4 RESULTS

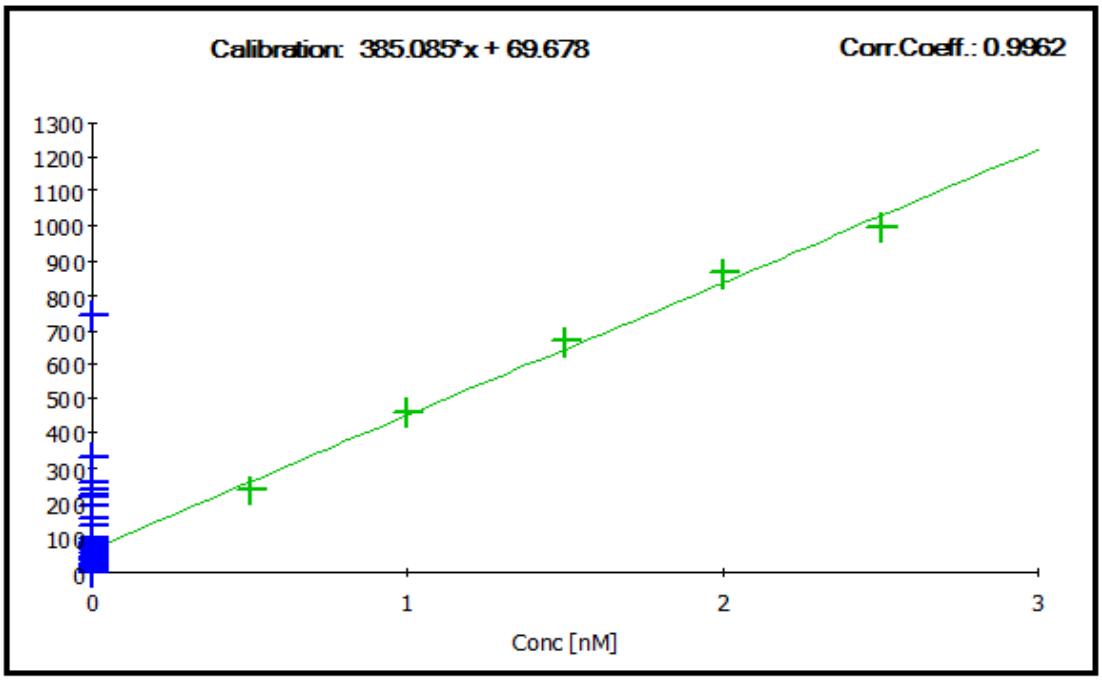
4.1 BIOCHEMICAL ANALYSIS

To standardize enzyme based diagnosis, we have primarily established normal range from leucocytes samples of normal healthy controls with no apparent disorder and growing normally with normal milestones. For prenatal diagnosis, we have established normal range using CT and/or AF samples from those pregnant women who have no family history of children with GM2 gangliosidosis (TSD, SD and GM2A) and referred to us for chromosomal abnormality.

4.1.1 Standard curve for Hexosaminidase-A (Hex-A) and total-Hexosaminidase (total-Hex) activity

The standard curve for measuring Hex-A and total-Hex activity was performed using 4-methylumbelliferone (4-MU) as substrate using spectrofluorometric method. 4-MU was obtained from SIGMA-ALDRICH (Catalog number: M1381). A *Figure 4.1* shows the standard curve for Hex-A and total-hex enzyme activity, which was linear from 0.5 to 2.5 nmol with 0.9962 correlation coefficient.

Figure 4.1: *4-MU standard curve for Hex-A and total-hex activity*



4.1.2 Measurement of Hexosaminidase-A (Hex-A) and total-Hexosaminidase (total-Hex) in Leucocytes

The enzyme activity was measured spectrofluorometrically using 4-MU specific synthetic sulfated substrate MUGS for Hex-A and MUG substrate for total-hex from leucocytes as described by (Wendeler and Sandhoff, 2009). The substrate was obtained from Melford Laboratories Ltd, UK. Initially, it was estimated from 50 normal healthy controls that include 32 male and 18 female in the age range of 1 year to 48 years to establish normal range as shown in *table 4.1*. Once normal range was established, we have investigated 409 cases from the pool of 1531 cases suspected with various LSDs referred to our centre.

The Hexosaminidase-A (Hex-A) activity for healthy controls and/or unaffected children is in the range of 80-635 nmol/hour/mg of protein towards MUGS substrate, while significant deficiency is in the range of 0.0-23 nmol/hour/mg of protein observed in children affected with TSD (*Table-4.1*). The mean Hex-A activity measured was significantly reduced in leucocytes obtained from TSD patients to be in the range of 2.6% of the mean control value ($p<0.01$) (*Table-4.1*). The total-hexosaminidase (total-Hex) activity for healthy controls and/or unaffected children is in the age range 714-2845 nmol/hour/mg of protein toward MUG substrate, while for TSD patient's it was found to be normal (761-2602 nmol/hour/mg of protein) similar to that range observed in unaffected children (*Table-4.1*). As an off shoot of the study we could also detect children with Sandhoff disease (SD) with reduced activity of Hex-A and total-Hex. Those with SD (GM2 Gangliosidosis), the activity of Hex-A and total-Hex were significantly reduced being less than 20% (Hex-A) and 10% (total-hex) of the mean concentration observed in healthy children of the same age group as shown in *table-4.2*.

Table 4.1: *Hexosaminidase-A (Hex-A) and total-Hexosaminidase (total-Hex) enzyme activity (nmol/h/mg protein) extracted from peripheral blood total leukocytes in healthy individual, unaffected and affected patients with TSD.*

Disease	**Enzyme**	**Sample types**					
Tay-Sachs disease	Hex-A	Leucocytes	**Activity in healthy Controls (Age range: 1 year-48 years)**				
			Mean±SD	***Range***	***N***		
			250.9±107.5	80-515	50		
			Activity in unaffected children (Age range: 1 months-9 years)				
			Mean±SD	***Range***	***N***		
			279.4±132.5	85-635	332		
			Activity in affected cases (Age range: 1.5 months-30 months)				
			Mean ± SD	***Range***	***N***	***% of Control***	***P***
			6.6±4.98	0.0-23	46	2.6	<0.01
	Total-Hex	Leucocytes	**Activity in healthy Control (Age range 1 year-48 years)**				
			Mean ± SD	***Range***	***N***		
			1770.9±544.4	714-2789	50		
			Activity in unaffected children (Age range: 1 months-9 years)				
			Mean±SD	***Range***	***N***		
			1812.1±602.5	728-2845	332		
			Activity in patients with deficiency of Hex-A (Age range: 1.5 months-30 months)				
			Mean ± SD	***Range***	***N***	***% of Control***	***P***
			1796.4±501.9	761-2602	46	100	>0.05

Table 4.2: *Hexosaminidase-A (Hex-A) and total-hexosaminidase (total-Hex) enzyme activity (nmol/h/mg protein) extracted from peripheral blood total leukocytes in healthy individual, unaffected and affected patients with SD*

Disease	**Enzyme**	**Sample types**					
Sandhoff disease	Hex-A	Leucocytes	**Activity in Controls (Age range: 1 year-48 years)**				
			Mean + SD	***Range***	***N***		
			250.9±107.5	80-515	50		
			Normal individual found (Age range: 1 months-9 years)				
			Mean+SD	***Range***	***N***		
			279.4±132.5	85-635	332		
			Activity in affceted cases (Age range: 8 months-3 years)				
			Mean + SD	***Range***	***N***	***% of Control***	***P***
			43.04±20.9	11.3-68.1	31	20	<0.01
	Total-hex	Leucocytes	**Activity in Control (Age range: 1 year-48 years)**				
			Mean + SD	***Range***	***N***		
			1770.9±544.4	714-2789	50		
			Normal individual found (Age range: 1 months-9 years)				
			Mean + SD	***Range***	***N***		
			1812.1+602.5	728-2845	332		
			Activity in affceted cases (Age range: 8 months-3 years)				
			Mean + SD	**Range**	**N**	**% of Control**	**P**
			189.4 ± 99.8	42.5-264	31	10	<0.01

4.1.3 Measurement of Hexosaminidase-A% (%Hex-A) and Hexosaminidase-total (total-Hex) activity in Leucocytes for carrier detection

The enzyme activity was measured spectrofluorometrically using synthetic 4-MU specific sulfated substrate MUG for %Hex-A to detect carrier status. Initially, it was estimated from 25 normal healthy controls that include 15 males and 10 female age range of 18 years to 48 years to establish normal range as shown in *table 4.3.* Once normal range was established, we have investigated 8 parents where proband was not alive but was earlier diagnosed by enzyme study and/or clinically suspected as a TSD.

The %Hex-A activity for normal was in the range of 63-79.4 % towards MUG substrate, while mild to moderate deficiency was in the range of 55-65 % was observed in all 8 TSD carriers parents (*Table 4.3*). Nonetheless carrier detection had some limitation due to overlapping activity between carrier and normal population.

Table 4.3: *Hexosaminidase-A% (%Hex-A) enzyme activity (nmol/h/mg protein) extracted from peripheral blood total leukocytes in healthy individual and TSD carriers*

Disease	Enzyme	Sample types			
Tay-Sachs disease for carrier detection	Hex-A%	Leucocytes	**Activity in Controls (Age range: 18 years-48 years)**		
			Mean±SD	**Range**	**N**
			70.1±5.1	63.0-79.4	25
			Activity in Carriers (Age range: 22 years-33 years)		
			Mean±SD	**Range**	**N**
			59.7±2.9	55.0-64.1	8

4.1.4 Measurement of Hexosaminidase-A (Hex-A) and Hexosaminidase-total (total-Hex) activity in CT/AF cells for prenatal diagnosis

The enzyme activity was measured spectrofluorometrically using synthetic 4-MU specific sulfated substrate MUGS for Hex-A and MUG substrate for total-hex from CT/AF as described by (Wendeler and Sandhoff, 2009). Initially, we have estimated 50 normal controls (25 CT and 25 AF) from those pregnant women who have any family history of GM2 gangliosdiosis (TSD, SD and GM2AP).

