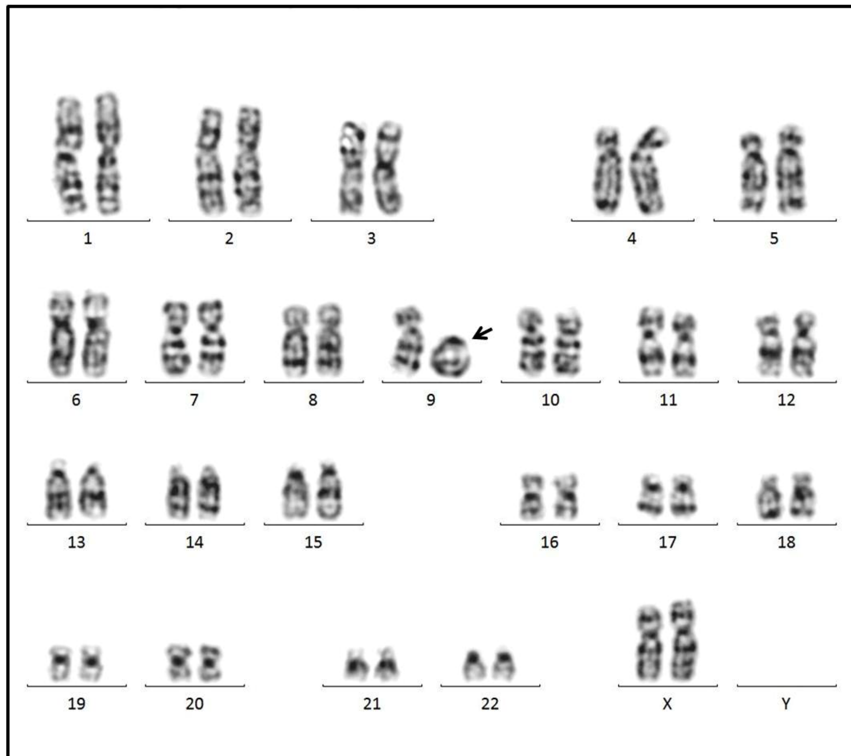
Fluorescence *in situ* hybridization (FISH) was performed in well spreaded metaphase slides to confirm the ring structure on chromosome 9 by using VYSIS LSI *BCR/ABL* Dual Color, Dual Fusion Translocation Probe (Abbott Molecular Inc., Des Plaines, IL, USA) according to the manufactures instructions.

RESULTS AND DISCUSSION

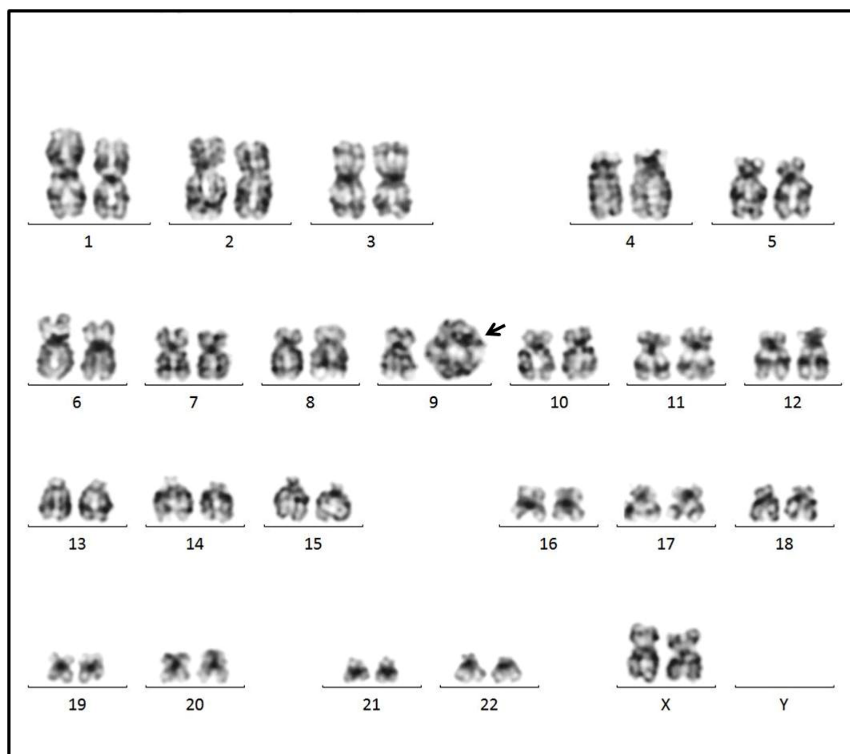
Chromosomal analysis at 450-band resolution revealed mainly a monocentric ring chromosome. Besides low frequencies of cells with dicentric ring and monosomy 9 were also observed. The karyotype was interptreted as 46,XX,r(9)(p24q34)[84]/46,XX,dic(9;9)(p24q34;p22q34)[3]/45,XX,-9[6]/46,XX[2] [Fig 1 (a-c)]. In FISH analysis, we observed presence of 9th chromosome specific *ABL* gene signals on the ring chromosome 9, confirming the formation of ring in chromosome 9. Chromosomal analyses of parents were unremarkable.

Ring chromosome 9 is a rare chromosomal abnormality. Because of the difference in the extent of the deleted regions on both p and q arms in ring chromosome formation, there was a disparity in phenotypic features in reported cases. Similar to the

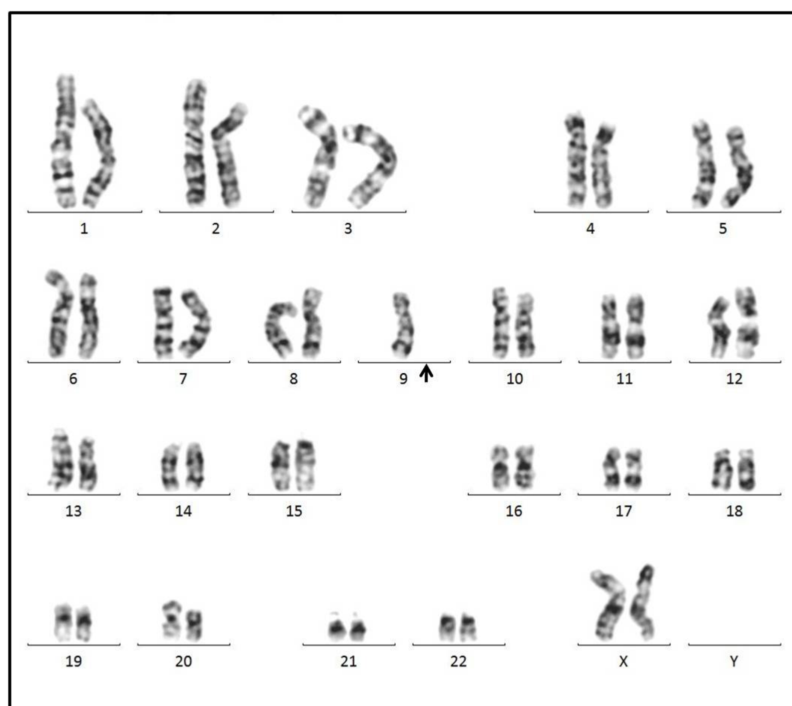
Figure 1. GTG-banded karyotype of proband showing



(A) Ring chromosome 9 [46,XX,r(9)(p24q34)]



(B) Dicentric chromosome 9 [46,XX,dic(9;9) (p24q34;p22q34)]



(C) loss of ring chromosome 9 [45,XX,-9]

previous reports, our proband showed dyanamic mosaicism. The abnormal phenotype of our proband is thought to be resulted from 'dyanamic mosaicism', which occurs in early embryonic divisions producing a mosaic pattern wherein some cells have one ring, others have no ring or have two rings.

Patients with ring chromosome 9 are often a combination of both 9q deletions and 9p deletions syndrome as they share clinical features, and the breakpoints are reported to lie in between 9p22p24 and 9q33q34 [2,3]. Similar to the other reports of r(9), deletion 9p and deletion 9q, our study subject also showed clinical manifestations such as microcephaly, low set ears, upward slant synophrys, retrognathia and global developmental delay. We conclude that combination of conventional molecular and cytogenetic techniques have become an indispensable tool to assess ring chromosome and health care management.

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PARENTAL KARYOTYPE IN UNRAVELING THE STRUCTURAL ABNORMALITIES IN CHILDREN WITH GENETIC DISORDERS

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INTRODUCTION

India is witnessing an accelerating shift towards non-communicable diseases and genetic disorders form a major group of the non-communicable diseases. Genetic disorders include chromosomal abnormalities, single gene disorder and polygenic disorders. Chromosomal abnormalities are alterations in the number or structure of the chromosomes causing a diverse phenotype. This is one of the leading causes of congenital anomalies which contribute to morbidity and mortality. Chromosomal abnormalities occur in 6% of all recognized congenital malformation. Similarly these chromosomal aberrations remain the leading cause of mental retardation/intellectual disability [1,2]. Genetic counseling is an integral part of management of these children and their families. Prenatal diagnosis in the family will help to prevent the recurrence of similar condition in successive pregnancy. All these interventions require a definitive genotypic diagnosis in the affected child. The awareness and information regarding theses condition is important in today's clinical practice since genetic services will be an integral part of primary care once our infant mortality reduces.

Developmental Delay/Mental retardation (DD/MR) can be categorized as syndromic, or nonsyndromic; and might be associated with dysmorphic features and multiple malformations. The etiology of DD/MR could be genetic or non genetic, where genetic disorders represent the major cause. Genetic disorders could be chromosomal, monogenic or polygenic; however chromosomal abnormalities have been documented as a single most common cause. The frequency of chromosome anomalies detected by karyotyping in patients with DD/MR was variable and estimated between 9% and 36% [3]. It also accounts for 30-40% of severe mental retardation, and 10% of mild mental retardation [4], Trisomy 21 remains the commonest single cause of mental retardation with its incidence 1:650 - 1:1000 live births [5].

Various structural abnormalities causing DD/MR and Multiple malformations (MM) can be sporadic or inherited from the parents. Similarly, inherited unbalanced chromosomal rearrangements are responsible for a large proportion of familial disorders [6]. On the other hand, apparently normal individuals carry balanced chromosomal rearrangements, which sometimes runs in family undetected throughout several generations until or unless there occur some genetic problems like children with congenital anomalies, mental retardation, dysmorphism, still birth, recurrent pregnancy loss etc. Structural rearrangements were detected in approximately 0.5% of new-born infants [5,7]. The delineation of these structural rearrangements can be done with advance high resolution molecular cytogenetic techniques like microarray and modifications of Fluorescent *In Situ* Hybridization (FISH). However they are very expensive to use in every case. Moreover for genetic counseling, the information regarding the karyotype in the parents are important. Identification of a derivative chromosome in a child with the presence of chromosomal material of unknown origin warrants the need for Parental Karyotyping [PK]. Screening of children with DD/MR/MM for genetic disorders is of great value, it will help in offering an appropriate counseling for those parents owing to minimize the number of affected children. The present study mainly focuses on the need for doing PK to delineate the structural rearrangements in children with MR/DD/MM/Dysmorphism.

