Investigation for Maternal Uniparental Disomy of Chromosome 7 in a Silver Russell Syndrome patient

Research Project submitted to the Central University of Punjab

For the award of

Master of Science

Life Sciences with Specialization in Human Genetics

In

Department of Human Genetics and Molecular Medicine

By

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Supervisor

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Centre for Human Genetics and Molecular Medicine

School of Health Sciences

Central University of Punjab, Bathinda

May, 2018

Central University of Punjab, Bathinda

Declaration

I declare that all the changes suggested by the external examiner in the Research project entitled "**Investigation for maternal Uniparental disomy of chromosome 7 in a Silver Russell Syndrome patient**" Submitted by me for the award of degree of Master of life science with specialization in Human Genetics in the Department of Human Genetics and Molecular Medicine has been incorporated in the research project.

Swati Gupta

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Certificate

I declare that the research project entitled "**Investigation for maternal Uniparental disomy of chromosome 7 in a silver Russell Syndrome patient**" has been prepared by me under the guidance of Dr. Preeti Khetarpal, Assistant Professor, Department of Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab. No part of this research project has formed the basis for the award of any degree or fellowship previously.

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Date: May, 2018

Certificate

I certify that Swati Gupta has prepared her research project entitled "**Investigation for maternal Uniparental disomy of chromosome 7 in a Silver Russell Syndrome patient**", for the award of M.Sc., degree of the Central University of Punjab, under my guidance. She has carried out this work at the Department for Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab.

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Date: May, 2018

ABSTRACT

INVESTIGATION FOR MATERNAL UNIPARENTAL DISOMY OF CHROMOSOME 7 IN A SILVER RUSSELL SYNDROME PATIENT

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Key words: Maternal Uniparental Disomy of chromosome 7, Silver-Russell Syndrome, Differential methylated regions, Polyacrylamide Gel Electrophoresis (PAGE), locus specific primers.

Silver-Russell syndrome (SRS) is a genetically heterogeneous disorder. Individuals with SRS show clinical features with varying severity. The major criteria for clinical diagnosis of SRS are intrauterine growth retardation (IUGR) accompanied with post natal growth retardation (PNGR) and relative macrocephaly, triangular face, feeding difficulties, fifth finger clinodactyly. Maternal Uniparental disomy of chromosome 7 had been implicated in 10% of SRS cases; in about 1% cases, structural chromosomal aberrations has been reported and in about 45% cases, epimutation have been detected in Imprinting control region (ICR1) of 11p region. Aetiology of remaining cases unknown. To investigate Maternal Uniparental Disomy of chromosome 7 in a Silver Russell Syndrome patient. The sample was collected of female patient suggested of SRS, clinically diagnosed with relative macrocephaly, mild facial asymmetry with postnatal growth retardation. The present study had been undertaken with an objective to detect maternal Uniparental disomy of chromosome 7 using locus specific primers to amplify STR loci by PCR. PCR products were visualized on 7.5% native Polyacrylamide Gel Electrophoresis (PAGE). On analysing the gel, matUPD7 was ruled out. Patient recruited in this study was an isolated cases with no previous incidence in the family.

Swati Gupta Dr. Preeti Khetarpal

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Swati Gupta

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LIST OF ABBREVIATIONS

Sr. No.	Full form	Abbrevations
1.	Silver Russell Syndrome	SRS
2.	Russell Silver Syndrome	RSS
3.	Imprinting control regions	ICR
4.	Uniparental disomy of chromosome 7	UPD 7
5.	Maternal UPD	MatUPD
6.	Paternal UPD	patUPD
7.	Mesoderm specific transcript	MEST
8.	Paternally expressed gene10	PEG10
9.	Growth factor receptor bound protein 10	GRB10
10.	Polymerase chain reaction	PCR
11.	Polyacrylamide gel electrophoresis	PAGE
12.	Methylation specific PCR	MS-PCR
13.	Intrauterine growth retardation	IUGR
14.	Postnatal growth retardation	PNGR
15.	Forkhead box p2	FOXP2
16.	Short tandem repeats	STR
17.	Variable number of tandem repeats	VNTR
18.	Differentially methylated regions	DMR

Student approval form

Name of the author	Swati Gupta
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Year of award	2018

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1. Introduction

Silver Russell Syndrome (SRS) is a type of rare congenital complex developmental disorder, diagnosed on the basis of clinical features i.e. pre and post natal growth retardation, relative macrocephaly, triangular faces, body asymmetry and also feeding difficulties. Additional clinical features include 5th finger clinodactyly, genital abnormalities, brachydactyly, mesophalangy, toes syndactyly and inguinal hernia (Saal, 2011).

Silver Russell Syndrome was described independently by H.K. Silver in 1953 (Silver *et al*., 1953) and Dr A. Russell in 1954 (Russell, 1954). It is also known as Russell-Silver Syndrome (RSS). Silver *et al* [1953] describes a 5 year old girl and 6 year old boy with congenital hemihypertrophy, shortness of stature and elevated urinary gonadotrophin. The children didn't had triangular face but they had macrocephaly. In 1954, Russell reported 2 girls and 3 boys with dwarfism, cranio facial dysostosis and short arms ratio.

Around 35-50% of cases of SRS have epimutations in 11p15 region (Saal, 2011). This region contains two imprinting control regions (ICR) adjacent to each other which plays an important role in the expression of the genes that play important role during fetal growth (Begemann *et al*., 2011). Maternal Uniparental disomy of chromosome 7 had been implicated in 10% of SRS cases (Eggermann *et al*., 2010). In rest of the cases mutation/epimutation is yet to be elucidated (Hoffmann *et al*., 2011).

There is a considerable overlap in clinical features of these two molecular subtypes of SRS. Birth length and birth weight are more reduced with characterized body features i.e. fifth finger clinodactyly and congenital anomalies are more commonly observed with chromosome 11p15 related SRS syndrome whereas delayed psychomotor developments are more frequent in patients with chromosome 7 related SRS syndrome (Mackay *et al*., 2015). Triangular facial shape is more pronounced in maternal UPD7 of SRS (Kotzot *et al*, 2008). Several types of rare chromosomal aberrations have been identified in a few cases of SRS (Fuke *et al*, 2013). The prevalence of SRS is 1 in 3000 to 100,000 people worldwide (Abu-Amero *et al*., 2008). The condition is equally prevalent in both the sexes with most of the cases being sporadic (Escobar *et al*., 1978; Preece *et al*,1997).

Uniparental disomy (UPD) is the inheritance of two homologous chromosomes from the same parent either from mother (matUPD) or father (patUPD) to the child. Inheritance of both the chromosomes could be from one parental pair (heterodisomy, hUPD) or two copies of the same chromosome from one parent (isodisomy, iUPD). Isodisomy have the chance to cause homozygosity for recessive mutation diseases i.e. cystic fibrosis (Binder *et al*., 2011). Four possible mechanisms for causing UPD namely trisomy rescue, gamete complementation, monosomy rescue and post fertilization errors had been proposed (Lapunzina and Monk, 2011).

UPD of chromosome 7 may cause the interruption in gene regulation among SRS patient (Saal, 2011). Chromosome 7 is a metacentric chromosome which spans about 158 million base pairs and signifies more than 5 % of the total DNA in cells. Approximate 1900 protein coding genes are present on chromosome 7 (Scherer *et al*., 2003). Three imprinted loci or the candidate SRS gene on chromosome 7 are GRB10 at 7p12, PEG10 at 7q21 and MEST at 7q32 which had been linked to the syndrome (Nakabayashi *et al*., 2002). Mesoderm-specific transcript (MEST) gene is expressed in all major fetal organs and tissues. PEG10 is paternally expressed gene 10 which is known to be essential for the embryonic development. Growth factor receptor bound protein 10 (GRB10) is highly imprinted gene derived from paternal allele, encoding a cytoplasmic adapter protein which helps in growth of various tissues in the body (Abu-Amero *et al.,* 2008).

Different techniques had been employed to identify genetic/epigenetic mutation among SRS patient. MatUPD7 can be detected by employed STR analysis using locus specific PCR primers and PAGE. Methylation specific PCR (MS-PCR) is another technique which can be utilized for the same.

Diagnosis of SRS is mainly based on clinical features but heterogeneous clinical features poses a challenge for its correct diagnosis. Therefore, it is essential to genetically characterize the patients to provide appropriate genetic counselling to the parents and management of affected child. Further, different genetic cohorts of SRS respond differently to management strategies. The management of SRS may include growth hormone therapy and individualized therapy depending on the clinical features (Wakeling *et al*., 2017). Hence the current project case was undertaken with the following objective.

Objective:

To investigate maternal Uniparental disomy of chromosome 7(matUPD7) in a Silver Russell Syndrome (SRS) patient.

2. Review of literature

Uniparental disomy (UPD) is the unusual situation in which a person receives both chromosomes from one parent and another chromosome for that pair from other parent is missing (Shaffer *et al*., 2001).

UPD results in many abnormalities affecting growth and development in the affected individual through parent-of-origin differences in gene expression. UPD is unequally observed in all chromosomes and occurrence of maternal UPD is three times more frequent than paternal UPD (Kotzot *et al*, 2000)

SRS was firstly described by Silver *et al* in 1953 and Russell in 1954 independently. Since the genetic causes for SRS is heterogeneous, individuals with SRS show complex clinical features with varying severity. (Riesewijk *et al*., 1998).

2.1. Clinical features associated with SRS

The diagnosis of SRS and supportive laboratory testing is done for individuals who have **three major criteria** or **two major and two minor criteria** (Saal, 2011). The following criteria are compiled from the studies (Netchine *et al*, 2007; Eggermann *et al*, 2009; Wakeling *et al*.,2017 ; Bartholdi *et al*., 2009; Bruce *et al*, 2005; Kotzot *et al*, 2008).