Table 4.4: *Hexosaminidase-A (%Hex-A) enzyme activity (nmol/h/mg protein) extracted from culrured trophoblstas (CT cells) and cultured amniotic fluid cells (AF cells)*

Disease	Enzyme	Sample types	Enzyme activity		
			Mean±SD	**Range**	**N**
Tay-Sachs disease	Hex-A	Cultured CVS/ Cultured trophoblasts (CT cells)	647.3±351.2	210-1621.2	25
		Cultured amniotic fluid cells/ Amniotic fibroblasts (AF cells)	622.1±331.8	218-1460.7	25

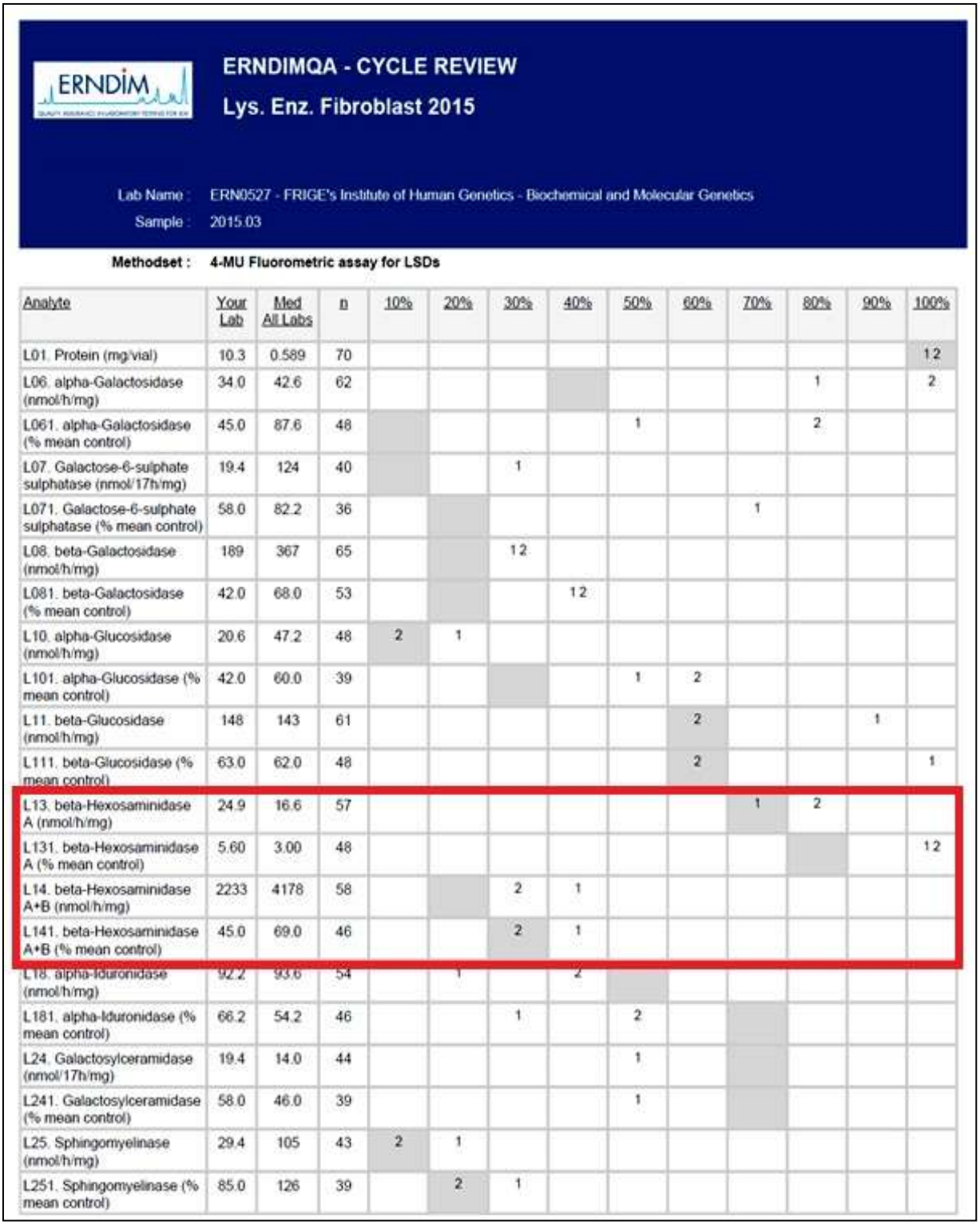
The normal Hex-A was in the range of 210-1621.2 nmol/hour/mg of protein from CT cells and 218-1460.7 nmol/hour/mg of protein from cultured AF cells towards MUGS substrate, while significant deficiency was identified in two fetus where Hex-A enzyme activity was 21.4 in CT

cells and 24.7 in AF cells from each one suggests present fetus is affected with TSD. The mean Hex-A activity measured was significantly reduced in CT/AF from fetus affected with TSD being nearly 3% of control mean (*refer table-4.18)*.

4.1.5 Quality Control

For ongoing evaluation of test performance, we have an ongoing participation in European external quality assessments program: European Research Network for Evaluation and Improvement of Screening, Diagnosis, and Treatment of Inherited Disorders of Metabolism (ERNDIM) (http://cms.erndimqa.nl/Home/Lysosomal-Enzymes.aspx). The internal audit program monitors operations throughout the testing process and the quality system. External assessments included regulatory inspections and proficiency testing programs.

In ERNDIM quality assurance program we are receiving six unknown samples of lyophilized fibroblasts for various LSDs enzymes study every year. During this quality assurance program we had found one sample of lyophilized fibroblasts with deficiency of Hex-A enzyme with normal enzyme activity of total-Hex suggesting TSD. We had submitted numerical enzyme activity values and percentage of the normal mean to online result submission portal, they curetted our results as shown in *figure 4.2 & 4.3*, which suggests that our results shows consistency with accurate diagnosis. This confirms that Hex-A enzyme assay is a reliable for diagnosis of TSD at our centre.



ERNDIM

ERNDIMQA - CYCLE REVIEW

Lys. Enz. Fibroblast 2015

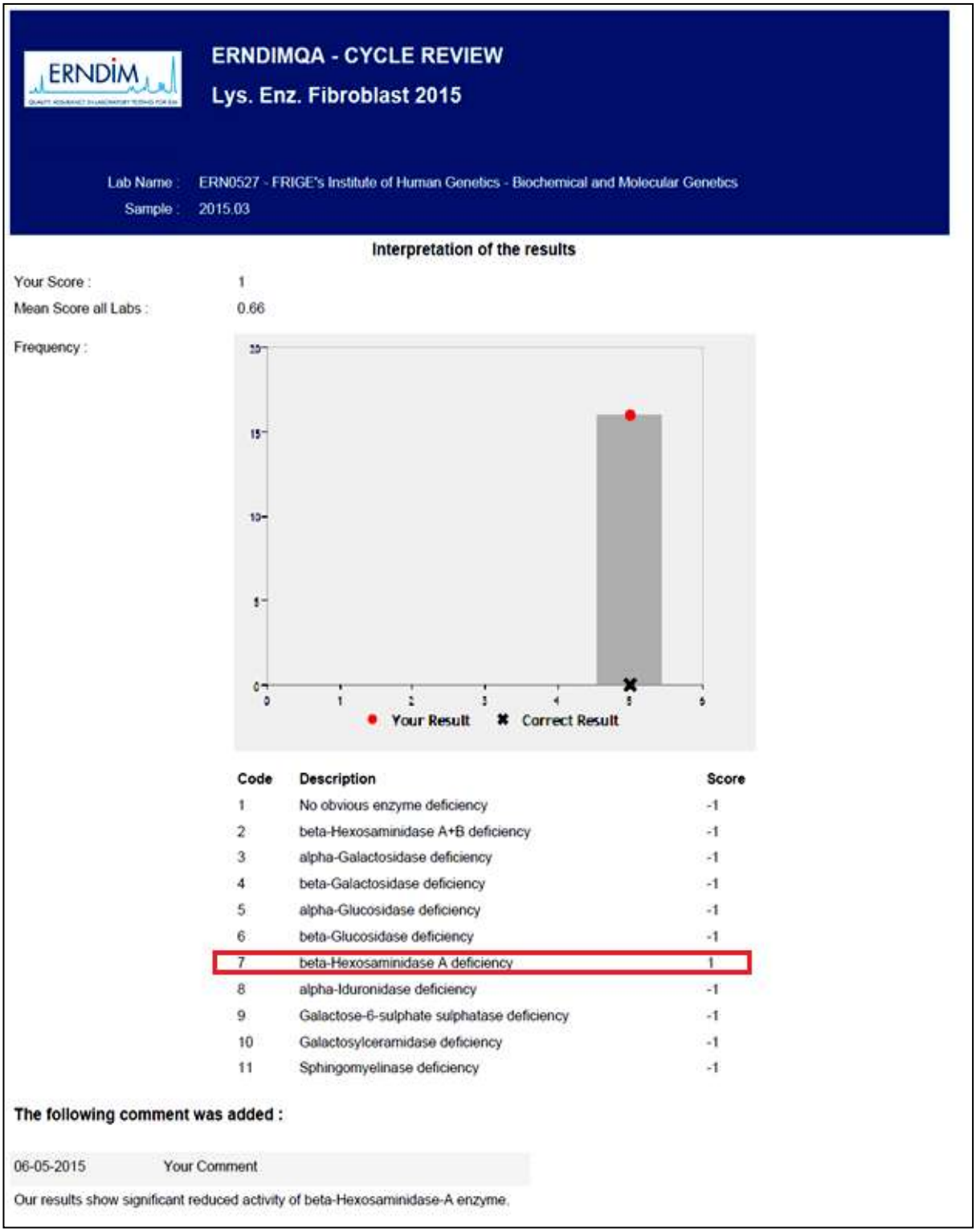
Lab Name : ERN0527 - FRIGE's Institute of Human Genetics - Biochemical and Molecular Genetics

Sample : 2015.03

Methodset : 4-MU Fluorometric assay for LSDs

Analyte	Your Lab	Med All Labs	n	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
L01. Protein (mg/vial)	10.3	0.589	70										12
L06. alpha-Galactosidase (nmol/h/mg)	34.0	42.6	62								1		2
L061. alpha-Galactosidase (% mean control)	45.0	87.6	48					1			2		
L07. Galactose-6-sulphate sulphatase (nmol/17h/mg)	19.4	124	40			1							
L071. Galactose-6-sulphate sulphatase (% mean control)	58.0	82.2	36							1			
L08. beta-Galactosidase (nmol/h/mg)	189	367	65			12							
L081. beta-Galactosidase (% mean control)	42.0	68.0	53				12						
L10. alpha-Glucosidase (nmol/h/mg)	20.6	47.2	48	2	1								
L101. alpha-Glucosidase (% mean control)	42.0	60.0	39					1	2				
L11. beta-Glucosidase (nmol/h/mg)	148	143	61						2			1	
L111. beta-Glucosidase (% mean control)	63.0	62.0	48						2				1
L13. beta-Hexosaminidase A (nmol/h/mg)	24.9	16.6	57							1	2		
L131. beta-Hexosaminidase A (% mean control)	5.60	3.00	48										12
L14. beta-Hexosaminidase A+B (nmol/h/mg)	2233	4178	58			2	1						
L141. beta-Hexosaminidase A+B (% mean control)	45.0	69.0	46			2	1						
L18. alpha-Iduronidase (nmol/h/mg)	92.2	93.6	54		1		2						
L181. alpha-Iduronidase (% mean control)	66.2	54.2	46			1		2					
L24. Galactosylceramidase (nmol/17h/mg)	19.4	14.0	44					1					
L241. Galactosylceramidase (% mean control)	58.0	46.0	39					1					
L25. Sphingomyelinase (nmol/h/mg)	29.4	105	43	2	1								
L251. Sphingomyelinase (% mean control)	85.0	126	39		2	1							

Figure 4.2: *ERNDIM quality control results (ERNDIMQA)*



ERNDIMQA - CYCLE REVIEW

Lys. Enz. Fibroblast 2015

Lab Name : ERN0527 - FRIGE's Institute of Human Genetics - Biochemical and Molecular Genetics

Sample : 2015.03

Interpretation of the results

Your Score : 1

Mean Score all Labs : 0.66

Frequency :

Code	Description	Score
1	No obvious enzyme deficiency	-1
2	beta-Hexosaminidase A+B deficiency	-1
3	alpha-Galactosidase deficiency	-1
4	beta-Galactosidase deficiency	-1
5	alpha-Glucosidase deficiency	-1
6	beta-Glucosidase deficiency	-1
7	beta-Hexosaminidase A deficiency	1
8	alpha-Iduronidase deficiency	-1
9	Galactose-6-sulphate sulphatase deficiency	-1
10	Galactosylceramidase deficiency	-1
11	Sphingomyelinase deficiency	-1

The following comment was added :

06-05-2015 Your Comment

Our results show significant reduced activity of beta-Hexosaminidase-A enzyme.