MATERIALS AND METHODS

Cytogenetic analysis was performed on the peripheral blood sample by Phytohemaglutinin stimulated lymphocyte microculture method followed by GTG-banding method to study the structural variations. For cytogenetic analysis 2 ml heparinized blood samples were collected cultured for 72hrs, harvested and the slides for metaphase study were prepared [8]; the slides were stained using Giemsa stain [9,10]. Metaphases were studied under oil immersion objective, captured and karyotyped using the ASI [Applied Spectral Imaging] software [USA]. For each sample, 20 metaphases were screened and 05 metaphases were karyotyped. All chromosomal abnormalities were recorded according to the International System for Human Cytogenetics Nomenclature [11].

RESULTS

The study is a retrospective data analysis done at the Genetic Lab of Child Development Centre, Government Medical College, Trivandrum. A total of 788 cases referred for Karyotyping during Nov 2013 to Nov 2016 (3years) with various clinical indications like MR/DD, dysmorphism, MM, Down phenotype, recurrent pregnancy loss (RPL), short stature, amenorrhea, ambiguous genitalia, infertility were included in the study. There was culture failure in 6 cases. So analysis was done in 782 cases.

Chromosomal Abnormalities [numerical and structural] were identified in 229 out of 782 cases (29%). These include aneuploidies (n=162), structural abnormalities (n= 61), XY female (n=5) and XX male (n=1). The percentage of structural abnormalities in various clinical indications were, 10% in Down syndrome patients, 9% in RPL, 8% in amenorrhea, 7.7% in infertility, 7 % in MR/DD/MM/Dysmorphism children and 6.3% in short stature. In the current study the structural abnormalities obtained in children with MR/DD/Dysmorphism/malformation (excluding the Down syndrome) were further analyzed. Children with MR were classified into three groups, MR with dysmorphism and malformations, MR with dysmorphism and MR without dysmorphism. The structural abnormalities were found to be high in children with MR/DD/MM (14/85cases; 16.4%), when compared to MR with dysmorphism (08/159 cases; 5%) and MR without dysmorphism (3 out of 113 cases; 2.6%). The structural abnormalities obtained in these groups include translocation, deletion, duplication, addition/derivative chromosome, ring chromosome etc

As the next step to delineate the structural abnormality in children with MR/DD/Dysmorphism/MM [n=25/357,7%], PK has been performed in all except 4 cases where the parental samples were not available/ willing to perform the test. Out of the 21 cases, 11 cases revealed one of the parents as a balanced translocation carrier [11/21, 52%]. Of the remaining 14 cases, FISH/Microarray/Spectral Karyotyping [SKY] helped to clarify the abnormalities in 4 cases. In 5 cases where the structural abnormality of the proband was deletion of chromosome, parents were found to be normal. So further delineation was not needed, as it might have occured *de novo*. In two cases where proband having apparently balanced translocation and PK is advised. In 3 cases, proband had a derivative chromosome with normal parental karyotype. These 5 cases have to be evaluated with advanced test.

DISCUSSION

The conventional karyotype is the starting point to investigate/ evaluate the cases with developmental delay and mental retardation with multiple malformations/ congenital anomalies. Usually, visible loss or gain of chromosome material will lead to abnormal development, resulting in a malformed phenotype [12]. Advanced genetic investigations like microarray and modification of FISH are required to delineate the structural abnormality in such cases. Karyotyping is an inexpensive technique which gives the complete genomic picture, and should be made a mandatory investigation in couples with repeated abortions or where there has been a birth of child with multiple congenital anomalies. This can help in better patient counseling and thus, better patient management. During reproductive counseling, it is important to explain the recurrence risk to the couples and also offer them prenatal genetic diagnosis.

In our study, the chromosomal abnormalities were described in 52% of the cases by parental karyotyping. Such parents have an increased risk of miscarriage or having a baby with a serious chromosomal abnormality. Identification of the abnormality will help in the genetic counseling and prenatal diagnosis. Cytogenetic analysis being less costly than the higher end techniques, parental karyotype should be considered as first step of investigation for all the children harboring a structural abnormality.

Our study concluded that chromosomal studies are a valuable diagnostic technique to evaluate cases with MR/DD/Dysmorphism/MM. Investigating parents for chromosomal abnormalities is important as the risk of inheritance is usually high in such cases. It helps to provide proper genetic counseling.

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SYMPOSIUM ON

CANCER GENETICS

CONVENTIONAL CYTOGENETICS IN CANCER DIAGNOSIS AND PROGNOSIS

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INTRODUCTION

Conventional Cytogenetics means the study of metaphase Chromosomes by G-Banding and it provides an overview of all chromosomal aberrations in a single cell. Chromosomal analysis requires five principal steps: 1) Cell culture 2) harvest of metaphases 3) Chromosome preparation 4) banding by using specific protocol and 5) analysis by light microscopy or karyotype assisted computer analysis [1,2].

Technology considerations

Chromosomal studies of malignancies pose a particular technical challenge. As the results are so unpredictable, there is not a single technique that can be guaranteed to work consistently and reliably. Therefore, every laboratory should adopt a slight variation of the basic protocol. Mitotically active cells from bone marrow, blood, Fine needle aspiration of lymphnodes, CSF, Pleural fluid and biopsies are suitable samples for culture. The most significant factors in obtaining a successful result is setting up multiple cultures to maximize the chances of obtaining optimal malignant cell divisions [3].

The common chromosomal abnormalities are numerical and structural viz., anueploidy, translocation, inversion, insertion, deletion, duplication, iso, ring, HSR and double minutes [4]. Molecular characterization of chromosomal abnormalities has led to the discovery of various mechanisms involved in the malignant transformations.

Cytogenetic nomenclature: In 1960, a group of cytogeneticists collaborated on a project to develop a system by which even complex numerical and/or structural abnormalities could be succinctly described. The resulting book would eventually come to be known as the International System for Human Cytogenetic Nomenclature (ISCN). Since its initial publication, the ISCN has been updated and revised several times (most recently in 2016) [5].

Cytogenetics in Malignancy: Cytogenetics play an important role in the identification of chromosomal abnormalities associated with many of the cancer (Malignancies) and it helps in diagnosis and prognosis, choice of therapy and relapse. Thus, it is an important investigation in management of cancer patients [1-4].

According to the WHO (2008), morphology, immunophenotypy & cytogenetics was the cassification system of Acute Leukemia [6]. Revised WHO (2016) classification of Myeloid neoplasm's and Acute Leukemia postulates and integrated approach that includes molecular analysis.

Chronic Myeloid Leukemia (CML): It is a myeloproliferative neoplasm originating from a clonal, pluripotent hematopoietic stem cell defined by the presence of the BCR-ABL1 fusion gene, usually as a result of t(9;22)(q34;q11.2). It is the first cancer in which a consistent chromosomal abnormality the Philadelphia (Ph) chromosome was detected [7]. Since the discovery of the Ph chromosome in CML, this has become a Cytogenetic Hallmark of disease diagnosis. About 90-95% of CMLs are characterized by reciprocal translocation of t(9;22)(q34;q11.2), remaining 5-10% are variants. Multiple additional abnormalities like additional Ph chromosome, -7, +8, i(17)(q10), -17/ +17, +19, +21 and mutations may occur during disease progression, have impact on the treatment decision and prognosis [Figure 1]. Recently,

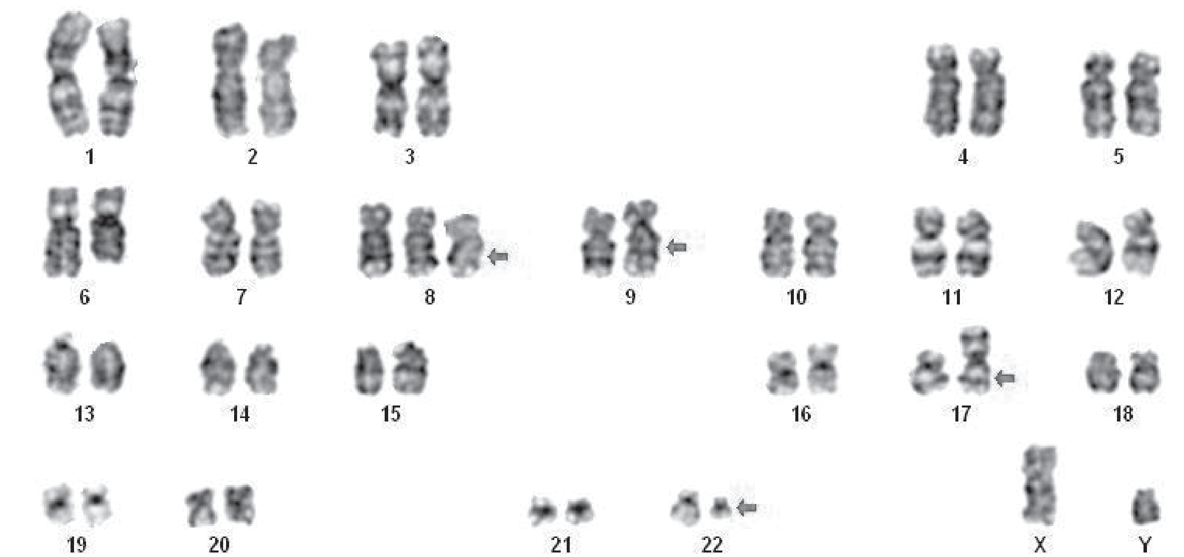

Figure 1. Karyotype of CML in blast crisis: 47,XY,+8,t(9;22)(q34;q11),i(17)(q10)

evaluation of Ph chromosome is also used in monitoring the treatment. However, the identification of genes BCR/ABL rearrangement underlying the Ph chromosome using highly sensitive molecular techniques (like FISH and RT-PCR) have revolutionized the field of cancer genetics and added precision.

Acute Myeloid Leukemia(AML): Extensive cytogenetic analysis of the leukemias has demonstrated an association of recurrent chromosomal aberrations with the clinical and biological diversity of AML and provided insight into the genetic changes that underline leukogenesis. The strong association of diagnostic karyotype with outcome, demonstrated in some large adult studies, has rendered cytogenetics the most valuable prognostic factor for treatment selection [8, 9, 10]. The single most important prognostic factor in AML is cytogenetics. Certain chromosomal abnormalities are associated with very good outcomes Viz., t(8;21),t(15;17) and inv(16). Normal karyotype, +8, +21, +22, del(7)(q), del(9)(q), abnormalities of chromosome 11 fall into an intermediate risk group. A number of other chromosomal abnormalities, -5, -7, abnormal 3q, t(9;22) and complex karyotype, known to associate with a poor prognosis and a high risk of relapse after treatment [1,3,8,9,10].

It is an independent prognostic variable and has been instrumental in predicting the likelihood of progression from MDS to AML. The most common isolated abnormalities of MDS are del (5q), monosomy 5, monosomy 7, del(7), trisomy8 and del(20q) [1,3].