- **The major criteria** for clinical diagnosis of SRS include IUGR and PNGR (intrauterine and postnatal growth retardation) below or equal to 2SD and triangular face, relative macrocephaly, asymmetry (limb, body or facial asymmetry), feeding difficulties, fifth finger clino or brachydactyly.
- **The minor criteria** are short arm span with normal upper to lower segment ratio, frontal bossing, motor delay, down-turned mouth corners, ear anomalies, teeth anomalies, and micrognathia, syndactyly of toes and squeaky voice, speech difficulties.
- **Supportive criteria** includes genitourinary anomalies (cryptorchidism), muscular hypotonia, hypoglycaemia and genital anomalies, high arched palate, pointed chin, café au lait spots or skin pigmentary changes, excessive sweating, prominent heels.

2.2. Genetic and epigenetic causes of SRS

Various genetic, epigenetic changes and chromosomal aberrations which are reported in association with SRS had been compiled in Table 2.1.

Table 2.1: Various genetic, epigenetic changes and chromosomal aberrations which are reported in association with SRS.

Sr. No	SRS subtype	Genetic Mechanism	Proportion of SRS attributed to this Genetic Mechanism	References
1	Epimutation in 11p15 region	Loss of ICR1 methylation of paternal 11p15.5	~35%- 50%	Eggermann *et al*, 2012, Gicquel *et al*, 2005, Demars *et al*, 2011, Beggermann *et al*, 2011, Hoffmann *et al*,2011
2	matUPD7 related SRS	Maternal UPD	~7%- 10%	Eggermann *et al*., 2012; Kotzot *et al*., 1995; Kotzot *et al*.,2000; Amero *et al*., 2008; Hoffmann *et al*,2011
3	Chromosomal aberrations related to SRS	Deletion/Duplication of Chr. 7,11	~1%- 2%	Hitchins *et al*., 2001 Binder *et al*., 2011
4.	Unknown	Unknown cause	~40%	Eggermann *et al*., 2012; Hoffmann *et al*.,2011

Chromosome 7 has been studied as a candidate locus when matUPD(7) shows phenotypic effect in patients with IUGR and PNGR. Most genes shows biallelic expression but imprinted genes are only active from one parental allele (Abu-Amero *et al*, 2008).

Different DMRs had been identified on chromosome 7 with specific locus are MAD1L1 (7p22), CARD11 (7p22), intergenic (7p21.2), RPS2P32 (7P15.3), HOXA4 (7p15.2), PRR15 (7p14.3), GLI3 (7p13), GRB10 (7p12.2), SGCE/ PEG10 (7q21.3), PON1 (7q21.3), SH2B2 (7q22), MEST (7q32), SVOPL (7q34), HTR5A (7q36.1), RARRES2 (7q36.1), Intergenic (7q36.2) [Jouppi *et al*.,2014].

Imprinting defects in GRB10, PEG10, MEST and FOXP2 have been suspected to cause SRS like phenotype. Mesoderm-specific transcript (MEST) gene is expressed in all major fetal organs and tissues. Growth factor receptor bound protein 10 (GRB10) encodes a cytoplasmic adapter protein which modulates coupling of a number of cell surface receptor tyrosine kinases with specific signalling pathways which helps in the growth of various tissues in the body. FOXP2 (forkhead box P2) gene is paternally expressed and responsible for development of verbal dysplaxia in many matUPD (7) cases [Feuk *et al*., 2006].

There are some Imprinted genes found in the region of MEST includes transcript of paternally expressed introne from the biallelically expressed gamma-2 COP (nonclathrincoatprotein) (COPG21T1), paternally expressed MEST1T1 (i.e PEG1-AS) and maternally expressed carboxypeptidase A4 (CPA4) [Abu-Amero *et al*., 2008].

Fuke *et al* (2013) examined 138 Japanese patients (66 males and 72 females) with SRS and identified H19-DMR epimutation in 43 cases. The patients of this subgroup had more number of reduced birth length and birth weight with more frequently occurring of body asymmetry and brachydactyly.

Epigenetics mechanisms of genomic imprinting includes various mechanisms namely, non-coding RNAs, Micro-imprinted domains and retro transposed genes, histone modification and chromatin modelling (Barlow and Bartolomei, 2014). Many studies had reported epigenetic changes in 11p15 region among SRS patient.

Kotzot *et al* (1995) examined thirty five patients with PNGR, and their parents with PCR markers for UPD7. Out of which four patients had maternal disomy, three had isodisomy and one had heterodisomy. In another study carried out by Eggermann *et al*., (1997), out of 37 patients three had maternal UPD case.

28 patients were found to be carrier of an IGF2/H19 hypomethylation in 11p15 and 5 showed UPD (7) mat and 1 with segmental UPD in 188 routine samples of SRS patients (Eggermann *et al*, 2009). Spengler *et al* (2010) identified maternal UPD7 in 2 patients and ICR1 at 11p15 in 1 patients.

2.3. Uniparental disomy and Silver Russell Syndrome

2.3.1 Genetic cause for Uniparental disomy

Mechanisms leading to UPD include (Lapunzina and Monk, 2011)

a) **Nullisomic gamete complementation:** Fertilization between a disomic germ cell from one parent and a nullisomic germ cell from other parent creates disomic zygote with UPD.

b) **Trisomic rescue:** Post zygotic loss of a homologue chromosomes from a fertilized ovum containing three copies of a chromosome to bring to a normal diploid chromosome, and the result lead to the formation of UPD.

c) **Monosomy rescue:** Fertilization between a normal haploid germ cell from one parent and a nullisomic germ cell from other parent produces monosomy zygote duplication of such chromosome may result in isodisomy UPD.

d) **Non-disjunction** during early mitotic division can result in Uniparental disomy.

2.3.2 Consequences of Uniparental disomy

For many chromosomes, parent-specific imprinting exists and UPD of these chromosomes leads to clinically recognizable syndrome. Five chromosomes have been shown to have a phenotypic effect due to Uniparental inheritance of imprinted regions of maternally derived chromosomes 7, 14, and 15 and paternally derived chromosomes 6, 11, 14 and 15 (Shaffer *et al*.,2001**).** It is currently unclear for some chromosomes, if there are any other phenotypic effects due to imprinting. In humans, almost all chromosomes have been implicated in UPD with the exception of chromosome 3, 12, 18, and 19 (Kotzot *et al*, 1995). FOXP2 expressed paternally might be responsible for developmental verbal dyspraxia in some mUPD7 patients (Feuk *et al*., 2006).

2.3.3 Uniparental disomy of chromosome 7 and Silver Russell syndrome

The concept of UPD was first suggested by Engel in 1980. UPD can be distinguished in two types Uniparental hetrodisomy and Uniparental isodisomy i.e. presence of two different homologous chromosome from the same parent or presence of two copies of identical chromosomes respectively (Eggermann *et al*, 2012).

Eggermann *et al*, (1997) screened 37 SRS families to determine the role of UPD in the etiology of SRS. It was reported that one case is maternal isodisomy, one case of maternal hetrodisomy and one case was showing partial isodisomy for 3STR markers.

UPD7 can be detected via polymorphic short tandem repeat (STR) markers by PCR or variable number of tandem repeat (VNTRs) by Southern blotting. A maternally imprinted gene PEG1/MEST was mapped on human chromosome region 7q32 which expresses paternally but biparental expression is seen in blood lymphocytes (Riesewijk *et al*, 1998).

The key candidate region on chromosome 7 that may be the causative factor in SRS are 7q32 having several imprinted genes such as PEG1/MEST DMR (paternally expressed), COPG2 (biallelically expressed) and CPA4(maternally expressed) and imprinted non coding RNAs(MESTIT, CIT1/COPG2IT1) (Eggermann *et al*, 2010). No mutation had been observed for CPA4 in 10 SRS patient but a girl born with assisted reproductive technology (ART) was observed with partial hypomethylation of MEST DMR (Eggermann *et al*, 2012).

Other candidate region is 7p11.2-p13 which harbours at least 1 imprinted gene (GRB10) playing an essential role in growth. Growth restriction is observed by over expression of GRB10 imprinted gene (Hitchins *et al*, 2002).

Joyce *et al* (1999) reported an interstitial inverted duplication in proximal 7p region of a mother and daughter both had same clinical features like SRS including short stature, low birth weight, facial asymmetry and also fifth finger clinodactyly.

2.4 Techniques to detect Uniparental Disomy

Routine cytogenetic analysis cannot identify UPD. UPD can be detected by genotyping with highly polymorphic microsatellite markers. The technique is fast and feasible with PCR (polymerase chain reaction) and PAGE (Polyacrylamide gel electrophoresis) [Kotzot *et al*, 1995]

Another method is **Methylation specific PCR (MS-PCR)** a rapid, qualitative and very sensitive technique. Following bisulphite conversion, PCR is performed using two sets of primers, which are designed to amplify either methylated or unmethylated alleles (Shen and Waterland, 2007) (Hoffmann *et al*, 2011).

It has advantage of being highly sensitive i.e. detects one methylated allele in a 1000 unmethylated allele. It can also be used on limited quality and quantity of DNA samples. There is no need of parent samples.

3. Material and methods

3.1 Subject

Blood sample was collected from the subject with suggestive clinical features of SRS. Blood sample of the parents with normal phenotype was also collected. Ethical clearance was obtained for the study from Institutional Ethics Committee (IEC), Central University of Punjab, Bathinda vide reference number CUPB/HSB/15/PD/20.