Figure 4.3: *Interpretation of ERNDIM quality control results (ERNDIMQA)*

4.1.6 Overall study of Tay-Sachs disease (TSD)

Present study has identified 46 cases of classical TSD from the pool of 1531 cases suspected with different LSDs as shown in *figure 4.4*. Diagnosis was confirmed by significant reduced activity of Hex-A with normal activity of total-Hex in leucocytes. In 2 cases Hex-A and total-Hex both the enzymes were normal and diagnosed as AB variant form of TSD (also called GM2 activator protein deficiency (GM2A deficiency)) by further molecular analysis (*Figure 4.4 & 4.5*). Carrier detection (% Hex-A) was carried out in 8 couples, where affected child was not alive but was earlier diagnosed by enzyme study and/or clinically suspected as a TSD. Furthermore, 31 cases had reduced activity of both the enzymes Hex-A along with total-Hex and final diagnosis was confirmed to be SD and molecular analysis was also carried out as an additional work as shown in supplementary documents. Prenatal diagnosis was also carried out in 8 cases using CT/AF cells having confirmed index case with TSD.

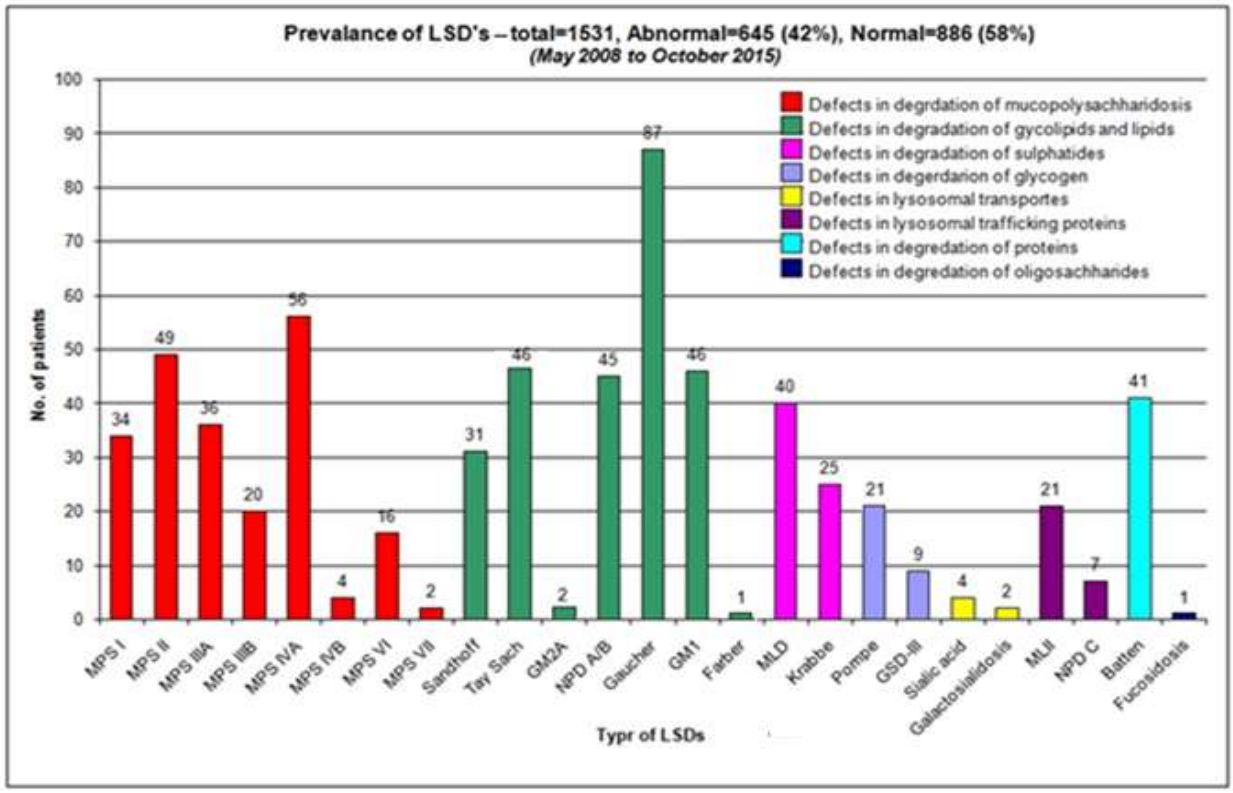


Figure 4.4: *Prevalence of LSD's in highly suspected cases-Our experience at FRIGE*

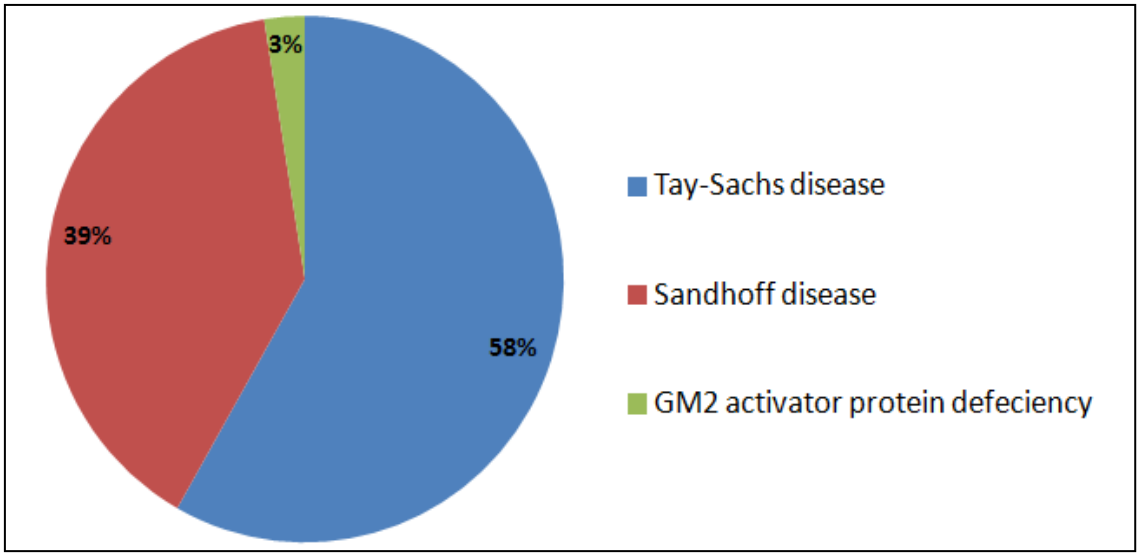


Figure-4.5: *Account for TSD, SD and GM2A in present study*

4.1.7 Analysis of TSD patients:

4.1.7.1 Provincial distribution of TSD patients:

We have found total 56 cases with TSD (46 cases with classical TSD, 2 cases with AB variant form of TSD and 8 carrier couples having history of affected child with TSD) from different parts of the India. It comprises of 42 (75%) patients from western, 11 (19.6%) from southern, 1 (1.8%) from northern and 2 (3.6%) from eastern India, as shown in *figure 4.6 and 4.10.*

4.1.7.2 Demographical profile of TSD patients:

The detailed demographic profile of all patients is in *table 4.5*, indicates out of 48 TSD patients, 30 were males and 18 were females in the age range of 1.5-30 months. Overall consanguinity in parents was observed in 24/56 (42.8%) cases of TSD.

4.1.7.3 Age of onset and Age at diagnosis:

The mean age of onset of TSD patients was 6.64±2.3 months (range=1.5-16) and the mean age at diagnosis was 16.28±5.96 months (range = 1.5-30 months). The median age of onset was 6 months and the median age at diagnosis was 14 months (*table 4.5*).

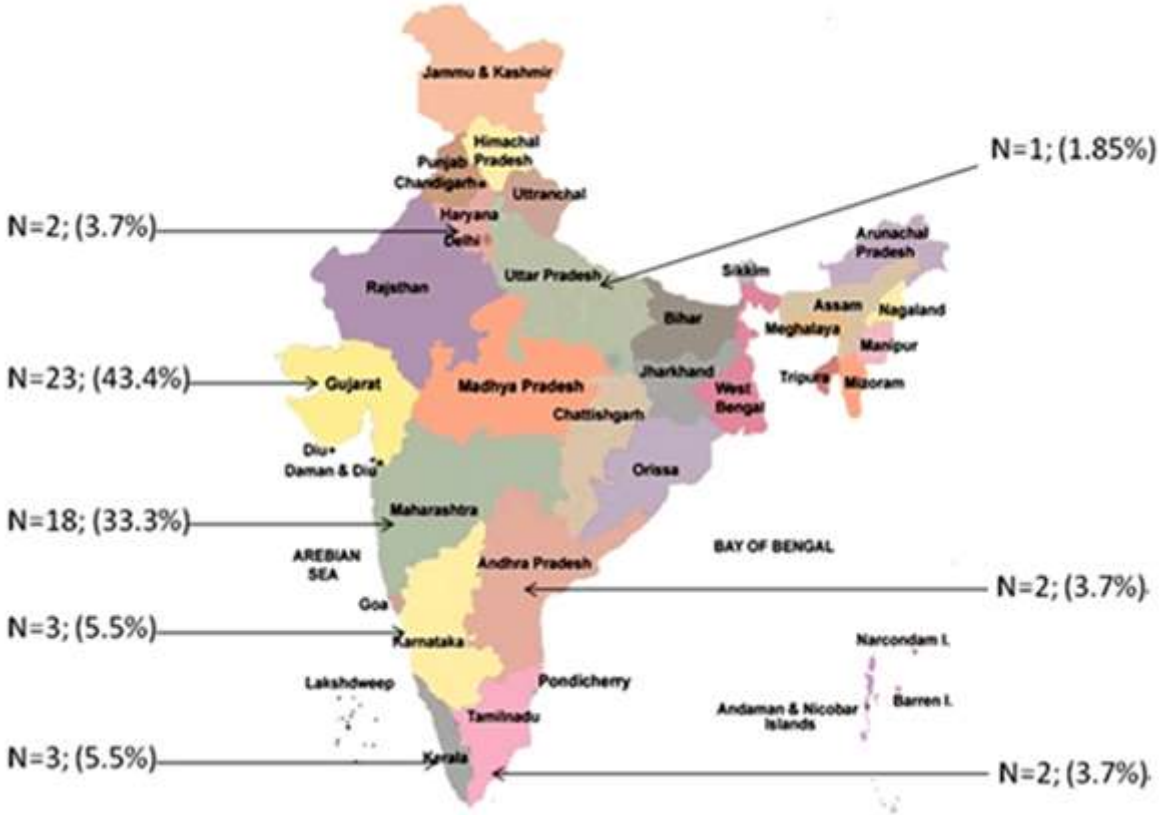


Figure-4.6: *Demographic distribution of TSD patients from various part of country*