Acute Lymphoblastic Leukemia (ALL): It is the most common pediatric malignancy accounting for 80% of all leukemias and one of the leading cause of death among children worldwide [1,2]. The peak incidence is between 2-5 years of age and approximately 90% contain clonal chromosomal abnormalities at diagnosis [1,3,4,9]. The most useful prognostic indicators in ALL are age, white blood cell count (WBC), immunological markers and cytogenetics. Chromosomal abnormalities in ALL are useful prognostic indicators as they are associated with specific immunologic phenotypes and characteristic outcomes [1,3,11]. Hyperdiploidy is a frequent phenomenon in pediatric B- cell ALL with good prognosis. Translocations are closely associated with the prognosis of Pediatric B-cell ALL. Presence of t(12;21)(p12;q22) is associated with good prognosis,

t(9;22)(q34;q11), t(1;19)(q23;p13) & t(v;11)(v;q23)V/MLL are associated with poor prognosis. t(10;14)(q26;q11) in Precursor T cell type and t(8;14)(q24;q32) in Burkett's leukemia/ lymphoma are associated with poor prognosis [1,3,11,12].

Chronic Lymphocytic Leukemia (CLL) is a low-grade leukemic lymphoma with clonal proliferation and accumulation of morphologically mature but immunologically incompetent lymphocytes of the B-cell lineage, and represents the most common type of leukemia among adults [1]. Chromosome 13q14 abnormalities are of little prognostic import, whereas trisomy 12, del(11)(q) and complex karyotype are indicative of poor prognosis and involvement of 14(q32) breakpoint with good prognosis [13].

Multiple Myeloma (MM) is a neoplastic plasma-cell disorder that is characterized by clonal proliferation of malignant plasma cells in the bone marrow microenvironment, monoclonal protein in the blood or urine, and associated organ dysfunction [14]. Cytogenetics shows abnormalities in 20-30% of cases because of limited proliferative nature of plasma cells in culture. Fluorescence *in situ* hybridization (FISH) can detect chromosomal aberration in 40-70%. Identifying genetic markers by cytogenetics, FISH and molecular methods is important for disease management and risk stratification. MM can be broadly divided into hyperdiploid and non-hyperdiploid subtypes. The latter represents cases with IgH translocations that are usually associated with more aggressive clinical features and shorter survival. The three essential translocations targeting IgH are t(11;14)(q13;q32), t(4;14)(p16;q32) and t(14;16)(q32;q23) [14,15].

Lymphoma is derived from the basic cells of lymphoid tissue, the lymphocyte, and histiocytes in any of their developmental stages [1]. Characteristic translocations are found to be associated with subtypes of B-cell non-Hodgkin lymphoma (NHL), for example t(8;14)(q24;q32) in Burkitt lymphoma, t(14;18)(q32;q21) in follicular lymphoma, and t(11;14)(q13;q32) in mantle cell lymphoma [16]. Only few recurrent cytogenetic aberrations have been identified in the T-cell NHL and the best known is the *ALK* gene translocation t(2;5)(p23;q35) in anaplastic large cell lymphoma. Cytogenetic and FISH aid in the diagnosis, correct classification, and evaluation of therapy for a variety of lymphomas [16].

Solid tumors: The combination of cytogenetics and molecular genetics is useful and ultimately serve as guidance for the diagnosis in some of the solid tumors. The majority of soft tissue tumors translocations associated fusion genes encode for aberrant transcription factors that cause transcriptional deregulation; examples include Ewing's sarcoma t(11;22)(q24;q12), synovial sarcoma t(X;18)(p11;q11), alveolar rhabdomyosarcoma t(2;13), Myxoid /round cell liposarcoma, and clear cell sarcoma. del(11)(p13p15) in wilm's tumor and del(1)(p32)/ double minutes in Neuroblastoma are diagnostic markers [17].

The high level of variation in culture success rate and condensed, ill defined, poorly spared, hardly banded, barley analyzable chromosomes pose a particular technical challenge in malignant sample. Cytogenetics has some limitation, this technique cannot identify the abnormalities beyond the 15kbp and it requires dividing cells. To overcome limitations of Cytogenetics the molecular technique has been emerged is FISH. These two techniques are complementary to each other in detection of abnormalities at diagnosis and during follow-up in management of cancer patients.

CONCLUSION

Thus, the discipline of Cytogenetics has grown up to the level of deciphering many types of leukemias, lymphomas and solid tumours in their diagnosis, prognosis, choice of therapy as well as remission status and distinguishing relapse from a new therapy-related leukemia. A number of new techniques are emerging. But reliable & less complex, less expensive methods such as Cytogenetics and FISH still play an important role in the diagnosis, prognosis & choice of therapy of cancer patients.

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MOLECULAR CYTOGENETICS IN CANCER DIAGNOSIS AND PROGNOSIS

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INTRODUCTION

Cancer can be defined as an abnormal growth of cells caused by multiple changes in gene expression leading to dysregulated balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host. High genome instability is one of the most important events in the malignant process. Gene mutations and numerical and/ or structural chromosomal aberrations are present in cancer cells and nonrandom chromosomal aberrations are associated with specific disease subtypes and have clear prognostic implications. There are different types of cancer, mainly haematological malignancies and solid tumours. In haematological malignancies chromosome abnormalities are very common and prognostication and treatment decision is made according to the type of chromosome abnormalities seen in these patients. According to the WHO classification chromosome abnormalities plays a major role in risk stratification. The study of human chromosomes has been going on for well over a 100 years. However, progress has been impeded by difficulties associated with the preparation of high-quality chromosomes from mammalian cells. It was not until 1956 that Tjio and Levan reported the correct number of human chromosomes [1]. In the 1960s, the addition of phytohaemagglutinin to blood lymphocyte cultures was found to stimulate cell division, significantly increasing the number of metaphase spreads [2,3]. These discoveries improved the analysis of human chromosomes and led to the realization that the analysis of chromosomes was indispensable to the study of human disease. After the observation of an extra copy of chromosome 21 (i.e. trisomy 21) in patients suffering from Down syndrome, other trisomies were found in the D and E group chromosomes and were linked to certain characteristic phenotypes that are associated with Patau syndrome (Trisomy 13) and Edwards syndrome (Trisomy18), respectively. Another milestone was the discovery of the Philadelphia chromosome and its association with chronic myelogenous leukaemia [CML] [4], which invigorated the field of cancer cytogenetics. The 1970s saw the introduction and successful application of a variety of staining techniques that gave chromosomes a banding pattern. Banding, by greatly improving the accuracy of chromosome analysis [5,6], permitted the analysis of many different tissues and diseases. Such banding patterns facilitated the analysis of sub chromosomal regions. High-resolution banding methodology made possible for the detection of chromosomal deletions and the identification of small translocations [7]. Classical cytogenetics represented by chromosomal banding techniques was successful in correlating karyotype abnormalities with diagnosis, prognosis, and response to therapy in hematological neoplasms. However, these techniques require a high rate of cell division and good chromosomal morphology, which represent challenges for the cytogeneticists, and a long period for assaying and analyzing, which usually is a challenge for physicians. Thus, it was not surprising that an outburst of progress in the cancer cytogenetics field followed the advent of molecular cytogenetic techniques such as, FISH, CGH and SKY/ M-FISH technologies [8,9]. Initially these technologies focused on research issues, but soon were applied to clinical questions and have proved sufficiently sensitive and reliable to fill in the gap between classic karyotyping and highly sensitive molecular techniques.

FISH (Fluorescence *In Situ* Hybridization)

The FISH technique was introduced in the late 1980s. It is a powerful technique used in the detection of chromosomal abnormalities [10]. The high sensitivity and specificity of FISH and the speed with which the assays can be performed have made FISH a pivotal cytogenetic technique that has provided significant advances in both the research and diagnosis of haematological malignancies and solid tumours. From a medical perspective, FISH can be applied to detect genetic abnormalities such as characteristic gene fusions, aneuploidy, and loss of a chromosomal region or a whole chromosome or to monitor the progression of an aberration serving as a technique that can help in both the diagnosis of a genetic disease or suggesting prognostic outcomes. FISH can also be applied to such research applications as gene mapping or the identification of novel oncogenes or genetic aberrations that contribute towards various cancers. The advantages of FISH technique are, it is very sensitive, easy to perform, the ability to detect such sequences in non-dividing cells (i.e. interphase cells), include the confirmation of chromosome breakpoints. FISH can be used to determine the origin of marker chromosomes and to confirm numerical and structural aberrations. Using banding techniques on highly extended chromosomes, the smallest chromosome abnormality detectable is ~2000–3000 kilobases (kb), whereas FISH probes can detect regions as small as 0.5 kb on metaphase chromosomes. The increased resolution provided by FISH has particular relevance in the study of microdeletion syndromes (e.g. Prader-Willi) because the size of the DNA region that has been deleted is often too small to be detected by conventional banding techniques. FISH probes are highly specific for their target or cDNA sequence, and can be divided into four main types: gene-specific probes, centromere specific, telomere specific and whole-chromosome painting probes. FISH could be used for identifying and confirming many chromosome abnormalities in Cancer [11]. In Leukemia FISH test is essential for classifying the patients in to different risk groups, like good, intermediate and poor. In Chronic Myelogenous Leukemia, the identification of BCR-ABL translocation is essential for deciding the treatment and prognostic prediction. For monitoring the treatment response at regular intervals FISH is to be performed. Similarly in ALL, AML, MDS, MM, Lymphoma and CLL, FISH test is essential for proper diagnosis and prognosis. In Bone marrow transplantation treatment, detection of chimerism and Minimal residual diseases in leukemia undergoing treatment, FISH has a major role. In solid tumours also FISH test has a pivotal role, like HER2, TOP2A, PI3K in Breast cancer, PTEN, TMPRESS-ERG Fusion, c-MYC in Prostate cancer, EGFR in colon cancer, HER2/ neu in Gastric cancer, MET, ALK, ROS1, PI3K, EGFR in Lung cancer etc.

Comparative Genomic Hybridization (CGH)

CGH is a molecular cytogenetic technique for analysing copy number variations (CNVs) relative to ploidy level in the DNA for a test sample compared to a reference sample, without the need of culturing cells [12,13]. CGH is based on quantitative two-colour FISH technique. The aim is to quickly and efficiently compare two genomic DNA samples arising from two sources which are mostly often closely related. This technique was originally developed for the evaluation of the differences between the chromosomal complements of solid tumors and normal tissue. It has an improved resolution of 2-4 megabases compared to the more traditional cytogenetic and FISH techniques. It has become an invaluable technique for studying chromosomal aberrations that occur in solid tumours and other malignancies [14]. A major advantage of the CGH technique is that only DNA from the tumour samples is needed for analysis; this avoids the often-difficult preparation of tumour metaphase chromosomes, which can have a poor morphology and resolution. Instead, karyotypically normal metaphase chromosomes are used to detect tumour associated chromosomal gains and losses. Another advantage of CGH is that formalin-fixed tissue sections can be used; thus, comparisons can be made between a phenotype and genotype, and genetic changes can be correlated with the clinical course of a disease. CGH is also useful for the analysis of chromosomal gains and losses in solid tumours. Recurrent patterns of gains and losses have been observed in the malignant tissue from virtually all human cancers. For example, using CGH to study pancreatic carcinomas, Armengo *et al.* found

recurrent gains on the chromosome arms 3q, 5p, 7p, 8q, 12p and 20q. Losses were observed on the chromosome arms 8p, 9p, 17p, 18q and 19p and on chromosome 21. Heselmeyer and colleagues studied tissue from the cervical epithelium at various stages of dysplasia. Their results showed that the gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma [15,16,17,18].