The overall methodology of the present study has been given in Figure 3.1

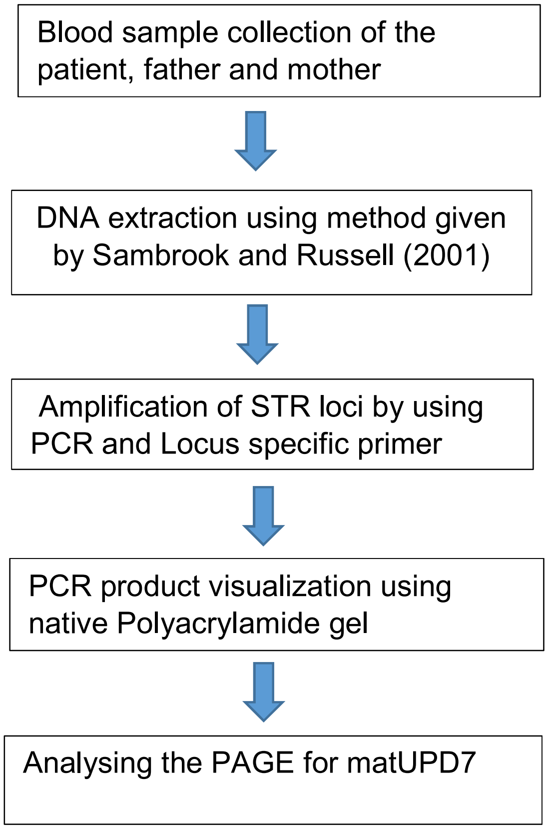

Fig.3.1: Overall work-flow of the study

3.2 Blood Sample collection

About 2 ml of blood sample was collected from both parents and a female patient (suspected case with SRS phenotype, aged 14 years) in EDTA sterile conditions (EDTA vial).

3.3 DNA Extraction

DNA was extracted from peripheral blood by Sambrook and Russell method (2001) and deproteinization was done with organic extraction as proposed by Adeli and Ogbonna (1990) with slight modifications.

i. The frozen blood sample in the vacutainer (EDTA vial) was thawed and kept at room temperature.
ii. 3ml of Lysis buffer 1 (1X) was added to 1ml of blood sample in an 15ml autoclaved centrifuge tubes.
iii. The sample was vigorously shaken and kept in ice for 15 minutes, followed by centrifugation of the tubes at 3000rpm for 15 minutes.
iv. Discard the supernatant.
v. Repeat the step ii, iii and iv thrice until a white pellet was obtained.
vi. By doing vortexing, add 1 ml of Lysis buffer-II, 56µl of 10% SDS and proteinase K with concentration of 25µl carefully to the pellet of the sample.
vii. Sample was incubated for 3 hours at 60ºC in water bath or keep the sample for overnight at 37ºC.
viii. Recover the sample from water bath and kept it at room temperature.
ix. PCA (25:24:1) was added and gently mixed for 15minutes till the emulsion formation. Now centrifuge it at 2500 rpm for 15minutes at room temperature (28ºC). Supernatant was transferred to the fresh and autoclaved polyprophylene tube.
x. CA (24:1) was added followed by gently mixing for 15-20 minutes. Centrifuge it at 2500 rpm for 15 minutes at room temperature (28ºC). Transfer the supernatant to the fresh and autoclaved polyprophylene tube.
xi. Repeat the step x for once.
xii. DNA precipitation was done by adding sodium acetate and chilled ethanol.by slow mixing DNA precipitated out. Keep it at -20ºC for complete precipitation.
xiii. DNA was transferred to the eppendorf tube along with 70% ethanol. Centrifuge at 1000 rpm for 12 minutes. Discard the supernatant and repeat step xiii.
xiv. DNA precipitate was allowed to air dry to remove traces of ethanol. Add 60 µl -80 µl of TE buffer for dissolution of DNA by keeping it at 37ºC in water bath for 3 hours.

3.3.1 DNA quality assessment

The quality assessment was done by Agarose gel electrophoresis after the dissolution of DNA in TE buffer (0.8% gel) [Fig 3.2]

i. Add 200mg of Agarose in 25ml of TAE buffer in a flask.
ii. Heat the flask for 1min in the oven till the Agarose dissolve. Cool it.
iii. Add 2 μl of Ethidium bromide to the solution.
iv. After proper mixing, pour the Agarose in casting tray. Set the comb and allow it to cool for 15-20 minutes.
v. After setting of the gel 2 μl of DNA sample was loaded along with 2 μl of loading dye in each well and run at 80 V for 30 minutes.

3.3.2 Quantification of DNA

Quantification of DNA was done by using Qubit fluorometer.

i. The qubit was calibrated by standard 1 and standard 2 solution provided in the kit.
ii. A mixture with 199 μl buffer and 1 μl dye was prepared to quantify DNA.
iii. In a sample tube containing 1μl of sample DNA, 199 μl of this mixture was added and centrifuge the tube for proper mixing of dye and DNA.
iv. DNA sample was quantified and the readings were recorded by clicking on read button displayed on qubit screen.

3.4 STR analysis

3.4.1 PCR amplification

Amplification of DNA was done using locus specific STR (Short Tandem repeats) primers. It used as genetic marker which is present abundant throughout the genome. Summary of primers used, PCR reaction mixture and conditions are given in Table 3.1, 3.2 and 3.3 respectively.

i. Reaction mixture was prepared of 50μl for each sample using Taq buffer, water, both primers (forward and reverse primers), dNTPs with Taq polymerase and sample DNA.
ii. Load the samples 2μl of PCR product along with 2μl loading dye in the wells of Agarose gel carefully.

iii. PCR products were analysed on 1.5 % agarose gel run at 80 V for 30 minutes.

Table3.1: The type of STR primer used with their respective annealing temperature and product size (Fuke *et al.*, 2013).

Sr. No.	STR	Primer Sequence	Annealing Temperature (aºC)	Product size (approximate)
1.	D7S531	F-AAACTGTGGTCCTGGCTG R-AAACTAGAGTCCTGGCCTGA	59	240-242
2.	D7S484	F- TGCGTTTGATCGAAGCAGA R-GCTGAGCAAGGCATTGTTT	61	98-100
3.	D7S519	F-ACAGCCAAGCATTTCTGCTG R-ACAGACCAGGACTCAGCCAG	63	255-257
4.	D7S672	F-ACATGAAGGTCTACCAGTAGCC R-CAATTTGGTTGGAGCAAGG	60.3	137-139
5.	D7S684	F-GCTTGCAGTGAGCCGAC R-GATGTTGATGTAAGACTTTCCAGCC	58	178-180
6.	D7S550	F-TCTCATCTGTGAATGCACTATC R-GCAGTTGGGTTATTTCAAGTC	57	192-194

Table3.2: PCR reaction mixture (10 µl)

Sr.no.	Reagents	Concentration	1X	2x	3X
1.	Taq Buffer	10X	1 µl	2 µl	3 µl
2.	dNTP’s	2.5Mm	0.75 µl	1.50 µl	2.25 µl
3.	Forward primer	10X	0.25 µl	0.50 µl	0.75 µl
4.	Reverse primer	10X	0.25 µl	0.50 µl	0.75 µl
5.	Distilled Water	-	6.75 µl	13.50 µl	20.25 µl
6.	Taq polymerase	1/µl	1 µl	2 µl	3 µl
7.	DNA template		0.5 µl	-	-

Table 3.3.: Polymerase chain reaction conditions

Sr. No.	PCR condition	Time	Temperature
1.	Initial Denaturation(Preheating)	5 min	94ºC
2.	Denaturation	45 sec	94ºC
3.	Annealing	30 sec	a ºC
4.	Extension	30 sec	72 ºC
5.	Final extension	7 min	72 ºC

Steps 2 to 4 were repeated for 32 times (32 cycles)

3.4.2 Native Polyacrylamide Gel Electrophoresis

Diameter of the pores can be adjusted by the concentration of acrylamide and bisacrylamide based on the DNA fragments size. It varies from base pairs to base pairs depending upon their size. For better separation of the DNA with >200bp and <200bp the concentration of acrylamide monomer used is 7.5% and 10% respectively. The polyacrylamide gel was prepared using reagents described in table below:

Table3.4: Volume of reagents used to cast polyacrylamide gel.

Sr. No.	Reagents	For 7.5% Gel	For 10% Gel
1	30% acrylamide	2.5ml	3.33ml
2	Water	3.5ml	2.67ml
3	5X TBE	2ml	2ml
4	50% Glycerol	2ml	2ml
5	10% APS	200µl	200 µl
6	TEMED	15 µl	15 µl

i. 7.5% polyacrylamide gel was prepared depending upon the size amplified products and let undisturbed for 60 minutes to polymerize.

ii. The electrophoresis apparatus was assembled and cleaned with 70% ethanol

iii. The gel was allowed to fix on casting cassette by binder clips for pre run of 30 minutes at 60V.

iv. Sample (5 µl of PCR product along with 5 µl loading dye) was loaded and run at 60V for 6-7 hours.

4. Results

4.1. Demographic and clinical features of the subject

Blood sample was collected from both parents and 14 year, female patient suggested of SRS. Relative macrocephaly, mild facial asymmetry with postnatal growth retardation was observed.

Table 4.1: Blood sample collection from patient, mother and father

Sr. no.	Subject	Age (years)	DNA quantity (ng/ µl)
1	Patient (female)	14	158.2
2	Mother	34	140
3	Father	38	358.5

4.2. Checking of the subject for SRS with matUPD7 under the study

Extracted DNA was quantified (Table 4.1) and its quality was analyzed on 0.8% Agarose gel (Fig. 4.1). In order to detect Uniparental disomy of chromosome 7, locus specific primers were used to amplify STR loci. PCR products were visualized on 7.5% native Polyacrylamide Gel Electrophoresis (PAGE). On analysing the gel, matUPD7 was ruled out.

FIGURES

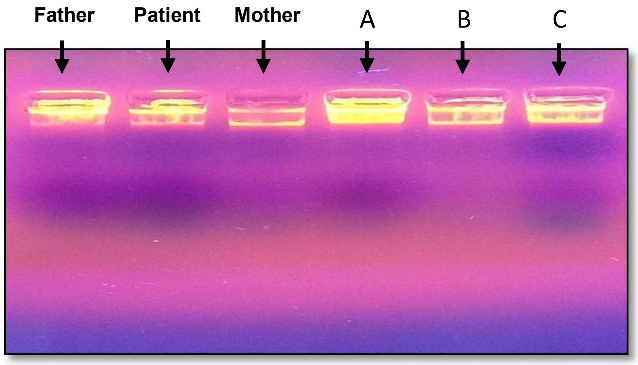

Fig 3.2: Quality assessment of extracted DNA using Agarose gel electrophoresis (0.8%) (From left) Father (F), Patient (P) and Mother (M). A, B and C are not the part of the study.