Table-4.5: *Demographic profile for TSD patients*

Total TSD patients	**Mean ± SD**	**Median (IQR)**
	TSD patients (n=48)	
Age at diagnosis (months)	16.26 ± 5.96	15
Age at onset (months)	6.64 ± 2.3	6
	Family history of TSD where affected child had died (n=8)	
Age at diagnosis (months)	13.56 ± 2.7	14
Age at onset (months)	6.28±0.95	6
Age of death (months)	21.43±3.05	20
Gender (n=48)	***Male***	***Female***
	30	18
Consanguinity (n=56)	***Yes***	***No***
	24	32

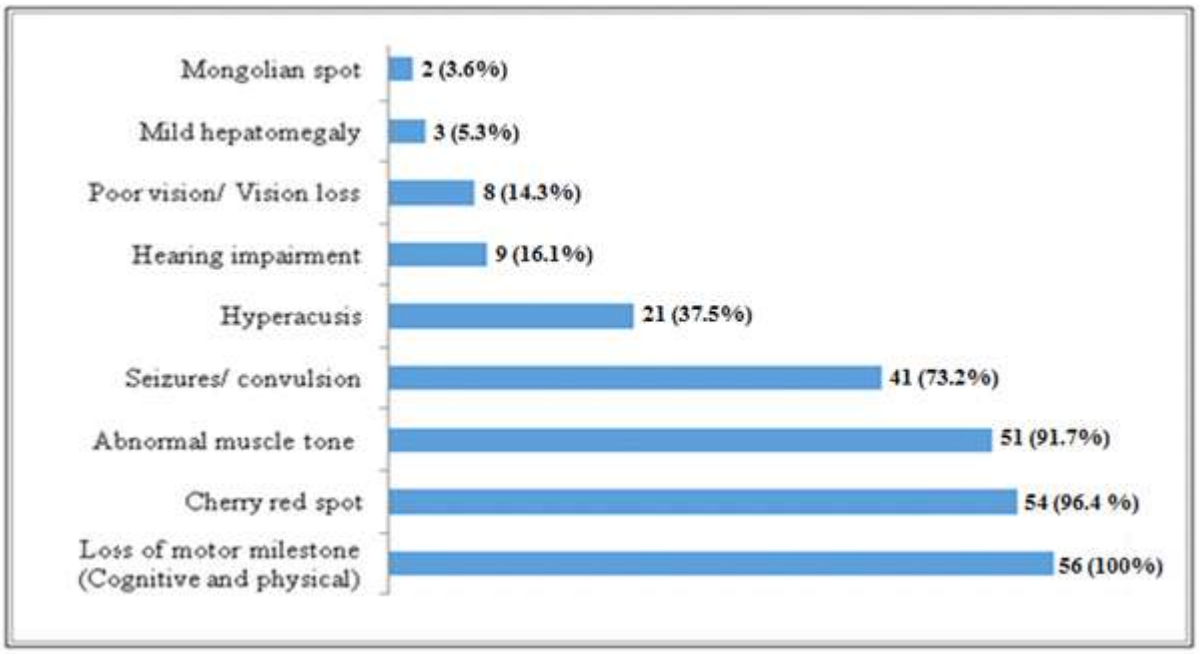
4.1.7.4 Clinical Profile of TSD patients

The detail clinical presentation of the patients studied at the time of diagnosis is shown in *figure 4.7*. The most common features present in all the TSD patients were regression of milestones (motor skills and cognitive regression) in 100% (56/56), cherry red spot in 96.4% (54/56), hypotonia in 91.7% (51/56), seizures in 73.2% (41/56), hyperaccusis in 37.5% (21/56), hearing impairment in 16.1% (9/56), poor vision and/ or vision loss in 14.3% (8/56) and mild hepatomegaly in (5.3%) 3/56. In addition, our study also demonstrated additional feature of presence of mongolian spots in 3.6% (2/56) of the cases.

The MRI brain was available in 31/56 cases and 24 cases showed characteristic findings of increased lessening of thalami and decreased attenuation of basal ganglia and isodense with white matter and 5 cases having dysmyelination also, while in 7 cases the MRI brain was normal.

The EEG study was available in 17 and all showed exaggerated startle response or hysparrhythmia.

Figure 4.7: *Clinical Profile of TSD patients (Total TSD patients; n=56)*



4.2 MOLECULAR ANALYSIS

OPEN ACCESS Freely available online

PLoS one

Identification of Novel Mutations in *HEXA* Gene in Children Affected with Tay Sachs Disease from India

Mehul Mistri[1☯], Parag M Tamhankar[2*☯], Frenny Sheth[1], Daksha Sanghavi[2], Pratima Kondurkar[2], Swapnil Patil[3], Susan Idicula-Thomas[3], Sarita Gupta[4], Jayesh Sheth[1*]

1 FRIGE Institute of Human Genetics, Ahmedabad, Gujarat, India, 2 ICMR Genetic Research Center, National Institute for Research in Reproductive Health, Mumbai, Maharashtra, India, 3 Biomedical Informatics Center, National Institute for Research in Reproductive Health, Mumbai, Maharashtra, India, 4 Department of Biochemistry, M.S. University, Vadodara, Gujarat, India

Abstract

Tay Sachs disease (TSD) is a neurodegenerative disorder due to β-hexosaminidase A deficiency caused by mutations in the *HEXA* gene. The mutations leading to Tay Sachs disease in India are yet unknown. We aimed to determine mutations leading to TSD in India by complete sequencing of the *HEXA* gene. The clinical inclusion criteria included neuroregression, seizures, exaggerated startle reflex, macrocephaly, cherry red spot on fundus examination and spasticity. Neuroimaging criteria included thalamic hyperdensities on CT scan/T1W images of MRI of the brain. Biochemical criteria included deficiency of hexosaminidase A (less than 2% of total hexosaminidase activity for infantile patients). Total leukocyte hexosaminidase activity was assayed by 4-methylumbelliferyl-N-acetyl-β-D-glucosamine lysis and hexosaminidase A activity was assayed by heat inactivation method and 4-methylumbelliferyl-N-acetyl-β-D-glucosamine-6-sulphate lysis method. The exons and exon-intron boundaries of the *HEXA* gene were bidirectionally sequenced using an automated sequencer. Mutations were confirmed in parents and looked up in public databases. *In silico* analysis for mutations was carried out using SIFT, Polyphen2, MutationT@ster and Accelrys Discovery Studio softwares. Fifteen families were included in the study. We identified six novel missense mutations, c.340 G>A (p.E114K), c.964 G>A (p.D322N), c.964 G>T (p.D322Y), c.1178C>G (p.R393P) and c.1385A>T (p.E462V), c.1432 G>A (p.G478R) and two previously reported mutations. c.1277_1278insTATC and c.508C>T (p.R170W). The mutation p.E462V was found in six unrelated families from Gujarat indicating a founder effect. A previously known splice site mutation c.805+1 G>C and another intronic mutation c.672+30 T>G of unknown significance were also identified. Mutations could not be identified in one family. We conclude that TSD patients from Gujarat should be screened for the common mutation p.E462V.

Citation: Mistri M, Tamhankar PM, Sheth F, Sanghavi D, Kondurkar P, et al. (2012) Identification of Novel Mutations in *HEXA* Gene in Children Affected with Tay Sachs Disease from India. PLoS ONE 7(6): e39122. doi:10.1371/journal.pone.0039122

Editor: Markus Schuelke, Charité Universitätsmedizin Berlin, NeuroCure Clinical Research Center, Germany

Received October 8, 2011; **Accepted** May 16, 2012; **Published** June 18, 2012

Funding: The work at FRIGE was supported by grant from Indian council of medical research (ICMR), project no BMS: 54/2010. The work at Genetic Research Center, NIRRH (National Institute for Research in Reproductive Health) was supported by intramural funds. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: jshethad1@gmail.com (JS); tamhankarp@nirrh.res.in (PMT)

☯ These authors contributed equally to this work.

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: http://www.journals.elsevier.com/molecular-genetics-and-metabolism-reports/

Expanding the spectrum of *HEXA* mutations in Indian patients with Tay–Sachs disease

Jayesh Sheth [a,*], Mehul Mistri [a], Chaitanya Datar [b], Umesh Kalane [b], Shekhar Patil [c], Mahesh Kamate [d], Harshuti Shah [e], Sheela Nampoothiri [f], Sarita Gupta [g], Frenny Sheth [a]

[a] Department of Biochemical and Molecular Genetics, FRIGE's Institute of Human Genetics, FRIGE House, Satellite, Ahmedabad 380015, India
[b] Department of Genetics, Clinical Geneticist, Sahyadri Medical Genetics and Tissue Engineering Facility (SMGTEF), Pune, India
[c] Department of Pediatric Neurology, Hiranandani Hospital, Mumbai, Maharashtra, India
[d] Department of Pediatric Neurology, KLES Prabhakar Kore Hospital, Belgaum, Karnataka, India
[e] Rajvee Child Neuro Hospital, Memnagar, Ahmedabad, India
[f] Department of Pediatric Genetics, Amrita Institute of Medical Science & Research Centre, AIMS Ponekkara PO, Cochin, Kerala, India
[g] Department of Biochemistry, Faculty of Science, M.S. University of Baroda, Vadodara, Gujarat, India

ARTICLE INFO

Article history:
Received 15 August 2014
Received in revised form 11 September 2014
Accepted 11 September 2014
Available online 29 September 2014

Keywords:
Tay–Sachs disease
HEXA gene
β-Hexosaminidase-A
Lysosomal enzyme

ABSTRACT

Tay–Sachs disease is an autosomal recessive neurodegenerative disorder occurring due to impaired activity of β-hexosaminidase-A (EC 3.2.1.52), resulting from the mutation in *HEXA* gene. Very little is known about the molecular pathology of TSD in Indian children except for a few mutations identified by us. The present study is aimed to determine additional mutations leading to Tay–Sachs disease in nine patients confirmed by the deficiency of β-hexosaminidase-A (<2% of total hexosaminidase activity for infantile patients) in leucocytes. The enzyme activity was assessed by using substrates 4-methylumbelliferyl-N-acetyl-β-D-glucosamine and 4-methylumbelliferyl-N-acetyl-β-D-glucosamine-6-sulfate for total-hexosaminidase and hexosaminidase-A respectively, and heat inactivation method for carrier detection. The exons and exon–intron boundaries of the *HEXA* gene were bi-directionally sequenced on an automated sequencer. 'In silico' analyses for novel mutations were carried out using SIFT, Polyphen2 and MutationT@ster software programs. The structural study was carried out by UCSF Chimera software using the crystallographic structure of β-hexosaminidase-A (PDB-ID: 2GJX) as the template. Our study identified four novel mutations in three cases. These include a compound heterozygous missense mutation c.524A>C (D175A) and c.805G>C (p.G269R) in one case; and one small 1 bp deletion c.426delT (p.F142LfsX57) and one splice site mutation c.459+4A>C in the other two cases respectively. None of these mutations were detected in 100 chromosomes from healthy individuals of the same ethnic group. Three previously reported missense mutations, (i) c.532C>T (p.R178C), (ii) c.964G>T (p.D322Y), and (iii) c.1385A>T (p.E462V); two nonsense mutations (i) c.709C>T (p.Q237X) and (ii) c.1528C>T (p.R510X), one 4 bp insertion c.1277_1278insTATC (p.Y427IfsX5) and one splice site mutation c.459+5G>A were also identified in six cases. We observe from this study that novel mutations are more frequently observed in Indian patients with Tay–Sachs disease with clustering of ~73% of disease causing mutations in exons 5 to 12. This database can be used for a carrier rate screening in the larger population of the country.