Multicolour Spectral karyotyping (SKY)/ M-FISH

SKY is a novel molecular cytogenetic FISH-based technique that allows colour karyotyping of human and mouse chromosomes. This technique that combines the power of conventional chromosome analysis with the specificity of FISH which limits analysis to specific chromosomes or regions of chromosomes, and CGH visualizes only those changes that result in variations in copy number, SKY permits the visualization of all chromosomes at one time, 'painting' each pair of chromosomes a different fluorescent colour. SKY entails a single multicolour FISH analysis, which can be used to yield 24 different-coloured chromosomes in a human metaphase spread. Before the development of this technique, molecular cytogeneticists analyzed chromosomes with staining techniques that produced a black-and-white banding pattern. However, the identification of all chromosomal aberrations in a complex karyotype was often not possible from such patterns. SKY has been applied to a variety of human malignancies and mouse model systems, and it has been highly effective in deciphering many complex karyotypic rearrangements which are sometimes difficult to identify using traditional banding analysis [19,20]. The applications of SKY for identifying chromosomal aberrations that are involved in human disease are manifold. SKY is suitable for the identification of subtle chromosomal aberrations, such as the translocation of telomeric chromatin, which is difficult to discern using banding alone, and the identification of small markers, which remain elusive after conventional banding analysis. This technique has also proven to be beneficial in elucidating the complex rearrangements that are observed in cancer genomes [21,22].

CONCLUSIONS

Cytogenetic research and diagnostics have greatly benefited from employing Cytogenetic and molecular cytogenetic techniques. In particular, the development of FISH technique has widened the plethora of applications considerably. The considerable gap in resolution between traditional cytogenetic techniques (mega base pairs) and molecular biology techniques (base pairs) has been closed to a large extent by molecular cytogenetics, which allows the assessment of genetic changes on chromosome preparations. The issue of sensitivity has been successfully tackled by devising new protocols for the detection of fluorescently tagged probes as small as 200 base pairs. The third defining technical parameter, multiplicity, has been solved by recent developments of colour karyotyping that allow the visualization of all human or mouse chromosomes simultaneously in different colours. One very important development in molecular cytogenetic techniques was the introduction of CGH and SKY, which has proved to be exceedingly valuable tool for the characterization of chromosomal imbalances in haematological malignancies and solid tumours. Together, these developments have contributed to the refinement of the field of molecular cytogenetics and have, in an unpredictable way, increased the flexibility with which experiments can be designed. Thus, the applications of these techniques have been extended beyond the mere diagnosis of chromosomal aberrations to functional and comparative basic research.

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ROLE OF EXOSOMES IN CANCER DIAGNOSTICS

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INTRODUCTION

Lung cancer is the main cause of cancer death worldwide killing over 1.2 million people each year [1-3]. This is mainly due to the lack of tests for early diagnosis [3,4]. The most common form of lung cancer is Non-small-cell lung cancer (NSCLC) (75-85%) [5]. Early detection of lung cancer significantly improves survival rate, thus its imperative to establish an early screening program [2]. The main focus in the clinic today in lung cancer management is targeted on the (a) identification of asymptomatic individuals at high risk for developing lung cancer and (b) individuals with indeterminate nodules following low dose spiral CT. There is an absolute consensus that blood (liquid biopsy) tests should be developed to improve diagnostic accuracy and facilitate screening in a public health setting. Current literature and our own studies indicate [6] that the cell free DNA and RNA in the plasma is protected by enclosure into vesicles or binding to protecting complexes. Also, one of the technical challenges regarding sensitivity and specificity of cancer-biomarker detection is to enrich for such cancer-DNA "carriers" and reduce noise originating from non-cancer cell breakdown. Exosomes are small membrane-bound microvesicles (40–150 nm) released from cells and containing a plethora of nucleic acids and proteins that are thought to facilitate microenvironment communication and control [7,8]. In cancer, exosomes change in both number and content affecting the microenvironment and inducing immune suppression, angiogenesis and premetastatic niche formation [9,10].

The small non-coding RNAs such as miRNA can control gene expression through RNA interference. There are approximately 120 miRNAs specific for certain genes found in exosomes. Some miRNAs are found bound to Argonaute (Ago) proteins, free from EVs. Ago proteins are able to associate with miRNAs which could be released during cell death. Their interaction with argonaute 2 (Ago2) protein makes them highly stable in the extracellular space [11,12]. A number of studies have shown exosomal miRNA to be involved in tumourigenesis (miR-17, miR-18, miR19a, miR-20, miR-19b-1, miR-93-1), metastasis, and stem cell differentiation (let-7) [13-15]. In different types of cancer, miRNAs expression is elevated. They could play a role as tumour suppressors or as oncogenes and promote or prevent the cancer growing. The presence of miRNA in exosomes provides an accessible means for noninvasive detection in the clinic [16,17].

In this pilot study, we analysed the qualitative and quantitative differences of the plasma exosomal content of lung cancer patients, in comparison to matched individuals with non-malignant lung disease. Our pilot cohort includes 120 individuals within four groups: (a) 30 lung adenocarcinoma, (b) 30 lung squamous cell carcinoma, (c) 30 chronic obstructive pulmonary disease (COPD) and (d) 30 no lung disease. The epigenetic and proteomic content of exosomes has been analysed in combination to clinicopathological information including NSCLC progression. The results from this study will assist us to determine which are the most promising exosome-related biomarkers for the early detection of lung cancer.

PATIENTS AND METHODS

Study design- In this study, plasma samples 50 cases of NSCLC and 47 age/ sex matched controls from the Liverpool Lung Project (LLP) were included. Appropriate ethical approval from the Liverpool Research Ethics Committee, ref 157/97, was obtained and all patients provided written informed consent.

Purification of exosomes from plasma- Plasma samples (2.0 ml) collected from the Liverpool Lung Project stored at -80°C were used for this study. The exosomes of the plasma were purified by the differential ultracentrifugation method, as described previously [18]. In brief, plasma samples were thawed and spun at 10,000 x g for 30 minutes. Superntants were then separated by ultracentrifugation at 110,000 × g for 70 min at 4°C, and the exosomal pellets were washed with phosphate-buffered saline (PBS) and stored at "80°C for Western blot and miRNA profiling analysis.

Transmission electron microscopy of exosomes- The 100,000 x g exosome-containing pellet was resuspended in 20µl of fixed solution containing 2.5% Glutaldehyde in Sorensen buffer (133mM Na_2HPO_4, 133mM KH_2PO_4) for at least 1 hour to overnight at 4°C. Approximately 5µl resuspended pellets were deposited on Formvar-carbon coated EM grids. The grids were covered and incubated for 20 min at room temperature. A Parafilm sheet was set up and 100µl drops of Sorensen buffer pipetted onto the sheet. The grids were transferred to the drops of PBS with exosome-side down to wash. The grids were then transferred to 50µl drop of 1% glutaldehyde for 5 mins followed by washing in 100µl drops of dH_2O for eight times. The grids were then incubated with 50µl uranyl-oxalate solution, pH7 for 1 minute and then contrasted and embedded in a mixture of 4% uranyl acetate and 2% methyl cellulose (1:9 ratio) for 10 minutes on ice. The grids were removed with stainless steel loop and excess fluid blotted onto Whatman paper. The grids were air-dried for 5 to 10 minutes on the loops. The grids were placed into dry storage boxes and observed under the electron microscope at 80 kV.

Western blot- Exosomes were lysed in Laemmli sample buffer (60 mMTris, pH 6.8, 4% SDS, 5% (w/v) glycerol). The lysates were then sonicated on ice and centrifuged to remove insoluble material. The resulting supernatants were collected, and the protein concentration was determined using a Bio-Rad protein assay kit according to the manufacturer's protocol. Approximately, 50 mM DTT and 0.05% bromophenol blue were added to the samples. Samples were incubated at 95°C for 5 min, and 25 µg of total protein was loaded onto 12% polyacrylamide gels containing 0.1% SDS. Gels were then transferred to 0.45 µM polyvinylidenedifluoride membrane (Millipore) and immunoblotted using an enhanced chemiluminescence system [19]. For quantification of Western blots, data are expressed graphically and represent the relative band intensity for each component detected following correction of any background. Signals were quantified using Chemidoc (Biorad), and several different exposure times were used to ensure that each image analyzed gave a linear response.

miRNA profile study

miRNA profiling was performed using plasma exosomes by HTG Molecular Diagnostics Inc. (Tucson, AZ). We analysed 2,083 human miRNA transcripts in a NGS-based assay using the HTG EdgeSeq technology. Expression levels of miRNAs were compared and visualized by the heatmap.

RESULTS

The molecular constituents of exosomes: In this pilot study we have investigated the qualitative and quantitative differences of the plasma exosomal content of lung cancer patients, in comparison to matched individuals with non-malignant lung disease. Our pilot cohort included 120 individuals within four groups: (a) 30 lung adenocarcinoma, (b) 30 lung squamous cell carcinoma, (c) 30 chronic obstructive pulmonary disease (COPD) and (d) 30 no lung disease. To determine the relevance of circulating exosome concentrations in non-small cell lung cancer (NSCLC), we prospectively isolated and characterized exosomes from the plasma of human