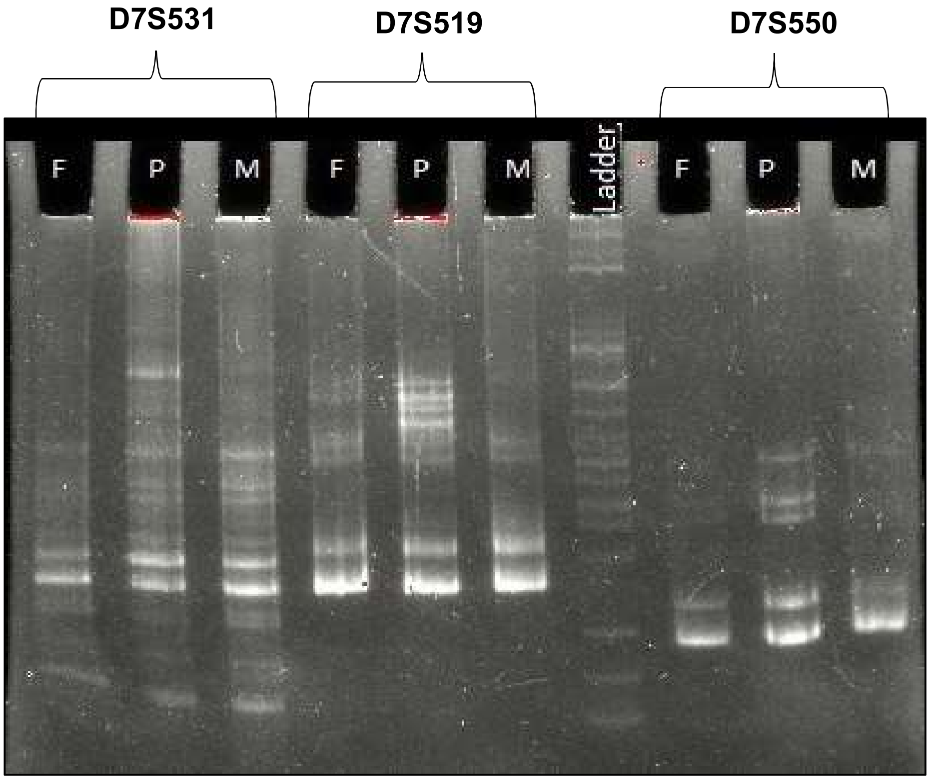

Fig 4.1: Polyacrylamide gel (7.5%) showing the bands for matUPD7 for STR primers of D7S531, D7S519, D7S550.

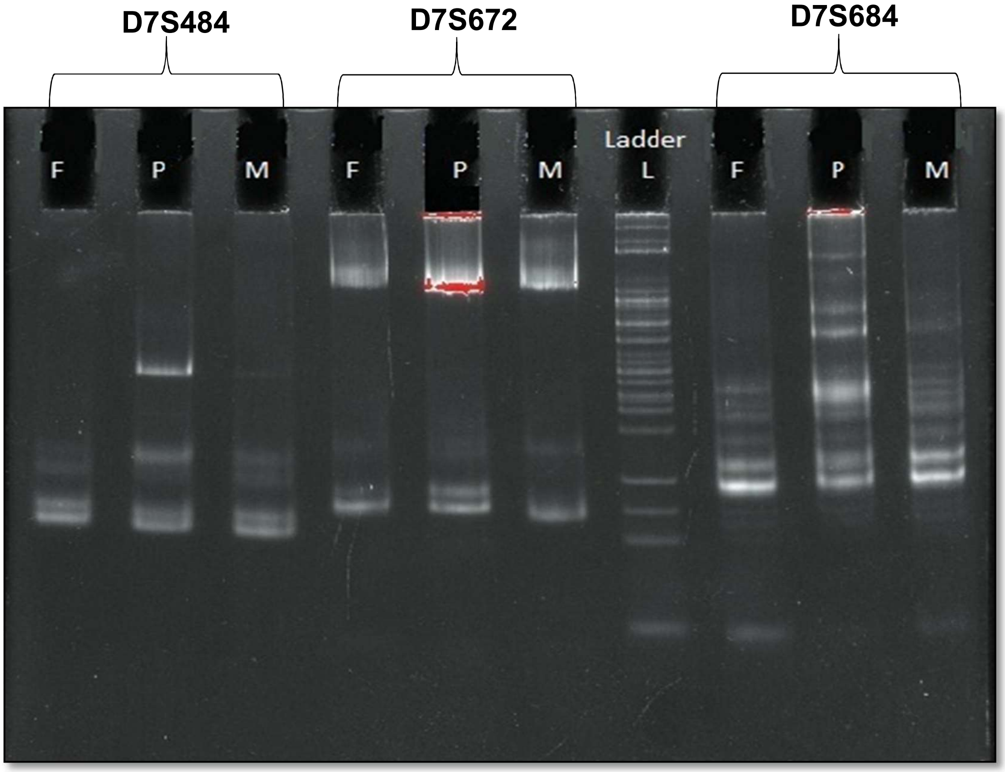

Fig 4.2: Polyacrylamide gel (7.5%) showing the bands for matUPD7 for STR primers of D7S484, D7S672, D7S684.

5. Discussion

5.1 Demographic and clinical features of the SRS subjects

Clinical diagnosis of SRS is mainly based on IUGR and PNGR. If a child have height less than third percentile, then it fulfils one major criteria for the diagnosis of SRS (Saal, 2011; Preece *et al*., 1999). It has been observed that matUPD7 is associated with mild SRS. SRS patients having learning and speech difficulties were likely to be associated with mUPD7 (Abu- Amero *et al*., 2008) and ICR1 hypomethylation was related with classical SRS, asymmetry, fifth finger clinodactyly and congenital anomalies (Wakeling *et al*., 2010).

5.2 Genetic investigation of SRS subject

In our study we investigated the suspected case of SRS of matUPD (7) by native polyacrylamide gel electrophoresis using locus specific primers. As only 10% of SRS is associated with matUPD7. However, It was reported in 5% (Netchine *et al*., 2007), 7% (Preece *et al*., 1999, Monk *et al*, 2000), 10% (Joyce *et al*., 1999; Eggermann *et al*., 1997) of cases. The subject had post natal growth retardation (PNGR) and mild facial asymmetry.

5.3 Conclusions

Patient recruited in this study was an isolated case with no previous incidence in the family.

5.4 Future perspectives

Investigations for epimutation in chromosome 11 at position p15.5 associated with SRS. Investigations for the duplication of 11p15 region which is implicated in1-2% of SRS. Molecularly confirmed cases of SRS may be helpful in management of the individuals with SRS and it might be helpful in genetic counselling to the parents of the affected child and also generating therapeutics in future.

SUMMARY

Silver-Russell syndrome (SRS) is a complex disorder. SRS individuals are diagnosed on the basis of clinical features identified. The major criteria for clinical diagnosis of SRS are intrauterine growth retardation (IUGR) accompanied with post natal growth retardation (PNGR) and relative macrocephaly, triangular face, feeding difficulties, fifth finger clinodactyly with other minor and supportive criteria. Maternal Uniparental disomy of chromosome 7 had been implicated in 10% of SRS cases. Investigation was done under my study was to detect maternal Uniparental disomy of chromosome 7(matUPD7) by using locus specific primers to amplify STR loci by PCR. The sample was collected from female patient suggested with SRS, clinically diagnosed with relative macrocephaly, mild facial asymmetry with postnatal growth retardation. Further, PCR products were visualized on 7.5% native Polyacrylamide Gel Electrophoresis (PAGE). The technique used in my study was fast and feasible. On analysing the native PAGE gel, matUPD7 was ruled out. Another method can also be used Methylation specific PCR (MS-PCR) which is also a rapid, qualitative and very sensitive technique. The patient suggested of SRS might have epimutation and duplication of chromosome 11p15 region associated with around 50% and 1-2% of SRS causes respectively.

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Appendix A

CENTRE FOR HUMAN GENETICS

SCHOOL OF HEALTH SCIENCES

CENTRAL UNIVERSITY OF PUNJAB, BATHINDA

CONSENT FORM

I have been explained the possible risks and benefits and have understood the purpose for which blood sample from me/my children is being sought by the centre for Human Genetics, School of Health Sciences, Central University of Punjab, Bathinda.

I am free from any pressure whatsoever and herby give my own consent/consent of my children (who are under 18 years of age today) to: (i) withdrawal of sample of about ....ml blood by veni-puncture; and (ii) to all types of analysis of my blood for non-profit research purposes for acquisition of knowledge for the benefit of mankind by Centre for Human Genetics and molecular medicine or their collaborators.

I will have the right to know the analyzed results for my sample (samples) and I am not giving my consent for disclosure of any personal information either direct or derived from the analysis of my sample (samples) to any one without my further consent.

I hereby give permission to the investigators to release the information obtained from me as result of participation in this study to the sponsors, regulatory authorities, Government agencies, and ethics committee. I understand that they may inspect my original records.

I am aware of the fact that I can opt out of the study at any time without having to give any reason and this will not affect my future treatment in the hospital. I am also aware that the investigators may terminate my participation in the study at any time, for any reason, without my consent.

I have been informed that my consent will be sought prior to any for-profit (including filing of patents) that may be taken by the Centre of Health Sciences or their collaborators on the basis of my blood sample.

Date:

Name: Sex: Age (Yrs):

Address:

Investigator Certificate

I certify that all the elements including the nature, purpose and possible risks of the above study as described in this consent document have been fully explained to the subject. In my judgment, the participant possesses the legal capacity to give informed consent to participate in this research and is voluntarily and knowingly giving informed consent to participate.