Molecular analysis was carried out in all enzymatically affected cases by screening of 5 common mutations [c.1277_1278insTATC, c.1421+1 G>C, 7.6 kb deletion, the pseudo-deficiency alleles (p.R247W and p.R249W)] followed by confirmative bi-directional sequencing using Sanger method.

4.2.1 Common variants study

The common mutations screening was carried out in all enzymatically affected 54 cases (46 affected child and 8 carrier couples) and c.1278insTATC mutation was homozygously detected in 5/54 cases while 1 with compound heterozygosity involving second allele c.964 G>A (p.D322N) in exon-9. These mutations were further confirmed by bidirectional sequencing. Whenever, the rest of the common mutations were not identified in any of our patients as shown in *table-4.6.*

Table 4.6: *Common mutations screening for TSD patients*

Exon/Intron	Type	Nucleotide Change[a]	Amino acid change[a]	Recurrence
Exon-1	Frameshift	c.1278insTATC	p.Y427IfsX5	6 Homozygous (n=5) Heterozygous (n=1)
Intron-12	Splice site	c.1421+1 G>C	-	0
UTR+Exon-1+Intron-1	Large deletion	7.6 kb deletion	-	0
Exon-7	Missense	c.739 C>T	p.R247W	0
Exon-7	Missense	c.745 C>T	p.R249W	0

a = numbered according to Gene Bank Accession NM_000520.4

4.2.2 Bi-directional sequencing of the *HEXA* gene to identify known/novel variants

4.2.2.1 Study of HEXA gene in enzymatically affected infantile/juvenile TSD

Further, the molecular analysis was carried out using bi-directional exon sequencing in 46 cases. Exon sequencing analysis revealed 29 variants (including c.1278insTATC) in 44 cases, 19 of which were novel, including 13 missense variants [c.340 G>A (p.E114K), c.524A>C (p.D175A), c.616G>C (p.V206L), c.637T>C (p.Y213H), c.788C>T (p.T263I), c.805G>C (p.G269R), c.964G>T (p.D322Y), c.964G>A (p.D322N), c.1121A>C (p.Q374P), c.1178C>G (p.R393P), c.1360 G>C (p.G454R), c.1385A>T (p.E462V) andc.1432G>A (p.G478R)], 2 nonsense variants [c.1421G>A (p.W474X) and c.1454G>A (p.W485X)], 2 small deletion [c.426delT (p.F142LfsX57) and c.898_905TTCATGAG (p.F300HfsX21)] and 2 splice site mutation c.459+4 A>C and c.460-1 G>A. Previously known 3 missense variants c.2T>C (p.M1T),

c.508C>T (p.R170W) and c.1495C>T (p.R499C), 2 nonsense variants c.316C>T (p.Q106X) and c.1528C>T (p.R510X), one 4bp insertion [c.1278insTATC (p.Y427IfsX5)] and 4 splice site variants c.459+5 G>A, c.805+1 G>C, c.1073+1 G>A, c.672+30 T>G was also identified (*Table 4.7 and Figure 4.8*). Mutation was not found in 1 case, while two patient was heterozygous for c.1385A>T (p.E462V) with unknown second allele. However the MLPA study will carry out to find out the second allele.

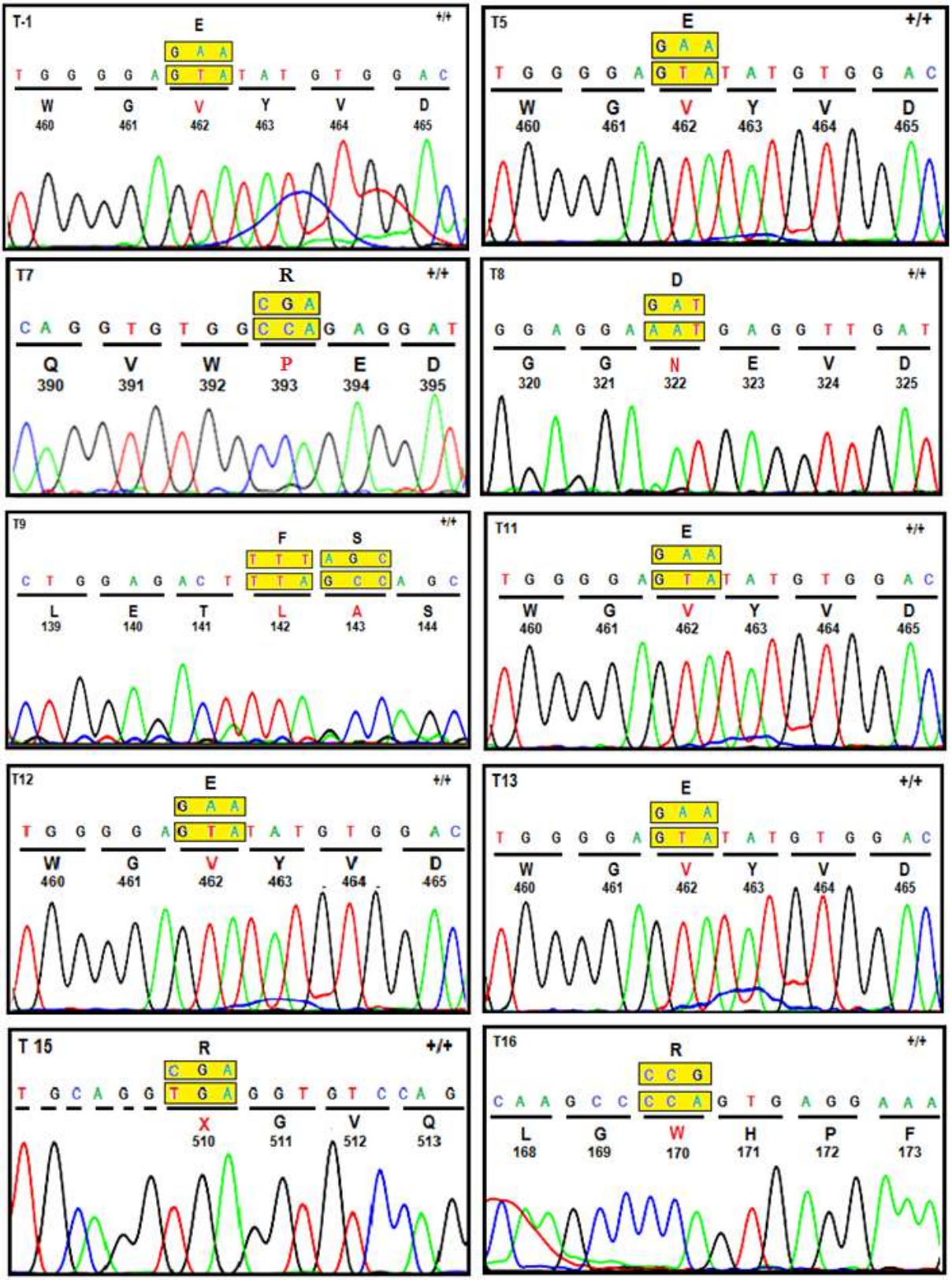
Table 4.7: *List of all HEXA sequence variants identified in TSD patients in present study*