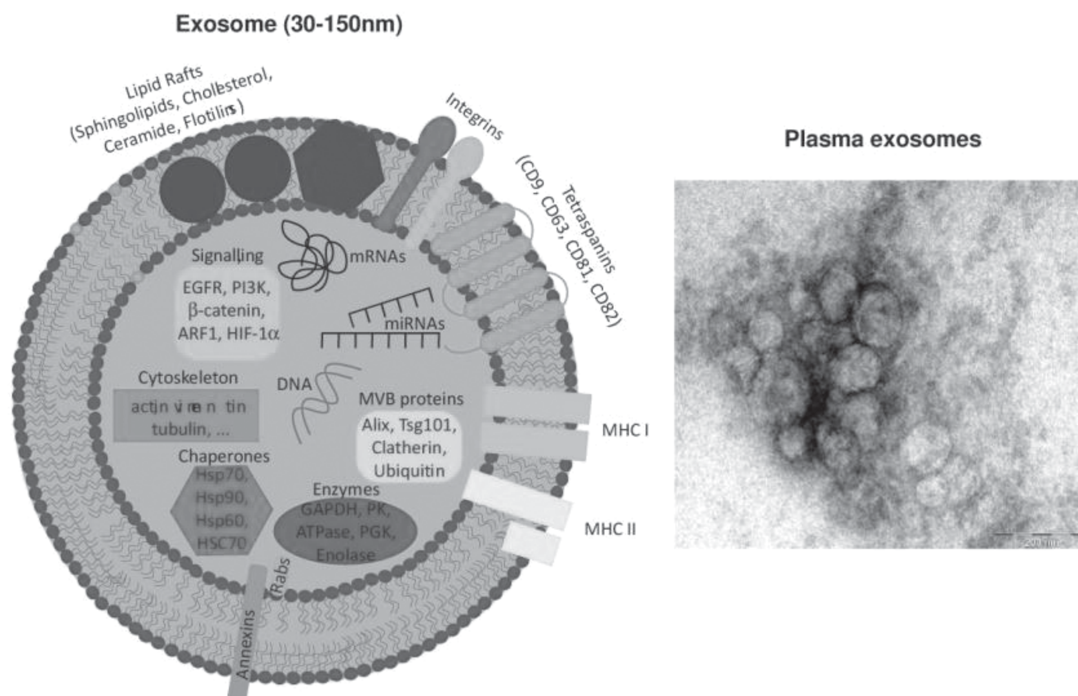

Figure 1. The molecular constituents of exosomes. (A) Exosomes are defined as a vesicle with a phospholipid-enclosed bilayer. They are composed of membrane proteins such as tetraspanins, lipid rafts, and MHC class proteins. The exosomal cargo is found in the lumen which includes nucleic acids (e.g. DNA, mRNA, and miRNAs) and soluble proteins (Hsps). (B) An electron microscopy image of plasma exosomes measuring approximately 30-150 nm in diameter.

subjects with different clinical stages of NSCLC. We isolated exosomes (30-150 nm particles) from plasma using differential ultracentrifugation and confirmed their identity by electron microscopy [Figure 1]. In addition, the exosome number and size distribution as quantified by NanoSight analysis did not alter compared to clinical stage (data not shown).

Analysis of protein expression in circulating exosomes from subjects with NSCLC: We confirmed the presence of known exosome markers, including Flotillin-2, CD63, Tsg101 and Alix [Figure 2]. In addition, we confirmed the absence of the mitochondrial protein, cytochrome C1 and the Endoplasmic reticulum protein, calnexin, both negative controls of exosomes. The protein levels of these markers did not change with clinical stage. In contrast, exosome protein concentration of CD9 was notably higher in case subjects at low and high stages versus both healthy control and COPD control subjects [Figure 2C]. The levels of EGFR in exosomes were elevated at low stage and significantly higher at late stage [Figure 2C]. Interestingly, EGFR levels in COPD control patients were significantly higher than healthy patients.

RNAseq analysis of plasma exosomes: We performed HTG EdgeSEQ on 97 plasma samples (50 cases and 47 age/ sex matched controls) from the Liverpool Lung Project (LLP). RNA sequencing targeted 2083 miRNA transcripts. PCA analysis of data shows similar patterns of commonality and the transcriptome level between cases and controls [Figure 3A]. Heatmap representation (row-scaled) showing similarities and dissimilarities among selected late stage and control samples [Figure 3B]. Approximately, 30 miRNAs were upregulated or downregulated in plasma exosomes from cases compared to age/sex matched controls. In our further studies, we implemented the LASSO penalized logistic regression, which provides model fitting and model building for generalized linear models, to select the

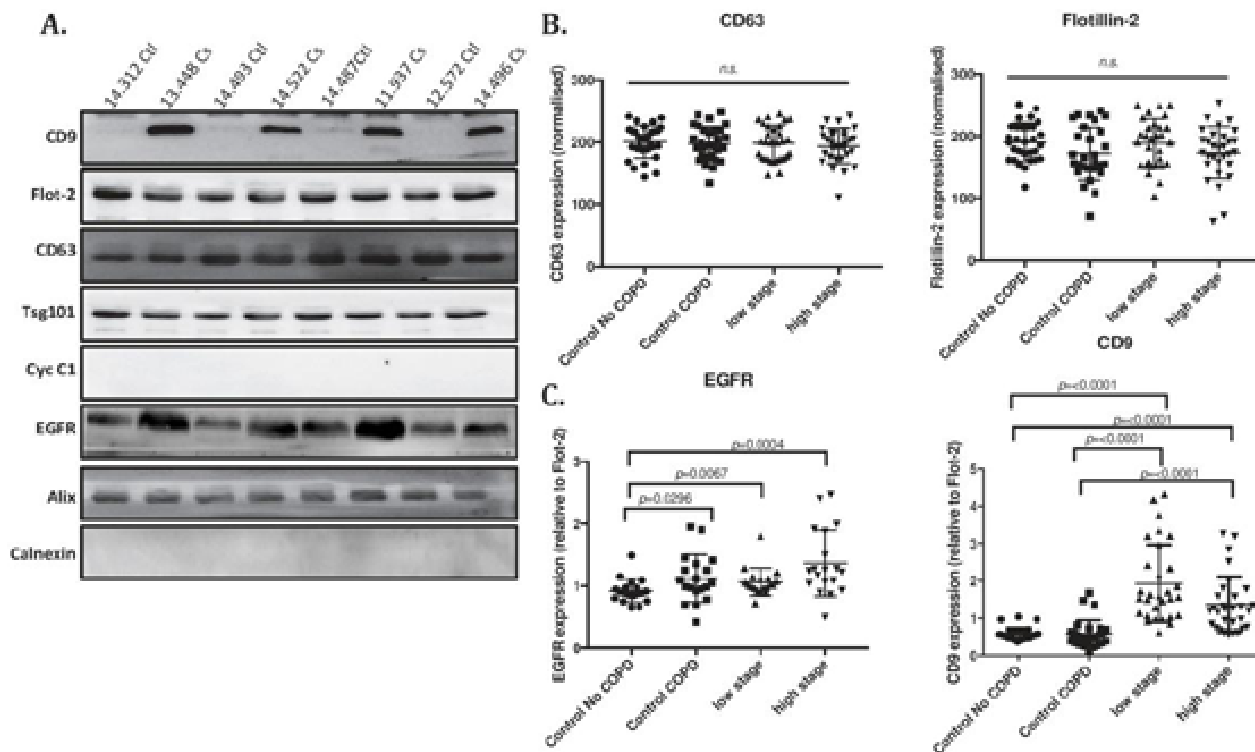

Figure 2. Analysis of protein expression in circulating exosomes form subjects with NSCLC. (A) Representative Western blot of CD9, Flotillin-2, CD63, Tsg101, Cytochrome C1 (Cyc C1), EGFR, Alix and Calnexin) in circulating exosomes isolated from the plasma of subjects with NSCLC (cs) and control (Ctl). (B) Statistical analysis of the Western blot densitometry of Flotillin-2 and CD63 in circulating exosomes. (C) Statistical analysis of the Western blot densitometry for CD9 and EGFR expression in circulating exosomes relative to Flotillin-2. Controls No COPD, n=30; Control COPD, n=30; low stage, n=30; high stage, n=30. *P* value was calculated by analysis of Mann-Whitney test.

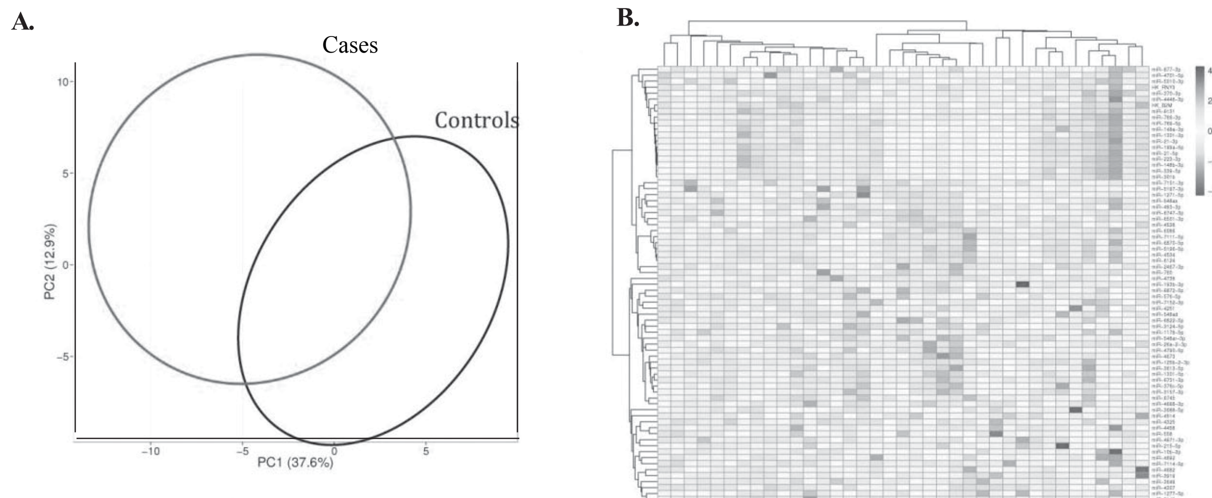

Figure 3. RNAseq analysis of plasma exosomes. (A) PCA analysis performed on the normalised dataset (Control n=20, Cases n=20). (B) Cluster heatmap of miRNAs upregulated (red) and downregulated (blue).

panel of miRNA that best predict lung cancer. We have identified four miRNAs that contributed to the best predicting model of lung cancer, with an AUC of 0.84 and a bias-corrected AUC of 0.82 (data not shown).

CONCLUSION

1. Western analysis of the plasma exosomes demonstrates differential expression of exosome surface antigens between cases and controls.
2. Analysed 2,083miRNAs in 97 (50 cases/47 controls) plasma samples.
3. Bioinformatic analysis has now indicated 4 targets with a very high potential to discriminate lung cancer cases from individuals with no malignancy.
4. his is the first such comprehensive miRNA screening of exosomes from lung cancer patients and controls. It provided an important profile analysis and suggested miRNA targets which may be used in early diagnosis.

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IDENTIFICATION OF DIFFERENT CYTOGENETIC GROUPS IN ACUTE MYELOID LEUKEMIA: COMBINED CONVENTIONAL AND MOLECULAR CYTOGENETIC STUDY

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INTRODUCTION

Acute Myeloid Leukemia (AML) is a highly heterogeneous disease commonly seen in adults, characterized by the proliferation and abnormal differentiation of myeloid progenitor cells in the bone marrow. It is a disease with high progression rate, will prompt death within 2- 3 months if untreated [1]. Cytogenetic analysis of AML is essential for classification, disease diagnosis, risk stratification and the information also critical for treatment guidance. Because of the heterogeneous nature of the disease, it is very important to identify the different types of AML. The commonly used French- American- British (FAB) classification of AML divide this disease into 8 different subtypes, M0- M7, based on the type of cell from which the leukemia developed and depending on the degree of maturity of the cell. The more recent World Health Organization (WHO) advanced a new classification for AML, used many different clinical and laboratory aspects of this disease, including immunophenotyping, cell morphology, cytology and genetics of the cells. The primary treatment of AML is chemotherapy, mostly using ADE (combination of Cytarabine, Daunorubicin Hydrochloride and Etoposide) for the past 40 years and researchers are endeavoring to enhance treatment outcome. Patients diagnosed as APL with t(15;17) responds well to the all- trans- retinoic acid (ATRA) based therapy.