Signature and Name of the Investigator: ________________ Dated: _________

भाग :II

__________________________________ ,पंजाब केन्द्रीय विश्वविद्यालय द्वारा जिस उद्देश्य से मुझ से दी कर व्याख्या मुझे की लाभ एवं जोखिम संभावित उसके हैं रहे जा मांगे नमूने के रक्त से बच्चों मेरे/ हूँ। चुका समझ भलीभांति उसे मैं और है गई

मैं मानव आनुवंशिकी केंद्र या इसके सहयोगियों द्वारा वेनी (i)पंचर द्वारा विधि (Venipuncture) बच्चों मेरे/मेराका लगभग .ली.मि .... रक्त निकालने लिए के ज्ञानार्जन में हित के मानवता (ii) और ; आयु जिनकी) बच्चों मेरे / मेरी स्वयं की करने विश्लेषण के प्रकार सभी के रक्त मेरे से उद्देश्य के अनुसन्धान आज 18 वर्ष से कम है है। नहीं दबाव कोई का प्रकार किसी पर मुझ और ;हूँ देती/देता सहमती की (

मुझे अपने नमूने नमूने अपने मैं और होगा अधिकार का जानने को परिणामों विश्लेषित से (नमूनों) (नमूनों)के विश्लेषण से प्रत्यक्ष या इससे उत्पन्न कोई भी सूचना आगे की सहमती के बिना किसी को भी प्रकट करने की सहमती नहीं दे रहा हूँ।

मैं एतदद्वारा इस अध्ययन में भाग लेने के परिणामस्वरूप मुझ से प्राप्त सूचना ,आयोजकों को (ओं) को अनुसंधानकर्ताओं की करने प्रकट को समिति नीतिशास्त्र तथा एजेंसियों सरकारी ,प्राधिकरणों नियामक भलीभांत मैं हूँ। देता अनुमतिि समझता हूँ कि वे मेरे मूल अभिलेखों का निरीक्षण कर सकते हैं।

मैं जानता हूँ कि मैं इस अध्ययन से किसी भी समय बिना कोई कारण बताए अपनी सहभागिता समाप्त कर सकता हूँ कि हूँ जानता भी यह मैं होंगे। नहीं प्रभावित ईलाज वाले होने में भविष्य मेरे इससे और ; अनुसंधानकर्ता किसी भी समय किसी भी कारण से मेरी सहमती के बिना इस अध्ययन में मेरी सहभागिता समाप्त कर सकते हैं।

मुझे सूचित किया जा चुका है कि ______________________ केंद्र या इसके सहयोगियों द्वारा मेरे रक्त नमूने के आधार पर किसी भी लाभ लिए के (सहित करवाने पंजीकृत पेटेंट) मेरी पूर्व सहमति ली जाएगी।

तिथि________________ :

नाम_________________ :लिंग ________________________ : आयु_______________ :(मे वर्षों)

पता :

____

अनुसंधानकर्ता प्रमाणपत्र-

मैं प्रमाणित करता हूँ कि उपरोक्त अध्ययन की प्रकृति संबंधित व्याख्या की जोखिम संभावित और उद्देश्य , लिए के करने प्रदान सहमति सूचित की सहभागिता में अनुसन्धान इस में राय मेरी है। गई दी कर को व्यक्ति पूर्ण और से रूप स्वैच्छिक वह और है रखता क्षमता कानूनी सहभागीज्ञान के साथ इसमें भाग लेने की सूचित सहमति दे रहा है।

अनुसंधानकर्ता के हस्ताक्षर एवं नाम__________________________ : :दिनांक ________________

ਭਾਗ ਦੂਸਰਾ:

ਮੈਨੂੰ ਸੰਭਾਵਤ ਖਤਰਿਆਂ ਅਤੇ ਫਾਇਦਿਆਂ ਤੋਂ ਜਾਣੂ ਕਰਵਾ ਦਿੱਤਾ ਗਿਆ ਹੈ ਅਤੇ ਮੈਂ ------------------------------------ ਕੇਂਦਰ, ਪੰਜਾਬ ਕੇਂਦਰੀ ਯੂਨੀਵਰਸਿਟੀ ਵਲੋਂ ਮੇਰੇ ਜਾਂ ਮੇਰੇ ਬੱਚਿਆਂ ਦੇ ਖ਼ੂਨ ਦੇ ਨਮੂਨੇ ਲਏ ਜਾਣ ਦਾ ਮੰਤਵ ਵੀ ਸਮਝ ਲਿਆ ਹੈ।

ਮੇਰੇ ਉਪਰ ਕਿਸੇ ਵੀ ਤਰ੍ਹਾਂ ਦਾ ਕੋਈ ਦਬਾਅ ਨਹੀਂ ਹੈ ਅਤੇ ਮੈਂ 1. ਨਾੜੀ ਵਿਚੋਂ ____ ਮਿਲੀਲੀਟਰ ਖ਼ੂਨ ਦਾ ਨਮੂਨਾ ਲੈਣ ਲਈ, ਅਤੇ 2. ------------------------------------ ਕੇਂਦਰ ਜਾਂ ਉਸਦੇ ਸਾਂਝੀਵਾਲਾਂ ਵਲੋਂ ਮਾਨਵਤਾ ਦੀ ਭਲਾਈ ਲਈ ਗਿਆਨ ਪ੍ਰਾਪਤੀ ਹਿੱਤ ਮੇਰੇ ਖ਼ੂਨ ਦੇ ਨਮੂਨੇ ਦੇ ਹਰ ਤਰ੍ਹਾਂ ਦੇ ਗੈਰ-ਮੁਨਾਫਾਕ੍ਰਿਤ ਖੋਜ ਅਧਿਐਨ ਲਈ ਆਪਣੀ ਸਹਿਮਤੀ/ਆਪਣੇ ਬੱਚਿਆਂ (ਜਿੰਨ੍ਹਾਂ ਦੀ ਉਮਰ ਅੱਜ ਦੇ ਦਿਨ 18 ਸਾਲ ਤੋਂ ਘੱਟ ਹੈ) ਦੀ ਸਹਿਮਤੀ ਦਿੰਦਾ ਹਾਂ।

ਮੈਨੂੰ ਆਪਣੇ ਨਮੂਨੇ (ਨਮੂਨਿਆਂ) ਦੇ ਅਧਿਐਨ ਦੇ ਨਤੀਜੇ ਜਾਣਨ ਦਾ ਹੱਕ ਹੋਵੇਗਾ ਅਤੇ ਮੈਂ ਅਗਲੇਰੀ ਅਗਾਊਂ ਸਹਿਮਤੀ ਹਾਸਲ ਕੀਤੇ ਬਗੈਰ ਸਿੱਧੀ ਜਾਂ ਨਮੂਨੇ (ਨਮੂਨਿਆਂ) ਦੇ ਅਧਿਐਨ ਰਾਹੀਂ ਹਾਸਲ ਕੀਤੀ ਗਈ ਕੋਈ ਵੀ ਨਿੱਜੀ ਜਾਣਕਾਰੀ ਜਨਤਕ ਕਰਨ ਦੀ ਸਹਿਮਤੀ ਨਹੀਂ ਦੇ ਰਿਹਾ।

ਮੈਂ ਖੋਜ ਕਰਤਾਵਾਂ ਨੂੰ ਇਸ ਅਧਿਐਨ ਵਿਚ ਮੇਰੀ ਸ਼ਮੂਲੀਅਤ ਸਦਕਾ ਮੇਰੇ ਤੋਂ ਪ੍ਰਾਪਤ ਜਾਣਕਾਰੀ ਸਪਾਂਸਰਾਂ, ਨਿਯਮਕ ਸੰਸਥਾਵਾਂ, ਸਰਕਾਰੀ ਏਜੰਸੀਆਂ ਅਤੇ ਸਦਾਚਾਰ ਕਮੇਟੀ ਨਾਲ ਸਾਂਝਾ ਕਰਨ ਦੀ ਇਜ਼ਾਜਤ ਦਿੰਦਾ ਹਾਂ। ਮੈਨੂੰ ਪਤਾ ਹੈ ਕਿ ਉਹ ਮੇਰੇ ਅਸਲੀ ਦਸਤਾਵੇਜ਼ਾਂ ਦੀ ਜਾਂਚ ਕਰ ਸਕਦੇ ਹਨ।

ਮੈਂ ਇਸ ਤੱਥ ਤੋਂ ਜਾਣੂ ਹਾਂ ਕਿ ਮੈਂ ਕਿਸੇ ਵੀ ਸਮੇਂ ਬਿਨ੍ਹਾਂ ਕੋਈ ਕਾਰਨ ਦੱਸੇ ਇਸ ਅਧਿਐਨ ਕਾਰਜ ਨੂੰ ਛੱਡ ਸਕਦਾ ਹਾਂ ਅਤੇ ਹਸਪਤਾਲ ਵਿਚ ਹੋਣ ਵਾਲੇ ਮੇਰੇ ਸੰਭਾਵੀ ਇਲਾਜ ਤੇ ਇਸਦਾ ਕੋਈ ਅਸਰ ਨਹੀਂ ਪਵੇਗਾ। ਮੈਨੂੰ ਇਹ ਵੀ ਜਾਣਕਾਰੀ ਹੈ ਕਿ ਖੋਜ ਕਰਤਾ ਕਿਸੇ ਵੀ ਸਮੇਂ, ਬਿਨ੍ਹਾਂ ਕੋਈ ਕਾਰਨ ਦੱਸੇ ਅਤੇ ਮੇਰੀ ਸਹਿਮਤੀ ਬਗੈਰ ਇਸ ਅਧਿਐਨ ਕਾਰਜ ਵਿਚ ਮੇਰੀ ਸ਼ਮੂਲੀਅਤ ਖਤਮ ਕਰ ਸਕਦੇ ਹਨ।