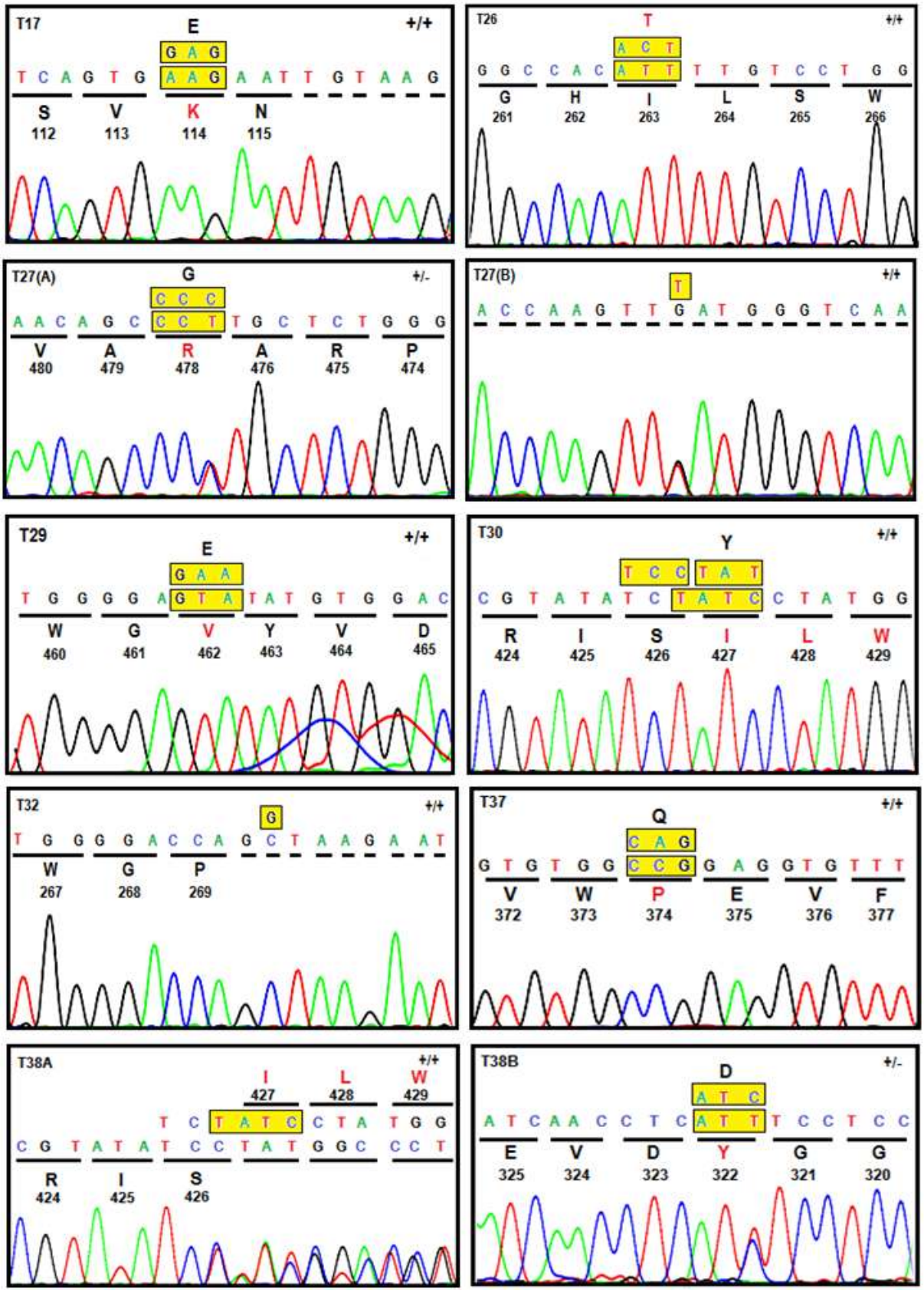
Patients ID	Exon/ Intron	Nucleotide change [a]	Amino acid change [a]	Types	Domain	Recurrence	HGMD Accession Number	References
T-87	Exon-1	c.2 T>C (CpG)	p.M1T	MS	Domain-I	1	CM930389	Harmon *et al.*, 1993 Present study (Unpublished data)
T-71[€]	Exon-1	c.316C>T	p.Q106X	NS	Domain-I	1	CM970715	Akerman *et al.*, 1997 Present study (Unpublished data)
T-17	Exon-2	c.340G>A	p.E114K	MS	Domain-I	1	CM125498	Present study Mistri *et al.*, 2012
T-9	Exon-4	c.426delT	p.F142LfsX57	FS	Domain-I	1	NA	Present study Sheth *et al.*, 2014b
T-69	Intron-4	c.459+4A>C	-	IV	Domain-I	1	NA	Present study Sheth *et al.*, 2014b
T-49	Intron-4	c.459+5G>A	-	IV	Domain-I	1	CS910443	Akli *et al.*, 1991
T-65	Intron-4	c.460-1G>A	-	IV	Domain-I	1	NA	Present study (Unpublished data)
T-16	Exon-5	c.508C>T (CpG)	p.R170W	MS	Domain-II	1	CM920331	Triggs-Raine *et al.*, 1991; Fernandes *et al.*, 1992; Akli *et al.*, 1993
T-45[¥]	Exon-5	c.524A>C	p.D175A	MS	Domain-II	1	NA	Present study Sheth *et al.*, 2014b
T-75	Exon-6	c.616G>C	p.V206L	MS	Domain-II	1	NA	Present study (Unpublished data)
T-81	Exon-6	c.637T>C	p.Y213H	MS	Domain-II	1	NA	Present study Sheth *et al.*, 2014b
T-27*	Intron-6	c.672+30T>G	-	IV	Domain-II	1	CS951438	Triggs-Raine *et al.*, 1995
T-26	Exon-7	c.788C>T	p.T263I	MS	Domain-II	1	NA	Present study Sheth *et al.*, 2014a
T-45[¥]	Exon-7	c.805G>C	p.G269R	MS	Domain-II	1	NA	Present study Sheth *et al.*, 2014b
T-32	Intron-7	c.805+1G>C	-	IV	Domain-II	1	CS951439	Ribeiro *et al.*, 1995
T-47	Exon-8	c.899_906delTTCATGAG	p.F300HfsX21	FS	Domain-II	1	NA	Present study Sheth *et al.*, 2014a

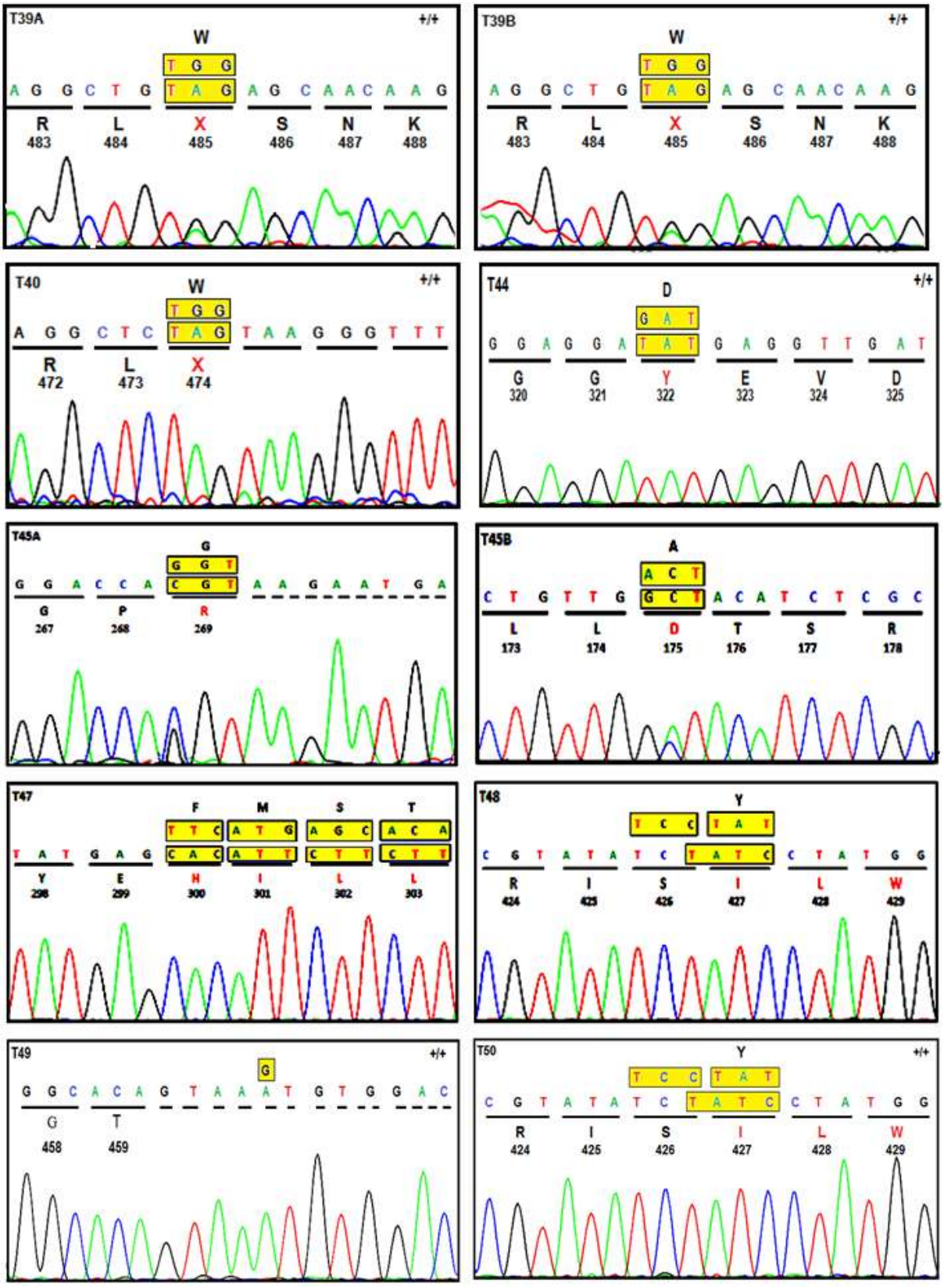
T-8 T-38[@] T-82	Exon-8	c.964G>A	p.D322N	MS	Domain-II	3	CM125496	Present study Mistri *et al.*, 2012
T-4 T-14 T-56 T-59	Exon-8	c.964G>T	p.D322Y	MS	Domain-II	3	CM1254897	Present study Mistri *et al.*, 2012
T-71[€]	Intron-9	c.1073+1G>A	-	IV	Domain-II	1	CS910444	Akli *et al.*, 1991
T-37	Exon-11	c.1121A>C	p.Q374P	MS	Domain-II	1	NA	Present study Sheth *et al.*, 2014a
T-7	Exon-11	c.1178G>C (CpG)	p.R393P	MS	Domain-II	1	CM125500	Present study Mistri *et al.*, 2012
T-30 T-38[@] T-48 T-50 T-60 T-73	Exon-11	c.1278insTATC	p.Y427IfsX5	FS	Domain-II	6	CI880091	Paw et al., 1990b Myerowitz and Costigan, 1988; Mules *et al.*, 1992; Kaufman *et al.*, 1997; Mc Dowell *et al.*, 1992
T-90	Exon-12	c.1360G>C	p.G454R	MS	Domain-II	1	NA	Present study (Unpublished data)
T-1 T-5 T-11 T-12 T-13 T-29 T-58[$] T-76 T-80[$] T-85 T-86	Exon-12	c.1385A>T	p.E462V	MS	Domain-II	11	CM125495	Present study Mistri *et al.*, 2012; Sheth *et al.*, 2014a; Sheth *et al.*, 2014b
T-40	Exon-12	c.1420G>A	p.W474X	NS	Domain-II	1	NA	Present study Sheth *et al.*, 2014a
T-27*	Exon-13	c.1432G>A	p.G478R	MS	Domain-II	1	CM125499	Present study Mistri *et al.*, 2012
T-39	Exon-13	c.1454G>A	p.W485X	MS	Domain-II	1	NA	Present study Sheth *et al.*, 2014a