Chromosomal abnormalities play a vital role in the disease progression. Both FAB and WHO classification use a variety of factors, especially cell cytogenetics, to classify AML as poor-risk, intermediate-risk, and better-risk disease [2]. Cytogenetic analysis of abnormal bone marrow blast cells is thus a cornerstone for disease characterization, determination of disease aggressiveness, response to treatment and prognosis. Treatment decisions have been made using this information either deciding on the aggressiveness of treatment, or in determining whether targeted therapy may influence the genetic or genomic aberration and specifically treat the individual's tumor [2]. Even though technology built up so far, diagnostic karyotype of the leukemic cells still remains as the strongest independent prognostic indicator [3].

Cytogenetic testing of bone marrow samples is widely commercially available, and results are consistent and interpretable. Fluorescence in-situ hybridization (FISH) can also identify and confirm cytogenetic abnormalities and should be done in addition to (but not instead of) routine cytogenetics.

MATERIALS AND METHODS

Study subjects: A total of 200 AML patients, who attended the Medical Oncology outpatient clinic of the Regional Cancer Centre, Thiruvananthapuram, during January 2016 to January 2017, formed the study subjects. All the cases were classified according to the FAB classification. Study approval was obtained from the Institutional Review Board and Human Ethics Committee. Bone marrow samples were collected from all the patients after getting informed consent. Among the 200 study subjects, 102 were males and 98 were females (male to female ratio, 1.04:1) with a mean age of 42.61 years (range, 16-74 years).

Cytogenetic analysis: Cytogenetic analysis was performed on metaphases from bone marrow aspirate by standard short- term culture procedure [4]. From

the GTG banded slides, 15-20 metaphases for each case were karyotyped and analysed using Band View, Applied Spectral Imaging (Israel) software. Karyotypes were described according to ISCN 2016 [5]. Patients were classified as having an abnormal, normal, or failed cytogenetic result.

Molecular cytogenetic analysis: Fluorescence *in situ* Hybridization (FISH) technique was performed to determine recurrent chromosomal abnormalities associated with specific FAB subtypes M2, M3 and M4 respectively using locus specific probes (Abott molecular/ Vysis, Des Plains, IL). For the confirmation of those cases, which were identified as sex chromosome deletion in their karyotype X/Y centromere specific probe was used. The slides were hybridized overnight following the abott molecular/ Vysis protocol. Spectral karyotyping was performed for the identification of complex chromosomal abnormalities, which were not revealed by karyotyping or FISH analysis, according to the standard protocol provided by Applied Spectral Imaging.

RESULTS

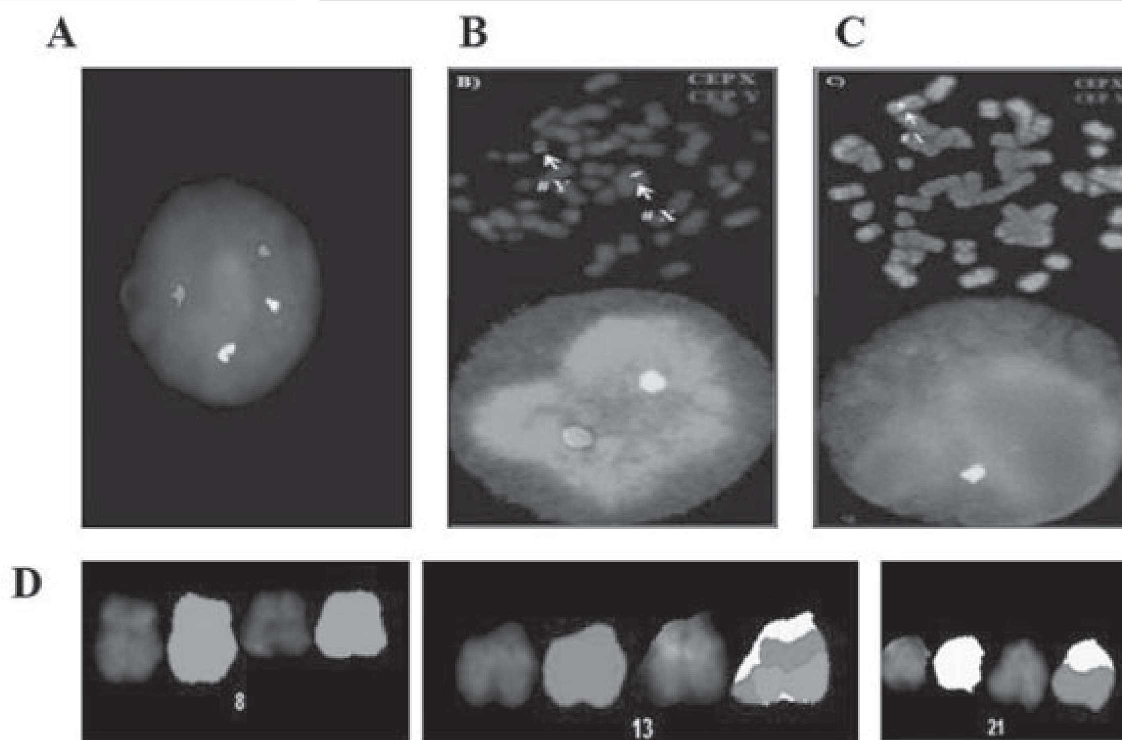
Conventional cytogenetic analysis: The cytogenetic profile of AML at diagnosis consistently remains a highly influential prognostic factor [6]. Conventional cytogenetic analysis was carried out in the bone marrow samples of the study subjects. All the 200 cases were readily classified according to FAB criteria. Among the patients, 65.5% had a normal karyotype and in 13 cases karyotype failed due to the unavailability of good quality metaphases. Frequencies of the various cytogenetic abnormalities identified in the remaining patients are shown in Table 1. Overall, karyotype analysis of the study subjects identified 16.5% cases as better, 76.5% as intermediate and 0.5% as poor- risk groups.

Table 1: Frequency of chromosomal abnormalities

Cytogenetics	No of Patients (n = 200)
Recurrent	33 (16.5%)
(8;21)	25
Sex chromosome deletion	7
t (15;17)	5
inv (16)	2
Numerical abnormalities	16 (8%)
Structutal abnormalities	6 (3%)

Molecular cytogenetic analysis (FISH & SKY): Fluorescence *In Situ* Hybridization (FISH) technique was employed in specific sub types of AML, which are associated with recurrent chromosomal abnormalities (AML- M2, M3 and M4). Specific AML-ETO, PML- RARA dual colour fusion probe and CBFB/MYH11 break apart probe were used for the identification of t(8;21), t(15;17) and inv(16) respectively. FISH analysis helped to identify 6 more cases with recurrent chromosomal abnormalities in which karyotype failed. Among the 25 cases with t(8;21) cases, sex chromosome deletion were observed in 7 cases. For the confirmation of sex chromosome deletion X/Y centromere specific probes were used. Metaphase FISH/SKY analysis helped to identify two complex abnormalities in which one cases was previously identified as with t(8;21) in the karyotype and AML- ETO single fusion in the interphase FISH analysis Figure 1].

It is widely accepted that certain cytogenetic abnormalities associated with specific subtypes of AML carry distinct responses to therapy. AMLs with normal cytogenetics, which represent a large proportion (almost 50%) of newly diagnosed cases belongs to intermediate- risk group. Only a small percentage of AML patients get a long term survival by the existing chemotherapy. Because of the inferior quality or inadequate number of metaphases, rarely recurrent abnormalities may get unnoticed. Also a certain percentage of patients possess additional chromosomal abnormalities along with the recurrent abnormalities. Thus for getting a clear picture of chromosomal abnormalities combined conventional and molecular cytogenetic analyses are required. In the present study, combined conventional and molecular cytogenetic analysis identified 38 cases (19%) as better and 2(1%) as poor risk groups.



(A) interphase cell showing single fusion signal for t(8;21)
(B) interphase and metaphase cell showing signal for XY
(C) -X/-Y signal
(D) Partial spectral karyotype image showing t(8;13;21)

DISCUSSION AND CONCLUSION

AML is a heterogeneous clonal disorder of haematopoietic progenitor cells and the most common malignant myeloid disorder in the adults. It is characterised by an abnormal increase in the number of immature myeloid cells in the bone marrow and an arrest in their maturation leading to an accumulation of blast cells in the bone marrow. AML is a highly heterogeneous disease and the heterogeneity can be seen in cytogenetic as well as molecular level [7]. Despite the fact that ~50% of AML patients are reported to have normal cytogenetics, several chromosomal abnormalities are closely and uniquely associated with distinct subtypes that has pivotal role in leukemiogenesis. Some of the recurrent abnormalities seen in AML patients are t(8;21), inv (16) and t(15;17). Both the molecular and conventional cytogenetic techniques are indispensible tool for both basic and clinical research, as well as diagnostics especially in leukaemia. Cytogenetic profiling of the patients is much important for treatment guidance. Recently several studies reported that the presence of gene mutations or additional chromosomal abnormalities can shift the risk group status of patients [8].

This study involved 200 cytopathologically confirmed AML patients with a mean age of 42.61 years. In case of inadequate number of metaphases or unavailability of good quality metaphases conventional cytogenetic analysis failed to identify chromosomal abnormalities in some cases. For those cases, FISH technique was used for the identification and confirmation of recurrent chromosomal abnormalities, where more number of cells could be analysed. For getting a clear picture of recurrent chromosomal abnormalities FISH technique have some advantage over karyotyping. But as it is specific for genes involved, other additional abnormalities cannot be identified. Sex chromosome deletion was

observed in 7 of the AML M2 patients, along with t(8;21). Even though M2 patients with t(8;21) included in the good prognostic group the additional abnormalities like sex chromosome deletion may shift the risk group status. Deletion of X chromosome (-X) does not seem to have any prognostic impact, whereas a recent study described a weak good prognostic impact for Y chromosome deletion (-Y) [8]. In brief, the results highlight diagnostic and prognostic significance of combined conventional and molecular cytogenetic analysis in AML patients which in turn helps for further refinement of risk stratification in AML. Thus combined cytogenetic and molecular studies in AML patients have prognostic, diagnostic and therapeutic implications.