ਮੈਨੂੰ ਜਾਣਕਾਰੀ ਦੇ ਦਿੱਤੀ ਗਈ ਹੈ ਕਿ ਮੇਰੇ ਖ਼ੂਨ ਦੇ ਨਮੂਨੇ ਦੇ ਅਧਾਰ ਤੇ ਕਿਸੇ ਵੀ ਲਾਭ ਵਾਲੇ ਕਾਰਜ (ਪੇਟੇਂਟ ਫਾਈਲ ਕਰਨ ਸਹਿਤ) ਨੂੰ ਕਰਨ ਤੋਂ ਪਹਿਲਾਂ -- ਕੇਂਦਰ ਜਾਂ ਉਸਦੇ ਸਾਂਝੀਵਾਲਾਂ ਵਲੋਂ ਮੇਰੀ ਸਹਿਮਤੀ ਹਾਸਲ ਕੀਤੀ ਜਾਵੇਗੀ।

ਮਿਤੀ:
ਨਾਮ: ਲਿੰਗ: ਉਮਰ (ਸਾਲ):
ਪਤਾ:

ਖੋਜਕਰਤਾ ਵਲੋਂ ਪ੍ਰਮਾਣ-ਪੱਤਰ

ਮੈਂ ਇਹ ਪ੍ਰਮਾਣਿਤ ਕਰਦਾ ਹਾਂ ਕਿ ਇਸ ਸਹਿਮਤੀ ਪੱਤਰ ਵਿਚ ਦਰਜ ਉਪਰੋਕਤ ਅਧਿਐਨ ਕਾਰਜ ਨਾਲ ਜੁੜੇ ਹੋਏ ਲੱਛਣਾਂ, ਮੰਤਵਾਂ ਅਤੇ ਸੰਭਾਵਿਤ ਖਤਰਿਆਂ ਸਮੇਤ ਸਾਰੇ ਤੱਥਾਂ ਤੋਂ ਭਾਗੀ ਨੂੰ ਜਾਣੂ ਕਰਵਾ ਦਿੱਤਾ ਗਿਆ ਹੈ। ਮੇਰੇ ਵਿਚਾਰ ਅਨੁਸਾਰ ਭਾਗੀ ਇਸ ਖੋਜ ਵਿਚ ਸ਼ਾਮਲ ਹੋਣ ਲਈ ਜਾਗਰੂਕ ਸਹਿਮਤੀ ਦੇਣ ਦੇ ਕਾਨੂੰਨੀ ਰੂਪ ਵਿਚ ਸਮਰਥ ਹੈ ਅਤੇ ਸਵੈਇੱਛਾ ਨਾਲ ਅਤੇ ਜਾਣਦੇ ਹੋਏ ਜਾਗਰੂਕ ਸਹਿਮਤੀ ਦੇ ਰਿਹਾ ਹੈ,

ਖੋਜਕਰਤਾ ਦੇ ਦਸਤਖਤ ਅਤੇ ਨਾਮ: _______________ ਮਿਤੀ: ________

CENTRE FOR HUMAN GENETICS
SCHOOL OF HEALTH SCIENCES,
CENTRAL UNIVERSITY OF PUNJAB, BATHINDA
PATIENT DOCUMENTATION FORM

1. Case No..............................Sr. No…………………………Date…………………..
2. Name...3.Sex...
4. Height (cm): ……………Weight (kg): …………… Date of Birth ……………….
5. Clinical symptoms shown by the subject:

i.	IUGR	Y/N
ii.	PNGR	Y/N
iii.	Delayed bone age	Y/N
iv.	Triangular face	Y/N
v.	Relative macrocephaly	Y/N
vi.	Frontal bossing	Y/N
vii.	Micrognathia	Y/N
viii.	Ear anomalies	Y/N
ix.	Downturned mouth corners	Y/N
x.	Skeletal asymmetry	Y/N
xi.	Hemihypertrophy	Y/N
xii.	Clinodactyly	Y/N
xiii.	Brachydactyly	Y/N
xiv.	Syndactyly	Y/N
xv.	Delayed psychomotor development	Y/N
xvi.	Speech difficulties	Y/N
xvii.	Feeding difficulties	Y/N

6. Any other abnormality/disease/disorder observed:

....

....

7. **Investigations:**

    a) Biochemical/Serological tests:
    b) Biopsy:
    c) Other findings:

8. Mother's name ....
Age.........................Education...
9. Father's name...
Age............................Education..
10. Occupation
Mother...
Father..
11. Name of the endogamous group...
12. Religion...
13. Any existing disorder/disease in the family
....
....
14. Correspondence address...
....
Phone..Email..
15. Permanent Address...
....
....
16. Ancestral village..
17. (a) Place of sample collection...
(b)Collected by...
18. (a) Collected in EDTA/Heparin/Any other coagulant…......................................
(b) Amount..
19. Transport conditions..
(b)Storage temperature...

20. Brief Pedigree

21. Remarks if any..
....

All the precautions have been taken to avoid all kinds of infections during blood sample collection.

Date.........................

Signature of Investigator......................

Appendix B

Buffers and reagents

Requirement for DNA extraction

i. Lysis Buffer I (10X): It was prepared by adding following in 100 ml of distilled water: Ammonium Chloride (NH_4Cl) 8.29g, Potassium Bicarbonate ($KHCO_3$) 1g, 0.5M EDTA 0.2 ml

ii. 0.5M EDTA (pH-8.0) was prepared by adding 9.305g EDTA disodium salt in 40ml of distilled water. Adjust the pH to8.0 with the help of NaOH pellet. And prepare the final volume to 50ml.

iii. Lysis Buffer II (1X): It was prepared in total 100 ml of distilled water by adding Tris HCL 0.500 ml, Sodium Chloride (NaCl) 1.68g, 0.5M EDTA 0.200 ml.

iv. 1 M Tris HCl (pH-8.0) was prepared by adding 6.05g tris base to 50 ml of distilled water.

v. 10% SDS: It was prepared by adding 10g SDS to 90 ml of distilled water.

vi. PCA (25:24:1): PCA was prepared by adding phenol, chloroform and isoamyl alcohol in ratio 25:24:1.

vii. Proteinase K

viii. CA (24:1): CA was prepared by adding chloroform and isoamyl alcohol in 24:1 ratio.

Reagents for Polyacrylamide gel electrophoresis (PAGE):

i. **5X TBE(stock)**: 2 g of TrIzma Base (Tris), 13.75 g of boric acid, and 10ml of EDTA. Add 300ml of distilled water. Dissolve them well by magnetic stirrer and filter it. Make final volume up to 500ml with distilled water, but it should be discarded when a precipitate forms. It is used for gel preparation.

ii. **1X TBE**: Add 100ml of 5X TBE in 400ml of distilled water to make 1X TBE. It is used for gel run and also for staining. Prevents denaturation of the small fragments of DNA by low voltage run from Joulic heating. Also acts as gel loading buffer

iii. **50% Glycerol:** Take 25ml glycerol, to make up volume of 50ml with distilled water. It Helps in the formation of crisp bands

iv. **Acrylamide: bisacrylamide (29:1**): for preparation of 50ml reagent, take 14.6g of acrylamide and 0.4g of bisacrylamide, make upto 50ml by adding distilled water, then filter it. Further, store at +4℃. Acrylamide is a neurotoxin and therefore must be handled carefully. Gloves must be worn when weighing out.

v. **10% APS**: 10% ammonium persulfate (w/v). 1g of APS in 10ml of distilled water, shake well. Can be stored at +4℃ for several months. It is used as a catalyst for the copolymerization of acrylamide and bisacrylamide.

Appendix C

Chemicals used

Sr. No.	Chemicals name	Catalogue no.	Make
1.	Acrylamide	A9099-500G	Sigma
2.	Agarose	612600502501730	Genei
3.	Ammonium chloride (NH4Cl)	RM717-500G	Himedia
4.	Bisacrylamide	146072-100G	Sigma
5.	Boric acid	L149751412	Himedia
6.	Chloroform	20077L25	Himedia
7.	dNTPs	61065240001730	Genei
8.	Ethanol	XK-13-011-00009	Analytical
9.	Ethidium bromide (EtBr)	E7637-5G	Himedia
10.	Ethylenediaminetetraacetic acid (EDTA)	0373100500	Lobachemie
11.	Potassium bicarbonate (KHCO3)	0532701000	Lobachemie
12.	Potassium chloride (KCl)	0534000500	Lobachemie
13.	Proteinase K	612150181001730	Merk
14.	Saturated phenol	P455-400ML	Sigma
15.	Sodium Chloride (NaCl)	MB023-500G	Himedia
16.	Taq buffer with 15Mm MgCl2	613600701001730	Genei
17.	Taq DNA Polymerase	610603400051730	Genei
18.	TEMED	T7024-25ML	Himedia
19.	Tris base	613600701001730	Himedia

Appendix D

Instruments used

Sr. No.	Instruments name	Make
1.	CO2 Incubator	Eppendorf
2.	Dancing Shaker	Tarsons
3.	Dry bath	Gnei
4.	Electrophoresis Power Supply	Genei
5.	Gel Documentation System	Biorad
6.	Ice flacking machine	Manitowoc USA
7.	Microwave	Samsung
8.	Qubit	Invitrogen
9.	Spinwinn	Tarsons
10.	Thermal Cycler Applied	Biosystem
11.	Thermobrite	Abott Molecular
12.	UV Transilluminator	Genei
13.	Vertica Laminar Airflow	Narang scientific
14.	Water bath	Genei
15.	Weighing Balance	Mettler Toledo

URKUND

Urkund Analysis Result

Analysed Document: SWATI 2018.docx (D38523470)
Submitted: 5/10/2018 8:31:00 AM
Submitted By: arora1205@gmail.com
Significance: 0 %

Sources included in the report:

Instances where selected sources appear:

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1. Introduction Silver Russell Syndrome (SRS) is a type of rare congenital complex developmental disorder, diagnosed on the basis of clinical features i.e pre and post natal growth retardation, relative macrocephaly, triangular faces, body asymmetry and also feeding difficulties. Additional clinical features include 5th finger clinodactyly, genital abnormalities, brachydactyly, mesophalangy, toes syndactyly and inguinal hernia. Silver Russell Syndrome was described independently by H.K. Silver in 1953 and Dr A. Russell in 1954. It is also known as Russell-Silver Syndrome (RSS). Silver et al [1953] describes a 5 year old girl and 6 year old boy with congenital hemihypertrophy, shortness of stature and elevated urinary gonadotrophin. The children didn't had triangular face but they had macrocephaly. In 1954, Russell reported 2 girls and 3 boys with dwarfism, cranio facial dysostosis and short arms ratio. Around 35-50% of cases of SRS have epimutations in 11p15 region. This region contains two imprinting control regions (ICRs) adjacent to each other which plays an important role in the expression of the genes that play important role during fetal growth.