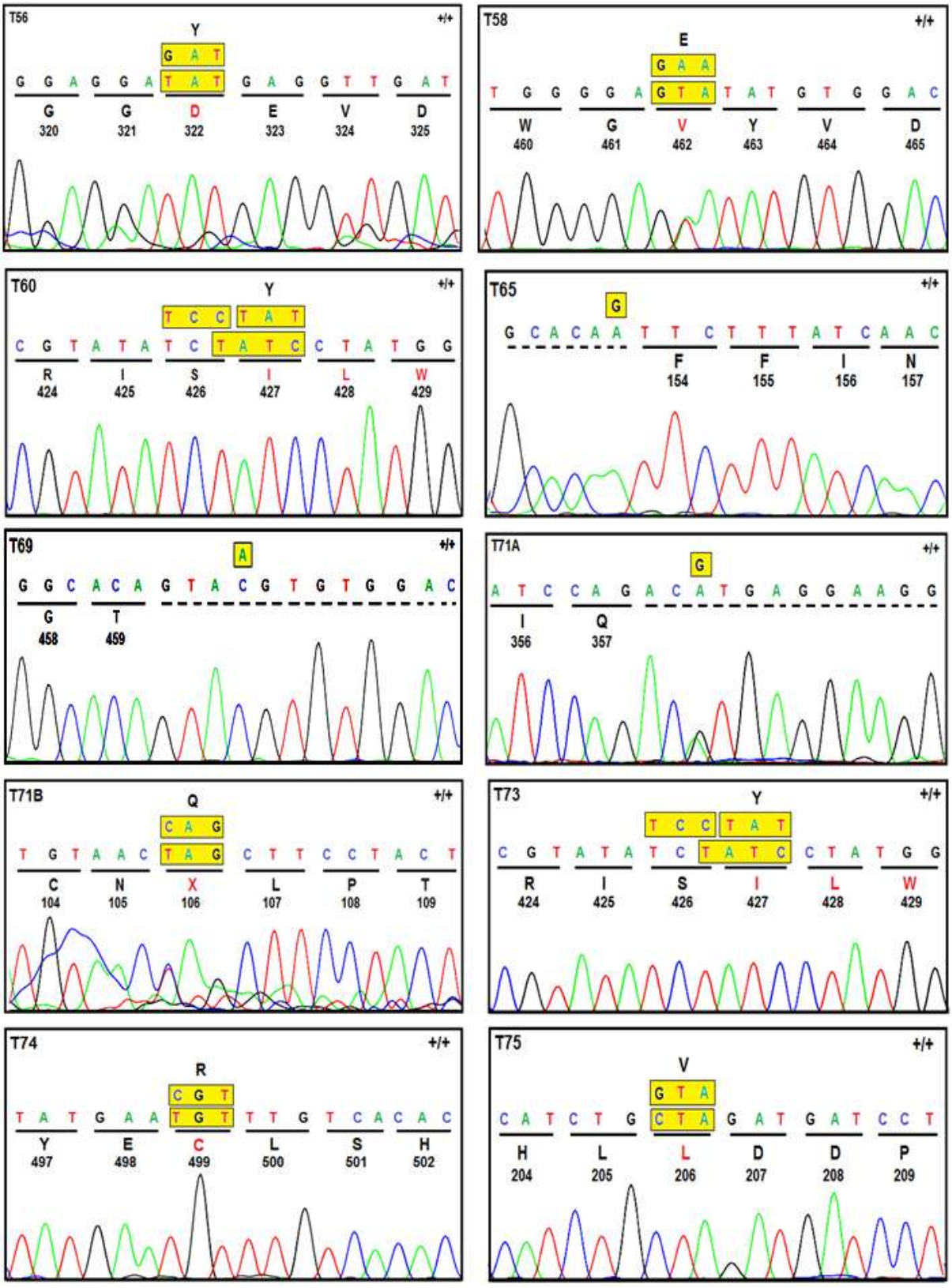
T-74	Exon-13	c.1495C>T (CpG)	p.R499C	MS	Domain-II	1	CM920341	Mules *et al.*, 1992; Akli *et al.*, 1993b; Ribeiro *et al*, 1995
T-15	Exon-14	c.1528C>T (CpG)	p.R510X	NS	Domain-II	1	CM113520	Kaya *et al.*, 2011; Jamali *et al.*, 2014; Sheth *et al.*, 2014a

¥, *, @, € Compound heterozygous; $ Compound heterozygous with Exon-1 deletion as shown in *table 4.9*


T-1
E
+/+
G A A
T G G G G A G T A T A T G T G G A C
W G V Y V D
460 461 462 463 464 465
T5
E
+/+
G A A
T G G G G A G T A T A T G T G G A C
W G V Y V D
460 461 462 463 464 465
T7
R
+/+
C G A
C A G G T G T G G C C A G A G G A T
Q V W P E D
390 391 392 393 394 395
T8
D
+/+
G A T
G G A G G A A A T G A G G T T G A T
G G N E V D
320 321 322 323 324 325
T9
F S
+/+
T T T A G C
C T G G A G A C T T T A G C C A G C
L E T L A S
139 140 141 142 143 144
T11
E
+/+
G A A
T G G G G A G T A T A T G T G G A C
W G V Y V D
460 461 462 463 464 465
T12
E
+/+
G A A
T G G G G A G T A T A T G T G G A C
W G V Y V D
460 461 462 463 464 465
T13
E
+/+
G A A
T G G G G A G T A T A T G T G G A C
W G V Y V D
460 461 462 463 464 465
T 15
R
+/+
C G A
T G C A G G T G A G G T G T C C A G
X G V Q
510 511 512 513
T16
R
+/+
C C G
C A A G C C C C A G T G A G G A A A
L G W H P F
168 169 170 171 172 173


T17
E
+/+
G A G
T C A G T G A A G A A T T G T A A G
S V K N
112 113 114 115
T26
T
+/+
A C T
G G C C A C A T T T T G T C C T G G
G H I L S W
261 262 263 264 265 266
T27(A)
G
+/-
C C C
A A C A G C C C T T G C T C T G G G
V A R A R P
480 479 478 476 475 474
T27(B)
T
+/+
A C C A A G T T G A T G G G T C A A
T29
E
+/+
G A A
T G G G G A G T A T A T G T G G A C
W G V Y V D
460 461 462 463 464 465
T30
Y
+/+
T C C T A T
C G T A T A T C T A T C C T A T G G
R I S I L W
424 425 426 427 428 429
T32
G
+/+
T G G G G A C C A G C T A A G A A T
W G P
267 268 269
T37
Q
+/+
C A G
G T G T G G C C G G A G G T G T T T
V W P E V F
372 373 374 375 376 377
T38A
+/+
I L W
427 428 429
T C T A T C C T A T G G
C G T A T A T C C T A T G G C C C T
R I S
424 425 426
T38B
D
+/-
A T C
A T C A A C C T C A T T T C C T C C
E V D Y G G
325 324 323 322 321 320


T39A
+/+
W
T G G
A G G C T G T A G A G C A A C A A G
R L X S N K
483 484 485 486 487 488
T39B
+/+
W
T G G
A G G C T G T A G A G C A A C A A G
R L X S N K
483 484 485 486 487 488
T40
+/+
W
T G G
A G G C T C T A G T A A G G G T T T
R L X
472 473 474
T44
+/+
D
G A T
G G A G G A T A T G A G G T T G A T
G G Y E V D
320 321 322 323 324 325
T45A
G
G G T
G G A C C A C G T A A G A A T G A
G P R
267 268 269
T45B
A
A C T
C T G T T G G C T A C A T C T C G C
L L D T S R
173 174 175 176 177 178
T47
F M S T
T T C A T G A G C A C A
C A C A T T C T T C T T
T A T G A G
Y E H I L L
298 299 300 301 302 303
T48
Y
T C C T A T
C G T A T A T C T A T C C T A T G G
R I S I L W
424 425 426 427 428 429
T49
+/+
G
G G C A C A G T A A A T G T G G A C
G T
458 459
T50
+/+
Y
T C C T A T
C G T A T A T C T A T C C T A T G G
R I S I L W
424 425 426 427 428 429


T56
+/+
Y
G A T
T A T
G G A G G A G A A G G T T G A T
G G D E V D
320 321 322 323 324 325
T58
+/+
E
G A A
G T A
T G G G G A T A T G T G G A C
W G V Y V D
460 461 462 463 464 465
T60
+/+
Y
T C C T A T
T A T C
C G T A T A T C C T A T G G
R I S I L W
424 425 426 427 428 429
T65
+/+
G
G C A C A A T T C T T T A T C A A C
F F I N
154 155 156 157
T69
+/+
A
G G C A C A G T A C G T G T G G A C
G T
458 459
T71A
+/+
G
A T C C A G A C A T G A G G A A G G
I Q
356 357
T71B
+/+
Q
C A G
T A G
T G T A A C T A G C T T C C T A C T
C N X L P T
104 105 106 107 108 109
T73
+/+
Y
T C C T A T
T A T C
C G T A T A T C C T A T G G
R I S I L W
424 425 426 427 428 429
T74
+/+
R
C G T
T G T
T A T G A A T G T T T G T C A C A C
Y E C L S H
497 498 499 500 501 502
T75
+/+
V
G T A
C T A
C A T C T G C T A G A T G A T C C T
H L L D D P
204 205 206 207 208 209

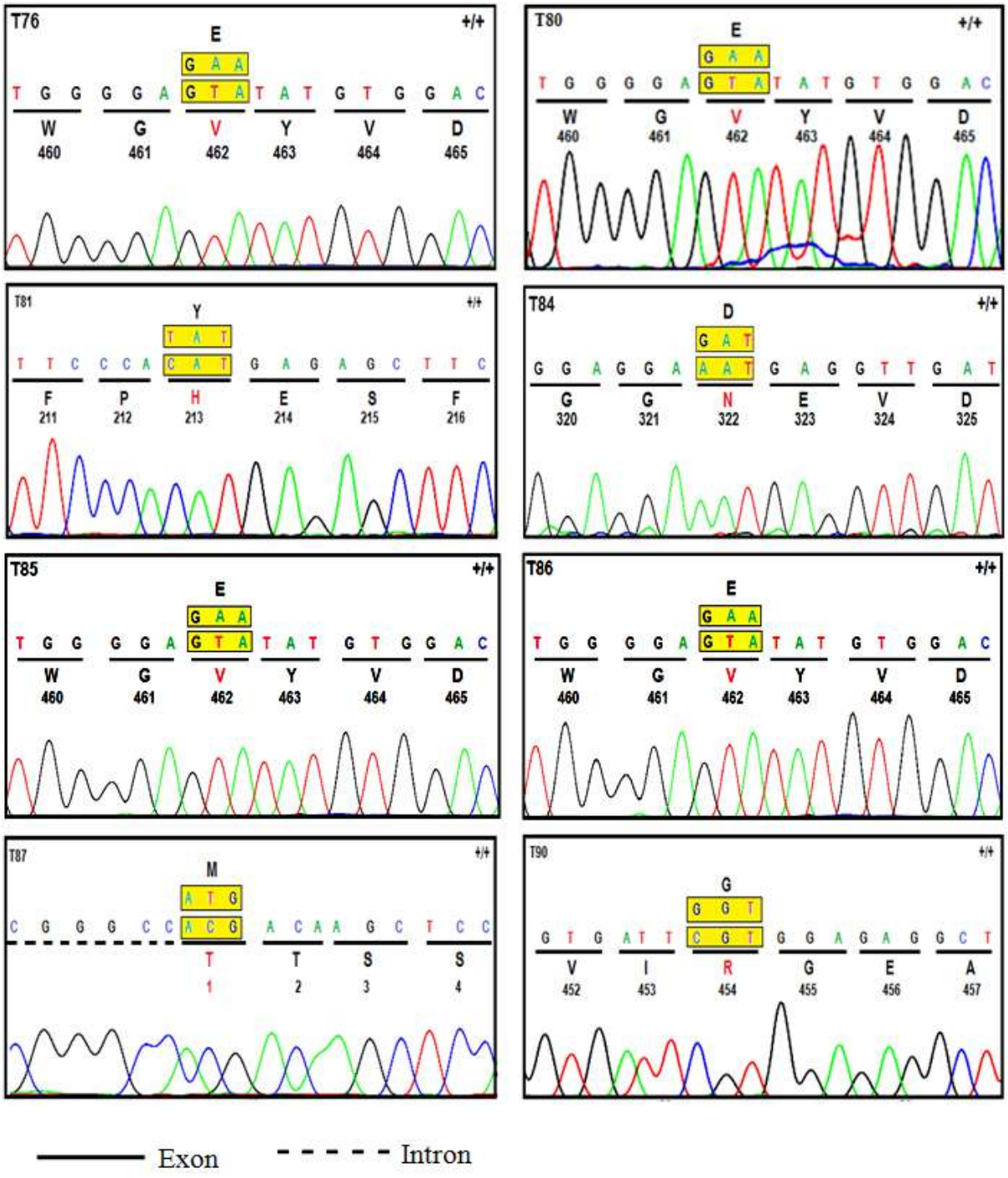


Figure 4.8: *Chromatogram of all TSD patients (Please refer table 4.15 for details of patients)*

4.2.2.2 Study of HEXA gene in carrier parents

Molecular analysis was also carried out in 8 carrier parents. The common mutations screening analysis was carried out and none of them had shown presence of common mutations. Furthermore, bi-directional exon sequencing study was carried out in all of them. Exon sequencing analysis revealed 7 mutations in 6 carrier parents, 4 of which were novel, including 1 missense mutation c.964 G>T (p.D322Y), 2 nonsense mutations [c.709C>T (p.Q237X) and c.1454G>A (p.W485X)], 1 parent was a compound heterozygous for novel splice site mutation c.459+4 A>C and known missense mutation c.532C>T (p.R178C). Also, previously reported missense mutation c.509G>A (p.R170Q) and splice site mutation c.805+1G>C mutations were also detected (*Table 4.8 and figure 4.9*). Mutation was not identified in 2 suspected carrier couples. For all, the presence of one mutant and one normal copy of this allele in both the parents suggest that the child would have had the mutation in the homozygous state, confirming TSD in the deceased child.