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THE IMPORTANCE OF CYTOGENETICS AND FLUORESCENT *IN SITU* HYBRIDIZATION (FISH) IN THE DETECTION OF BCR-ABL FUSION GENE IN CML PATIENTS

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INTRODUCTION

Chronic Myeloid Leukemia (CML) is classified as one of the myeloproliferative neoplasm which exhibit excessive proliferation of immature myeloid cells. The disease occurrence is higher in older age but it can occur at any age. In India it is the most common adult leukemia. There is a slight male predominance, the median age at diagnosis lies in between 55-60 years (30%-60%). The crucial genetic event in CML is the generation in a hematopoietic stem cell of a t(9;22)(q34;q11), reciprocal chromosomal translocation between the long arms of chromosome 9 and 22 results in a shortened chromosome 22, known as the Philadelphia chromosome (Ph) and found in over 90% of CML patients [1]. Nowadays it is estimated that the CML cases have at least one Ph even in their chronic phase. The chimeric gene BCR/ABL on chromosome 22 and a reciprocal ABL/ BCR on chromosome 9 are the molecular consequences of this translocation [2]. The amino terminal end of Breakpoint Cluster Region (BCR) gene on chromosome 22 is fused to most of the c Abelson 1 (c-ABL 1) proto-oncogene on chromosome 9,thereby creating an 8.5 kb BCR-ABL chimeric mRNA encoding a 210-k Da hybrid protein (p210 BCR-ABL) [3].The later gene, which is situated on ninth chromosome although transcriptionally active, does not appear to have any functional role in CML and no ABL/BCR protein has, as yet, been identified [4]. Two other BCR/ABL proteins other than p120 like p190 and p230, generated by variant fusion genes, are only occasionally detected in classic CML [5].The chronic phase lasts several years and results in the accumulation of myeloid precursors cells within the bone marrow, peripheral blood and extra medullary regions [6]. The accelerated phase lasts 4 to 6 months and is characterized by an increase in disease burden and in the frequency of progenitor/precursor cells rather than terminally differentiated cells. The blast crisis have much more similarity with acute leukemia and is characterized by the rapid expansion of a population of myeloid or lymphoid differentiation-arrested blast cells. The transition from chronic phase to blast crisis is unavoidable before the invention of targeted drugs but the introduction of tyrosine kinase inhibitors like imatinibmesylate (gleevec) is able to postponed for several years in most patients with CML [7,8]. Only a small proportion of patients are getting resistance to imatinib. During blast crisis secondary genetic abnormalities such as 17p+, 16 q+, +8, hyper diploid, double Ph, t(3;21) will accumulate due to the action of BCR/ABL and the changes in the gene expression will leads to the differentiation arrest [9].

Philadelphia chromosome (Ph Chromosome):
An abnormal shortened chromosome 22, termed as Philadelphia chromosome; which is the result of a reciprocal translocation of genetic material between chromosome 9 and 22 t(9;22) (q34.1;q11.21). Ph chromosome is present in 90-95% of patients with the clinical haematological features of CML. The discovery of Ph chromosome in Philadelphia in the year 1960 was a land mark. It was the first consistent chromosome abnormality found in any kind of malignancy. The discovery led to the identification in CML cells of the BCR-ABL fusion gene and its corresponding protein. As the result of reciprocal

translocation, two fusion genes are generated; BCR-ABL on the chromosome 22 and ABL-BCR on the chromosome 9.The BCR- ABL fusion gene product functions as a constitutively activated tyrosine kinase, it has a domain that can add phosphate groups to its tyrosine residues but does not require activation by other cellular proteins. Thus it activates a number of cell cycle controlling proteins and enzymes, accelerates cell division, inhibits DNA repair. Ph chromosome is the hallmark of CML.

Treatment of CML: There are different types of treatments for patients with CML; they include Chemotherapy, Immunotherapy, Stem cell transplantation, Donor lymphocyte infusion, Surgery and Targeted therapy. CML cells contain an oncogene- BCR-ABL, which encodes a type of protein P210, with an enhanced tyrosine kinase activity. Drugs known as tyrosine kinase inhibitors (TKIs), which target BCR-ABL protein are the standard treatment for CML. These drugs are less likely to affect normal cells, so their side effects are generally not as severe as those seen with other drugs, such as traditional chemotherapy drugs or Interferon alpha. ImatinibMesylate (Gleevec), Dasatinib (Sprycel) and Nilotinib (Tasigna) have been approved as initial therapy for chronic phase and advanced phases of CML.

STUDY SUBJECTS AND METHODS

A total of 210 cytopathologically confirmed CML patients, who attended the medical oncology clinic of the Regional Cancer Centre, Trivandrum, from January 2015 to May 2017 were included in the study. The patients belonged to the age of 17 to 75 years which includes 120 males and 90 females with a mean age 45 years. Bone marrow aspirate withdrawn at the time of disease diagnosis after getting informed consent was used for classical cytogenetic analysis after short term culture. Harvesting and GTG banding were performed as per the standard procedure [10]. Karyotypes were described according to ISCN 2016. Twenty metaphases were karyotyped using Cytovision software (Cytovision,USA). FISH analysis was performed to determine BCR-ABL gene fusion using locus specific probes (Abott Molecular/ Vysis, Des Plains, IL).

Conventional Cytogenetics: Bone marrow samples were collected in heparinised vaccutainer. Direct bone marrow culture technique was performed using RPMI medium along with colchicine for 1 to 2 hours at 37°C in an incubator. After incubation period, hypotonic treatment was performed using 0.075M KCl solution for 20 minutes, after centrifugation and decanting of the supernatant. The cells were fixed and washed using Carnoy's fixative (3 methanol: 1 acetic acid mixture). Slide preparation was performed with the appropriately diluted clear pellet suspension dropped from a height of one to three feet to a chilled slide. The slides were allowed to dry at room temperature, aging was done by keeping the slides at 60°C overnight and GTG banding was performed and stained with geimsa stain. The slides were scanned under 10 X Research microscope and focused in 100x under oil immersion, good quality metaphases (at least 20) were karyotyped with the help of Cytovision software, following ISCN (International System for Human Cytogenetic Nomenclature).

FISH (Fluorescence *In Situ* Hybridisation): FISH is a molecular cytogenetic technique which was used for confirming the presence of BCR-ABL fusion gene. It was performed in the unbanded, unstained cytogenetic slide. It is a two day procedure. First day pre-treatment protocol was performed on the sample loaded slide and the probe was added, co-denatured and hybridized in an humidified chamber at 37°C overnight. Second day washed and counterstained the slides with DAPI and kept in the dark for at least 30 minutes. Scanning was performed under oil immersion objective of a florescent microscope connected with a camera which is attached to a FISH software system (Cytovision USA). Signals were captured and analysis was done. BCR gene was labelled with spectrum Green and ABL was labelled with spectrum Red. Presence of two green signals and two red signals in cells were considered as BCR-ABL fusion negative (lacking (9;22)) and one green, one red and two yellow signals were considered as BCR-ABL fusion positive. At least 100 cells were scored.

RESULTS

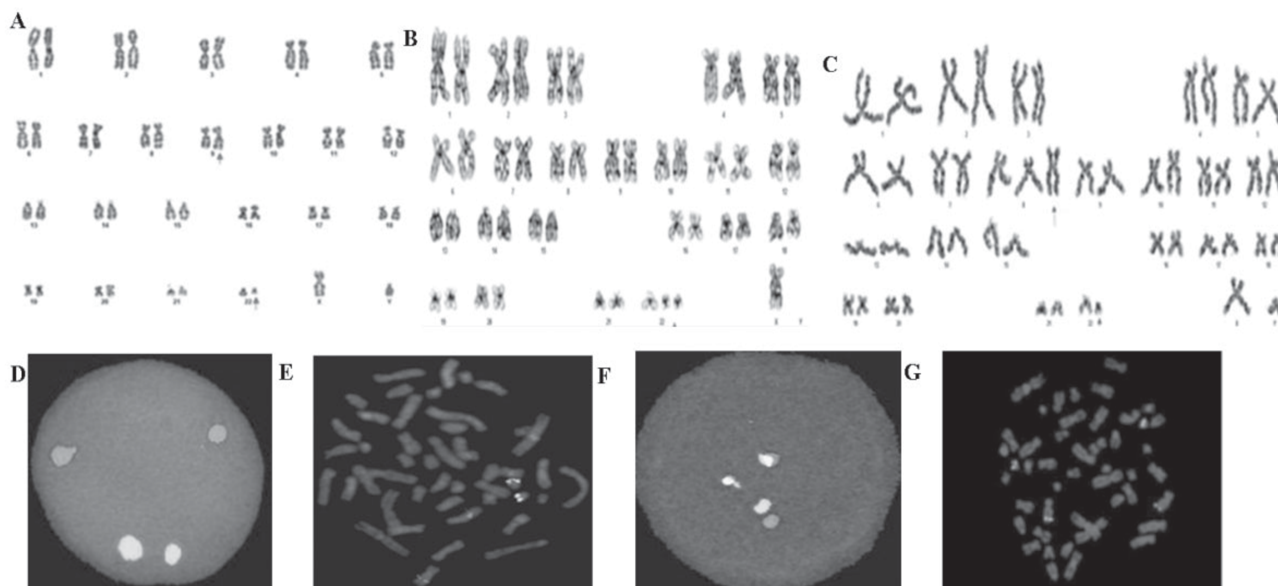
Cytogenetic analysis and FISH: The study population include a total of 210 cytopathologically confirmed CML patients. Conventional cytogenetic analysis identified Ph chromosome in 190 patients and normal karyotype in 5 cases. Analysis was failed due to the unavailability of good quality metaphases in 15 cases. The cytogenetic profiling identified in 210 CML cases were depicted in Table 1. FISH analysis was performed in 20 cases, where cytogenetic analysis was failed and other 5 cases with normal karyotype. FISH analysis using specific BCR-ABL DC DF probe, identified BCR-ABL fusion gene in 13 cases, among them, 2 cases were previously identified as normal karyotype and in 11 cases conventional analysis was failed. Karyotype and FISH images observed were shown in Figure 1.

DISCUSSION AND CONCLUSION

Chronic Myeloid Leukemia (CML) is a haematopoietic stem cell disorder, characterized by the presence of Philadelphia Chromosome (Ph), [t(9;22)(q34;q11)]. The Ph chromosome results in the formation of the BCR-ABL fusion gene, which is

Table 1.Cytogenetic and FISH findings in the study subjects

		No of cases
Cytogenetics Karyotype (N- 210)	46,XX,Ph+	78
	46,XY,Ph+	109
	46,XX	2
	46XY	3
	47,XY,t(9;22), der(22) t(9;22)	1
	47,XX,t(9;22),+8	1
	47,XY,t(9;22),+8	1
	Karyotype failure	15
FISH(N- 20)	BCR-ABL Positive	13
	BCR-ABL Negative	7



(A) 46 , X Y, t (9; 22) (q 34; q 11) (B) double Ph (C) 46, X Y, Ph, + 8
(D) & (E) Interphase & metaphase FISH image showing the absence of BCR - ABL fusion gene
(F) & (G) Interphase & metaphase FISH image showing the presence of BCR - ABL fusion gene

Figure 1. Showing Karyotype and FISH analysis

a constitutively activated tyrosine kinase. Ph chromosome is the hallmark of CML. This study clearly shows the importance of cytogenetics and FISH technique for the detection of BCR-ABL fusion gene in CML patients. 210 cytopathologically confirmed CML patients with an age range of 17-75 years were karyotyped. Cytogenetic analysis identified Ph chromosome in 190 cases and 5 cases with normal karyotype. In 15 cases, good metaphases were not available; therefore analysis could not be done. In CML case, where the Ph chromosome cannot be identified cytogenetically, molecular analysis may reveal molecular events associated with the Ph chromosome such as BCR-ABL fusion transcript and thus establish the diagnosis. The present study shows a male predominance as reported in many other studies [11]. Fluorescence *In Situ* Hybridization (FISH) is a molecular cytogenetic technique used for the detection and confirmation of the gene rearrangement. It is an accurate and sensitive method for the identification of the fusion transcript, since the probes are specific for the respective genes. In this study FISH was performed in 20 cases, where Ph chromosome could not be detected by conventional cytogenetic technique, which includes the 5 cytogenetically Ph negative cases and 15 samples where good metaphases were not obtained. When we performed FISH analysis BCR-ABL fusion signals were observed in 2 Ph negative cases, 11 cases where good quality metaphases were not available. So FISH analysis could be used for confirming the BCR-ABL fusion gene in Ph negative cases and for detecting BCR-ABL fusion gene in poor quality metaphase cases. FISH analysis could be used for monitoring treatment response in patients under treatment, and treatment dose could be adjusted according to the percentage of BCR-ABL fusion gene. FISH could be used to confirm the presence of BCR-ABL fusion gene in cases with complex chromosome translocation which may lead to a false report by conventional karyotyping. FISH could be performed in peripheral blood sample also which may help the clinician where bone marrow could not be aspirated.