Maternal uniparantal disomy of chromosome 7 had been implicated in 10% of SRS cases. In

rest of the cases mutation/epimutation is yet to be elucidated. There is a considerable overlap in clinical features of these two molecular subtypes of SRS. Birth length and birth weight are more reduced with characterized body features i.e. fifth finger clinodactyly and congenital anomalies are more commonly observed with chromosome 11p15 related SRS syndrome whereas delayed psychomotor developments are more frequent in patients with chromosome 7 related SRS syndrome. Triangular facial shape is more pronounced in maternal UPD7 of SRS. Several types of rare chromosomal aberrations have been identified in a few cases of SRS. The prevalence of SRS is 1 in 3000 to 100,000 people worldwide. The condition is equally prevalent in both the sexes with most of the cases being sporadic. Uniparental disomy (UPD) is the inheritance of two homologous chromosomes from the same parent either from mother (matUPD) or father (patUPD) to the child. Inheritance of both the chromosomes could be from one parental pair (heterodisomy, hUPD) or two copies of the same chromosome from one parent (isodisomy, iUPD). Isodisomy have the chance to cause homozygosity for recessive mutation diseases i.e. cystic fibrosis. Four possible mechanisms for causing UPD namely trisomy rescue, gamete complementation, monosomy rescue and post fertilization errors had been proposed. UPD of chromosome 7 may cause the interruption in gene regulation among SRS patient. Chromosome 7 is a metacentric chromosome which spans about 158 million base pairs and signifies more than 5 % of the total DNA in cells. Approximate 1900 protein coding genes are present on chromosome 7. Three imprinted loci or the candidate SRS gene on chromosome 7 are GRB10 at 7p12, PEG10 at 7q21 and MEST at 7q32 which had been linked to the syndrome. Mesoderm-specific transcript (MEST) gene is expressed in all major fetal organs and tissues. PEG10 is paternally expressed gene 10 which is known to be essential for the embryonic development. Growth factor receptor bound protein 10 (GRB10) is highly imprinted gene derived from paternal allele, encoding a cytoplasmic adapter protein which helps in growth of various tissues in the body. Different techniques had been employed to identify genetic/epigenetic mutation among SRS patient. MatUPD7 can be detected by employed STR analysis using locus specific PCR primers and PAGE. Methylation specific PCR (MS-PCR) is another technique which can be utilized for the same.

Diagnosis of SRS is mainly based on clinical features but heterogeneous clinical features poses a challenge for its correct diagnosis.

Therefore, it is essential to genetically characterize the patients to provide appropriate genetic counselling to the parents and management of affected child. Further, different genetic cohorts of SRS respond differently to management strategies. The management of SRS may include growth hormone therapy and individualized therapy depending on the clinical features. Hence the current project case was undertaken with the following objective. Objective: To investigate maternal Uniparental disomy of chromosome 7(matUPD7) in a Silver Russell Syndrome (SRS) patient.

2. Review of literature Uniparental disomy (UPD) is the unusual situation in which a person receives both chromosomes from one parent and another chromosome for that pair from other parent

is missing.

UPD results in many abnormalities affecting growth and development in the affected individual through parent-of-origin differences in gene expression. UPD is unequally observed in all chromosomes and

occurrence of

maternal UPD is three times more frequent than paternal UPD. SRS was firstly described by Silver et al in 1953 and Russell in 1954 independently. Since the genetic causes for SRS is heterogeneous, individuals with SRS show

complex clinical features with varying severity. 2.1.

Clinical features associated with SRS The diagnosis of SRS and supportive laboratory testing is done for individuals who have three major criteria or two major and two minor criteria. The following criteria are compiled from the studies. •

The major criteria for clinical diagnosis of SRS include IUGR and PNGR (intrauterine and postnatal growth retardation) below or equal to 2SD and triangular face, relative macrocephaly, asymmetry (limb, body or facial asymmetry), feeding difficulties, fifth finger clino or brachydactyly. • The minor criteria are short arm span with normal upper to lower segment ratio, frontal bossing, motor delay, down-turned mouth corners, ear anomalies, teeth anomalies, and micrognathia, syndactyly of toes and squeaky voice, speech difficulties. • Supportive criteria includes genitourinary anomalies (cryptorchidism), muscular hypotonia, hypoglycaemia and genital anomalies, high arched palate, pointed chin, café au lait spots or skin pigmentary changes, excessive sweating, prominent heels.

2.2. Genetic and epigenetic causes of SRS Various genetic, epigenetic changes and chromosomal aberrations which are reported in association with SRS had been compiled in Table 2.1.

Chromosome 7 has been studied as a candidate locus when matUPD(7)

was found in some patients with IUGR and PNGR. Most genes shows biallelic expression but imprinted genes are only active from one parental allele. Different DMRs had been identified on chromosome 7 with specific locus are MAD1L1 (7p22), CARD11 (7p22), intergenic (7p21.2), RPS2P32 (7P15.3), HOXA4 (7p15.2), PRR15 (7p14.3), GLI3 (7p13), GRB10 (7p12.2), SGCE/ PEG10 (7q21.3), PON1 (7q21.3), SH2B2 (7q22), MEST (7q32), SVOPL (7q34), HTR5A (7q36.1), RARRES2 (7q36.1), Intergenic (7q36.2). Imprinting defects in GRB10, PEG10, MEST and FOXP2 have been suspected to cause SRS like phenotype. Mesoderm-specific transcript (MEST) gene is expressed in all major fetal organs and tissues. Growth factor receptor bound protein 10 (GRB10) encodes a cytoplasmic adapter protein which modulates coupling of a number of cell surface receptor tyrosine kinases with specific signalling pathways which helps in

the

growth of various tissues in the body. FOXP2 (forkhead box P2) gene is paternally expressed and responsible for development of verbal dysplaxia in many maternal upd (7) cases. There are some Imprinted genes found in the region of MEST

includes transcript of

paternally expressed introne from the biallelically expressed gamma-2 COP (nonclathrincoatprotein) (COPG21T1), paternally expressed MEST1T1 (i.e PEG1-AS) and maternally expressed carboxypeptidase A4 (CPA4). Fuke et al (2013) examined 138 Japanese patients (66 males and 72 females) with SRS and identified H19-DMR epimutation in 43 cases. The patients of this subgroup had more

number of reduced birth length and birth weight with more frequently occurring of

body asymmetry and brachydactyly. Epigenetics mechanisms of genomic imprinting includes various mechanisms namely, non-coding RNAs, Micro-imprinted domains and retro transposed genes, histone modification and chromatin modelling. Many studies had reported epigenetic changes in 11p15 region among SRS patient. Kotzot et al (1995) examined thirty five patients with PNGR, and their parents with PCR markers for UPD7. Out of which four patients had maternal disomy, three had isodisomy and one had heterodisomy. In another study carried out by Eggermann et al., (1997), out of 37 patients three had maternal UPD case. 28 patients were found to be carrier of an IGF2/H19 hypomethylation in 11p15 and 5 showed UPD (7) mat and 1 with segmental UPD in 188 routine samples of SRS patients. Spengler et al (2010) identified maternal UPD7 in 2 patients and ICR1 at 11p15 in 1 patients. 2.3. Uniparental disomy and Silver Russell Syndrome 2.3.1 Genetic cause for Uniparental disomy Mechanisms leading to UPD include a) Nullisomic gamete complementation: fertilization between a disomic germ cell from one parent and a nullisomic germ cell from other parent creates disomic zygote with UPD. b) Trisomic rescue: Postzygotic loss of a homologue chromosomes from a fertilized ovum containing three copies of a chromosome to bring to a normal diploid chromosome, and the result lead to the formation of UPD. c) Monosomy rescue: fertilization between a normal haploid germ cell from one parent and a nullisomic germ cell from other parent produces monosomy zygote duplication of such chromosome may result in isodisomy UPD. d) Non-disjunction during early mitotic division can result in Uniparental disomy. 2.3.2 Consequences of Uniparental disomy For many chromosomes, parent-specific imprinting exists and UPD of these chromosomes leads to clinically recognizable syndrome. Five chromosomes have been shown to have a phenotypic effect due to Uniparental inheritance of imprinted regions of maternally derived chromosomes 7, 14, and 15 and paternally derived chromosomes 6, 11, 14 and 15. It is currently unclear for some chromosomes, if there are any other phenotypic effects due to imprinting. In humans, almost all chromosomes have been implicated in UPD with the exception of chromosome 3, 12, 18, and 19. FOXP2 expressed paternally might be responsible for developmental verbal dyspraxia in some mUPD7 patients. 2.3.3 Uniparental disomy of chromosome 7 and Silver Russell syndrome The concept of UPD was first suggested by Engel in 1980. UPD can be distinguished in two types Uniparental hetrodisomy and Uniparental isodisomy i.e. presence of two different homologous chromosome from the same parent or presence of two copies of identical chromosomes respectively. Eggermann et al, (1997) screened 37 SRS families to determine the role of UPD in the etiology of SRS. It was reported that one case is maternal isodisomy, one case of maternal hetrodisomy and one case was showing partial isodisomy for 3STR markers. UPD7 can be detected via polymorphic short tandem repeat (STR) markers by PCR or variable number of tandem repeat (VNTRs) by Southern blotting. A maternally imprinted gene PEG1/MEST was mapped on human chromosome region 7q32 which expresses paternally but biparental expression is seen in blood lymphocytes. The key candidate region on chromosome 7 that may be the causative factor in SRS are 7q32 having several imprinted genes such as PEG1/ MEST DMR (paternally expressed), COPG2 (biallelically expressed) and CPA4(maternally expressed) and imprinted non coding RNAs(MESTIT, CIT1/COPG2IT1). No mutation had been observed for CPA4 in 10 SRS patient but a girl born with assisted reproductive technology (ART) was observed with partial hypomethylation of MEST DMR. Other candidate reqion is 7p11.2-p13 which harbours at least 1 imprinted gene (GRB10) playing an essential role in growth. Growth restriction is observed by over expression of GRB10 imprinted gene. Joyce et al (1999) reported an interstitial inverted duplication in

proximal 7p region of

a mother and daughter both had same clinical features like SRS including short stature, low birth weight, facial asymmetry and

also

fifth finger clinodactyly. 2.4 Techniques to detect Uniparental Disomy Routine cytogenetic analysis cannot identify UPD. UPD can be detected by genotyping with highly polymorphic microsatellite markers. The technique

is fast and feasible with PCR (polymerase chain reaction) and PAGE (Polyacrylamide gel electrophoresis).

Another method is Methylation specific PCR (MS-PCR) a rapid, qualitative and very sensitive technique. Following bisulphite conversion, PCR is performed using two sets of primers, which are designed to amplify either methylated or unmethylated alleles. It has advantage of being highly sensitive i.e. detects one methylated allele in a 1000 unmethylated allele. It can also be used on limited quality and quantity of DNA samples. There is no need of parent samples.

3. Material and methods 3.1 Subject Blood sample was collected from the subject with suggestive clinical features of SRS. Blood sample of the parents with normal phenotype was also collected. Ethical clearance was

obtained

for the study from Institutional Ethics Committee (IEC), Central University of Punjab, Bathinda

vide reference number

CUPB/HSB/15/PD/20. 3.2 Blood Sample collection

About 2 ml of blood sample was collected from both parents and a female patient (suspected case with SRS phenotype, aged 14 years) in EDTA sterile conditions (EDTA vial). 3.3 DNA Extraction DNA was extracted from peripheral blood by Sambrook and Russell method (2001)

and deproteinization was done with organic extraction as proposed by

Adeli and Ogbonna (1990) with slight modifications. i. The frozen blood sample in the vacutainer (EDTA vial) was thawed and kept

at room temperature. ii. 3ml of Lysis buffer 1 (1X) was added to 1ml of blood

sample in a 15ml autoclaved centrifuge tubes. iii. The sample was vigorously shaken and kept in ice for 15 minutes, followed by centrifugation of the tubes at 3000rpm for 15 minutes. iv. Discard the supernatant. v. Repeat the step ii, iii and iv thrice until a white pellet was obtained. vi. By

doing

vortexing, add 1 ml of Lysis buffer-II, 56µl of 10% SDS and proteinase K with concentration of 25µl carefully to the pellet

of

the sample. vii. Sample was incubated for 3 hours at 60^0C in water bath or keep the sample for overnight at 37^0C. viii. Recover the sample

from water bath and kept it at room temperature. ix. PCA (25:24:1) was added and gently mixed for 15minutes till the

emulsion formation. Now centrifuge it at 2500 rpm for 15minutes at room temperature (28^0C). Supernatant was transferred to the fresh and autoclaved polyprophylene tube. x. CA (24:1) was added followed by gently mixing for 15-20 minutes. Centrifuge it at 2500 rpm for 15 minutes at room temperature (28^{0}C). Transfer the supernatant to the fresh and autoclaved polyprophylene tube. xi. Repeat the step x for once. xii.

DNA precipitation was done by adding sodium acetate and chilled ethanol.by slow mixing DNA precipitated

out. Keep it at -20^{0}C for complete precipitation. xiii. DNA was transferred to the eppendorf tube along with 70% ethanol. Centrifuge at 1000 rpm for 12 minutes. Discard the supernatant and repeat step xiii. xiv. DNA precipitate was allowed to air dry to remove traces of ethanol. Add 60 μl -80 μl of TE buffer for dissolution of DNA by keeping it at 37^{0}C in water bath for 3 hours. 3.3.1 DNA quality assessment The quality assessment was done by Agarose gel electrophoresis

after the dissolution of DNA in TE buffer (0.8% gel) [Fig 3.2] i. Add 200mg of Agarose in 25ml of TAE buffer in a flask.

ii. Heat the flask for 1min in the oven till the Agarose dissolve. Cool it. iii. Add 2 μl of Ethidium bromide to the solution. iv. After proper mixing, pour the Agarose in casting tray. Set the

comb and allow it to cool for 15-20 minutes. v. After setting of the gel 2 μl of DNA sample was loaded along with 2 μl of loading dye in each well

and run

at 80 V for 30 minutes. 3.3.2 Quantification of DNA Quantification of DNA was done by using Qubit fluorometer.

i.

The qubit was calibrated by

standard 1 and standard 2 solution provided in the kit. ii. A mixture with 199 μl buffer and 1 μl dye was prepared

to quantify DNA.

iii. In a

sample tube containing 1μl of sample DNA, 199 μl of this mixture was added and centrifuge the tube for proper mixing of dye and DNA. iv. DNA sample was quantified and the readings were recorded by clicking on read button displayed on qubit screen. 3.4 STR analysis 3.4.1 PCR amplification Amplification of DNA was done using locus specific

STR (Short Tandem repeats) primers. It used as genetic marker which is present abundant throughout the genome.

Summary of primers used, PCR reaction mixture and conditions are given in Table 3.1, 3.2 and 3.3 respectively.

i. Reaction mixture was prepared of 50µl for each sample using Taq buffer, water, both primers (forward and reverse primers), dNTPs with taq polymerase and sample DNA. ii. Load the samples 2µl of PCR product along with 2µl loading dye in the wells of Agarose gel carefully. iii. PCR products were analysed on 1.5 % agarose gel run at 80 V for 30 minutes. 3.4.2

Native Polyacrylamide Gel Electrophoresis Diameter of the pores can be adjusted by the concentration of acrylamide and bisacrylamide based on the DNA fragments size. It varies from base pairs to base pairs depending upon their size. For better separation of the DNA with <200bp and >200bp the concentration of acrylamide monomer used is 7.5% and 10% respectively. The polyacrylamide gel was prepared using reagents described in table below:

i. 7.5%

polyacrylamide gel was prepared depending upon the size amplified products and let undisturbed for 60 minutes to polymerize. ii. The electrophoresis apparatus was assembled and cleaned with 70% ethanol iii. The gel was allowed to fix on casting cassette by binder clips for pre run of 30 minutes at 60V. iv. Sample (5 µl of PCR product along with 5 µl loading dye) was loaded and

run

at 60V for 6-7 hours. v. The gel apparatus was disassembled and transferred the gel to the staining tray. Ethidium bromide (EtBr) was added to it

with concentration of 2µl. vi.

The gel was allowed to stain for 20 minutes on dancing shaker and was then transferred to the Gel documentation system for observtion.

4. Results 4.1. Demographic and clinical features of

the

subject Blood sample was collected from both parents and 14 yeared, female patient suggested of SRS. Relative macrocephaly, mild facial asymmetry with postnatal growth retardation was observed. 4.2.

Checking of the subject for SRS with matUPD7 under the study Extracted DNA was quantified (Table 4.1) and its quality was analyzed on 0.8% Agarose gel (Fig. 4.1). In order to detect Uniparental disomy of chromosome 7, locus specific primers were used to amplify STR loci. PCR products were visualized on 7.5% native Polyacrylamide Gel Electrophoresis (PAGE). On analysing the gel, matUPD7 was ruled out.

5.

Discussion 5.1

Demographic and clinical features of the SRS subjects Clinical diagnosis of SRS is mainly based on

IUGR and PNGR. If a child have height less than third percentile, then it fulfils one major criteria for the diagnosis of SRS. It has been observed that matUPD7 is associated with mild SRS. SRS patients having learning and speech difficulties were likely to be associated with mUPD7 and ICR1 hypomethylation was related with classical SRS, asymmetry, fifth finger clinodactyly and congenital anomalies. 5.2 Genetic investigation of SRS subject In our study we investigated the suspected case of SRS of matUPD (7) by native polyacrylamide gel electrophoresis using locus specific primers. As only 10% of SRS is associated with matUPD7. However, It was reported

cases in 5%, 7%, 10%.

The subject had post natal growth retardation (PNGR) and mild facial asymmetry. 5.3 Conclusions Patient recruited in this study was an isolated case with no previous incidence in the family. 5.4 Future perspectives Investigations for epimutation in chromosome 11 at position p15.5 associated with SRS. Investigations for the duplication of 11p15 region which is implicated in1-2% of SRS. Molecularly confirmed cases of SRS may be helpful in management of the individuals with SRS and it might be helpful in genetic counselling to the parents of the affected child and also generating therapeutics in future.

SUMMARY Silver-Russell syndrome (SRS) is a complex disorder. SRS individuals are diagnosed on the basis of clinical features identified. The major criteria for clinical diagnosis of SRS are intrauterine growth retardation (IUGR) accompanied with post natal growth retardation (PNGR) and relative macrocephaly, triangular face, feeding difficulties, fifth finger clinodactyly with other minor and supportive criteria. Maternal

Uniparental

disomy of chromosome 7 had been implicated in 10% of SRS cases. Investigation was done under my study was to detect maternal Uniparental disomy of chromosome 7(matUPD7) by using locus specific primers to amplify STR loci by PCR. The sample was collected from female patient suggested

with

SRS, clinically diagnosed with relative macrocephaly, mild facial asymmetry with postnatal growth retardation. Further, PCR products were visualized on 7.5% native Polyacrylamide Gel Electrophoresis (PAGE). The technique used in my study was fast and feasible. On analyzing the native PAGE gel, matUPD7 was ruled out. Another method can also be used Methylation specific PCR (MS-PCR) which is also a rapid, qualitative and very sensitive technique. The patient suggested of SRS might have epimutation and duplication of chromosome 11p15 region associated with around 50% and 1-2% of SRS causes respectively.

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