According to WHO classification, the cases without the presence of BCR-ABL gene may not be considered as CML and could not be treated with Imatinibmesylate (targeted therapy). At diagnosis, conventional cytogenetics can detect t(9;22) in about 95% of cases of CML; however an additional 2-3% of cases with submicroscopic translocations can be recovered by applying molecular methods. FISH could be used as a convincing complementary test for cytogenetically Ph negative cases and cases with non-analyzable metaphases. Whether the results of both cytogenetics and FISH are negative, then an alternative diagnosis should be considered. Cytogenetic analysis is a gold standard method for detecting all the chromosomal abnormalities in metaphases, whereas FISH could be used for the detection and confirmation of specific abnormalities only, it could be performed in interphase cells also. In brief, the present study explores the significance of conventional cytogenetics and FISH technique in the specific diagnosis of CML patients.

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GENOMIC ANALYSIS OF miRNAS IN THE DIAGNOSIS AND MANAGEMENT OF PROSTATE CANCER

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INTRODUCTION

The most commonly diagnosed non-cutaneous malignancy in men is prostate cancer which is also considered as the second largest cancer, causing death. It is estimated that one in six men would be diagnosed with prostate cancer during their lifetime [1]. Prostate cancer is prevalently diagnosed in older men and it is found that about 6 cases in 10 are diagnosed in men aged 65 or older, and it is rare before age 40. The average age at the time of diagnosis is found to be 66. Combination of a Digital Rectal Examination (DRE) and a Prostate Specific Antigen (PSA) and a trans rectal ultrasound guided prostate biopsy (TRUS) is the conventional procedure for prostate cancer detection. PSA, a predominantly used biomarker for the detection of prostate cancer, is limited by its lack of sensitivity and specificity for prostate cancer which is found to cause false-negative (15%) and false-positive (66%) diagnosis [2]. All these data disprove PSA to be an ideal biomarker for prostate cancer detection [1]. The other diagnostic tests are invasive and lack early detection of cancer as well. Therefore novel and minimal invasive detection for prostate cancer is needed which may be achieved with the help of microRNAs(miRNAs or miRs)

miRNAs are a family of small non-coding RNA species, that have been implicated in the control of many fundamental cellular and physiological processes such as cellular differentiation, proliferation, apoptosis and stem cell maintenance. miRNAs regulate gene expression by the sequence-selective targeting of mRNAs, leading to translational repression or mRNA degradation [3]. Amplification or over expression of miRNAs can down-regulate tumor suppressors or other genes involved in cell differentiation, thereby contributing to tumor formation by stimulating proliferation, angiogenesis, and invasion; they act as oncogenes and upregulate oncogenic miRNAs. Thereby miRNAs pose as novel biomarkers for cancer detection [4]. For miRNA identification, clinical data could be collected from TCGA database which is a web-based platform for cancer researchers to search, download, and analyze data sets generated by TCGA. The portal contains all TCGA data sets pertaining to clinical information, genomic characterization, and high-throughput sequencing analysis of the tumor genome.

MATERIALS AND METHODS

Patient cohorts and miRNA data sets: The following meta-analysis is based on the data generated from TCGA research network. At the time of analysis 500 cases were enrolled of which 490 were alive and the rest were dead. miRNA sequencing information were available for 490 cases and the dataset had the expression value for 1046 noted miRNAs.

Screening of differently expressed miRNAs and clustering: We screened the differently expressed miRNA between cancer and non- cancer patient samples of prostate cancer by using TCGA biolinks package in R environment with $p < 0.05$. Multi experiment viewer tool was used to generate the hierarchical clustering heat map for the above generated data.

Identification of miRNAs associated with patient survival: Cut off scores for the significant differentially expressed miRNAs were achieved by Receiver Operating Characteristic (ROC) curve plotted using SPSS software package. The highest score for each

miRNA with maximum sensitivity and specificity was selected and this was used to categorize the patients into high expression and low expression group.

The generated data was plotted using Kaplan-Meier survival analysis to determine whether the selected miRNAs had significant influence on the vital status of the patients. Then the miRNAs were given as input to PROGmiR, a web based tool that allows to study prognostic properties of miRNAs to cross check the results from Kaplan Meier survival analysis. The gene targets for these miRNAs were identified by miRTarBase – an experimentally validated miRNA-target interactions database.

KEGG signal pathway and GO annotation of 3-miRNAs predicted genes: In order to further explore the 3 miRNAs signature in biological function and mechanism, we analysed the potential targets of the three miRNAs through KEGG pathway and GO annotation analysis using DAVID and GO term analysis (PANTHER database) respectively. As a result, gene ontology of those target genes and their metabolic pathway were identified.

RESULTS

Identification of signature miRNAs differently expressed in prostate cancer: Data of 448 prostate adeno-carcinoma patients and 52 controls were downloaded using TCGA biolinks package of R. Data on miRNA expression and clinical profile information were also downloaded from the same portal. miRNA expression levels were compared between the normal and the tumor samples. We found that 185 miRNAs were significantly ($p < 0.05$) differentially expressed between the two groups. Hierarchical clustering revealed that 147 miRNAs were up regulated and 38 were down regulated [Figure 1].

Validation of miRNAs associated with cancer patients' survival: Of the above 185 miRNAs those with missing expression data were filtered and 69 miRNAs were considered for further analysis. These 69 miRNAs were considered for future analysis. These 69 miRNAs were subjected to ROC analysis to set the cut off value to classify 500 patients into high and low expression groups. miRNAs with significant ROC cut off values were subjected to Kaplan Meier survival analysis followed by PROGmiR. This gave rise to 3 miRNAs significantly associated with patient survival. The 3 miRNAs were found to be hsa-mir-133b, hsa-mir- 20a and hsa- mir-17. Further validation of the miRNAs with respect to staging of tumor or tumor recurrence was not possible due to the lack of such details in the TCGA dataset.

Pathway analysis of the targets of the 3 miRNAs: To probe into the function and mechanism of these 3 miRNAs the targets for these miRNAs were retrieved from the miRTarBase. We found that miRNA- 133b had 30 targets; miRNA- 17 had 78 targets and miRNA-20a, 55 targets.

Figure 1 Hierarchal clustering of differentially expressed miRNAs in normal and tumor samples

The potential targets were then analyzed using DAVID (**D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery). The results indicated that the genes play a significant role in bladder cancer, pancreatic cancer, melanoma, glioma, hepatitis- B infection, chronic myeloid leukemia, HTLV-I infection, non-small cell lung cancer and small cell lung cancer. The role of the genes in prostate cancer found from DAVID included G1/S progression, cell proliferation, cell cycle progression, cell proliferation survival, apoptosis inhibition, cell survival and impaired G1 and G2 arrest reduced apoptosis genomic instability [Table 1].

GO annotation also revealed that many genes regulated by the 3 miRNAs were involved in key functions like tyrosine kinase receptor activity, serine/ theronine kinase activity, cytosine receptor binding, ATP binding etc.

DISCUSSION AND CONCLUSION

Management of prostate cancer is still a challenge. The use of PSA as a marker for detection of prostate cancer is not able to clearly determine the patients requiring surgical intervention. Hence, there is a need for the identification of an efficient non-invasive biomarker for determining the prognosis and severity of prostate cancer. miRNAs have been shown to regulate multiple genes involved in many cancers like head and neck squamous cell carcinoma, breast cancer etc. However the differential expression pattern of miRNAs in a large cohort study like TCGA has not been attempted yet. In the present study, we have analysed the miRNA expression patterns of normal (n = 498) and prostate tumor sample (n = 53) and identified 3 miRNAs (hsa-mir-133b, hsa-mir- 20a and hsa- mir-17) significantly associated with survival prognosis.

As miRNAs influence multiple genes in many pathways, we performed KEGG pathway analysis through DAVID and GO term analysis. It was found that many target genes of the short listed 3 miRNAs participated in tumorigenesis related pathways and were oncogenic [Table 1]. The key pathways included role in bladder cancer, pancreatic cancer, melanoma, glioma, hepatitis-B infection, chronic myeloid leukemia, HTLV-I infection, non-small cell lung cancer and Small cell lung cancer (121 genes, $p<0.05$).

Thus it was seen that the 3 miRNAs played a significant role in tumor progression. An extensive literature analysis was also done and the 3 miRNAs were the staging of tumor. Nevertheless, the miRNAs identified in the study have been proven to be differentially expressed at various stages of cancer and also as a discriminating biomarker between the normal and

Table 1. Role of the genes in prostrate cancer: p value -(7.9E-10)

ROLE OF GENES	GENES
G1/ S progression	E2F, RB
Cell proliferation	cyclin D1, p21,EGFR, AKT, PTEN
Cell cycle progression	p21, AKT, PTEN, EGFR
Cell proliferation survival	Ras, EGFR, PTEN
Apoptosis inhibition	EGFR, AKT, PTEN
Cell survival	BCL2, AKT, PTEN, EGFR
Impaired G1 and G2 arrest reduced apoptosis genomic instability	AKT, MDM2, PTEN, EGFR

reported to act as efficient biomarkers not only in the tumor samples but were differentially expressed in other body fluids like serum and plasma [5-9]. A major drawback which was encountered in the present study was that the number of patients with "alive" and "dead" vital status was not matching. The number of patients with vital status "dead" was only 10 as compared to the large number of "alive" patients. Hence few other miRNAs which might influence the survival prognosis would have been missed. Similarly, the TCGA cohort for prostate cancer also lacked information on tumor samples.

Hence further exploration of these miRNAs in future studies with clinical samples belonging to various stages of prostate cancer warrants its application as a diagnostic biomarker and a therapeutic strategy against prostate cancer.

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