Table 4.8: *List of all HEXA sequence variants identified in TSD carrier parents*

Couple	Exon/ Intron	Nucleotide change	Amino acid change	Types	Domain	Recurrence	HGMD Accession Number	References
T-43*	Intron-4	c.459+4A>C*	-	IV	Domain-I	1	NA	Present study Sheth *et al.*, 2014a
T-88	Exon-5	c.509G>A (CpG)	p.R170Q	MS	Domain-II	1	CM920330	Nakona *et al.*, 1990
T-43*	Exon-5	c.532C>T* (CpG)	p.R178C	MS	Domain-II	1	CM900126	Tanaka *et al.*, 1990a
T-33	Exon-7	c.709C>T	p.Q237X	NS	Domain-II	1	NA	Present study Sheth *et al.*, 2014b
T-6	Intron-7	c.805+1G>C	-	IV	Domain-II	1	CS951439	Ribeiro *et al.*, 1995
T-4	Exon-8	c.964G>T	p.D322Y	MS	Domain-II	1	CM125497	Present study Mistri *et al.*, 2012
T-28	Exon-13	c.1454G>A	p.W485X	MS	Domain-II	1	NA	Present study Sheth *et al.*, 2014a

* Mutations was found in form of compound heterozygous in one parents

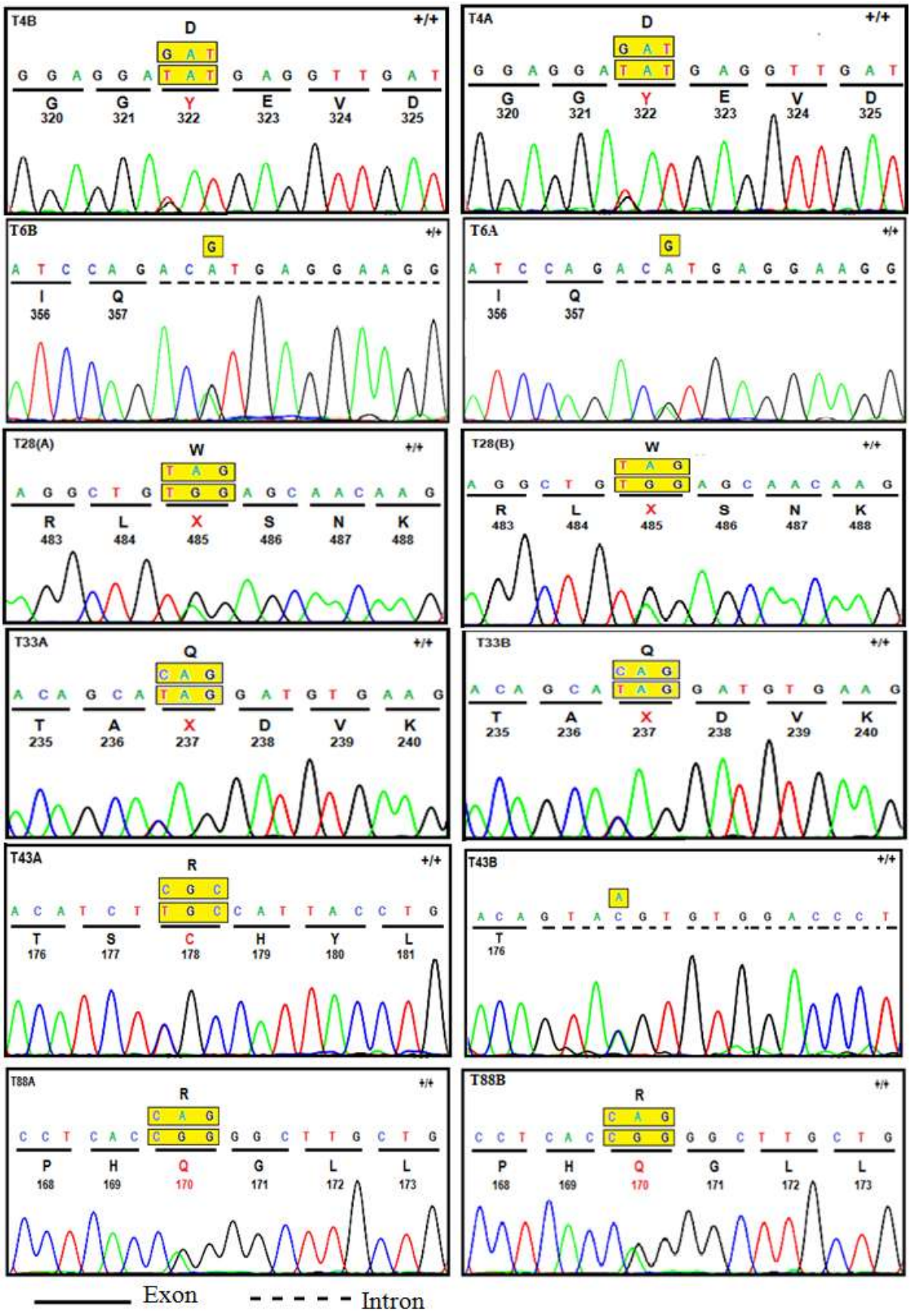


Figure 4.9: *Chromatogram of Carrier parents [please refer table 4.15 for details]*

4.2.3 Multiple ligation probe analysis (MLPA) study of *HEXA* gene

Multiplex ligation-dependent probe amplification (MLPA) study was carried out in 4 patients for deletion/duplication analysis where mutation was not identified and/or single allele was identified in common mutations screening and bi-directional sequencing of *HEXA* gene. In order to analyze the presence of large deletions of the *HEXA* gene non-detectable by sequencing, we developed a synthetic probe set for MLPA analysis. The MLPA analysis showed the presence of a homozygous deletion of exon-1 to -2 in one patient [*Table 4.9; Figure 4.10(a)*] and a heterozygous deletion of exon-1 with p.E462V as a second allele in *HEXA* gene was identified in two patient [*Table 4.9; Figure 4.10(b) & 4(c)*], while one patient showed a normal pattern [*Table 4.9; Figure 4.10(d)*].

Table 4.9: *MPLA analysis for deletion/duplication analysis of HEXA gene*

Patient ID	Deletions/ Duplications	No of Exons Deleted/ Duplicated	**MLPA probe ratio (Dosage quotient)	Clinical relevance	References
T-46	Homozygous deletions	2 (Exon 2 and 3)	0.0	Yes	Present study
T-58[$] T-80[$]	Heterozygous deletion	1 (Exon 1)	0.5	Yes	Present study
T-65	Normal	None	None	-	-

[$] Compound heterozygous with c.1385 A>T (p.E462V) mutation as shown in *table 4.15*

*The exon numbering is based on the *HEXA* mRNA reference sequence NM_000520.4 nomenclature in the NCBI Gen Bank database.

**MLPA ratios (dosage quotient) of below 0.7 or above 1.3 are indicative of a deletion (copy number change from two to one) or duplication (copy number change from two to three), respectively. A dosage quotient of 0.0 indicates a homozygous deletion, 0.35 to 0.65 indicates heterozygous deletion, 1.35 to 1.55 indicates heterozygous duplication and 1.7 to 2.2 indicates homozygous duplication.

Figure 4.10(a): *MLPA analysis suggests homozygous deletion of exon 2 & 3 in HEXA gene*

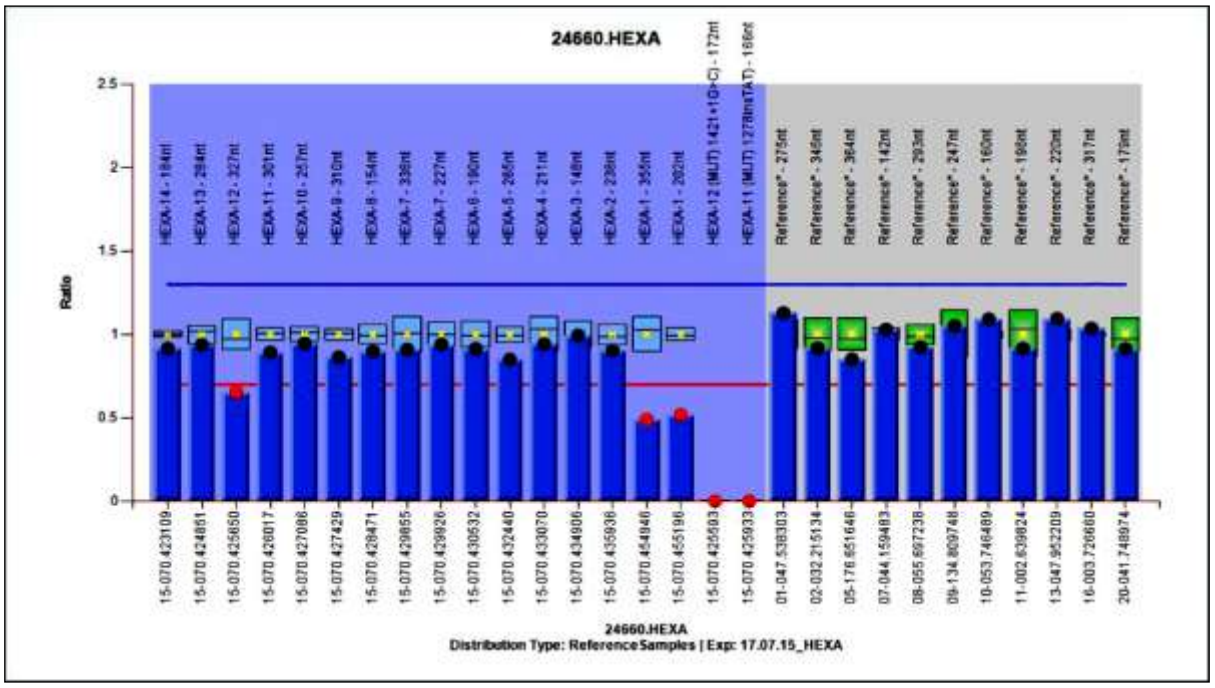


Figure 4.10(b): *MLPA analysis suggests heteroygous deletion of exon 1 in HEXA gene*

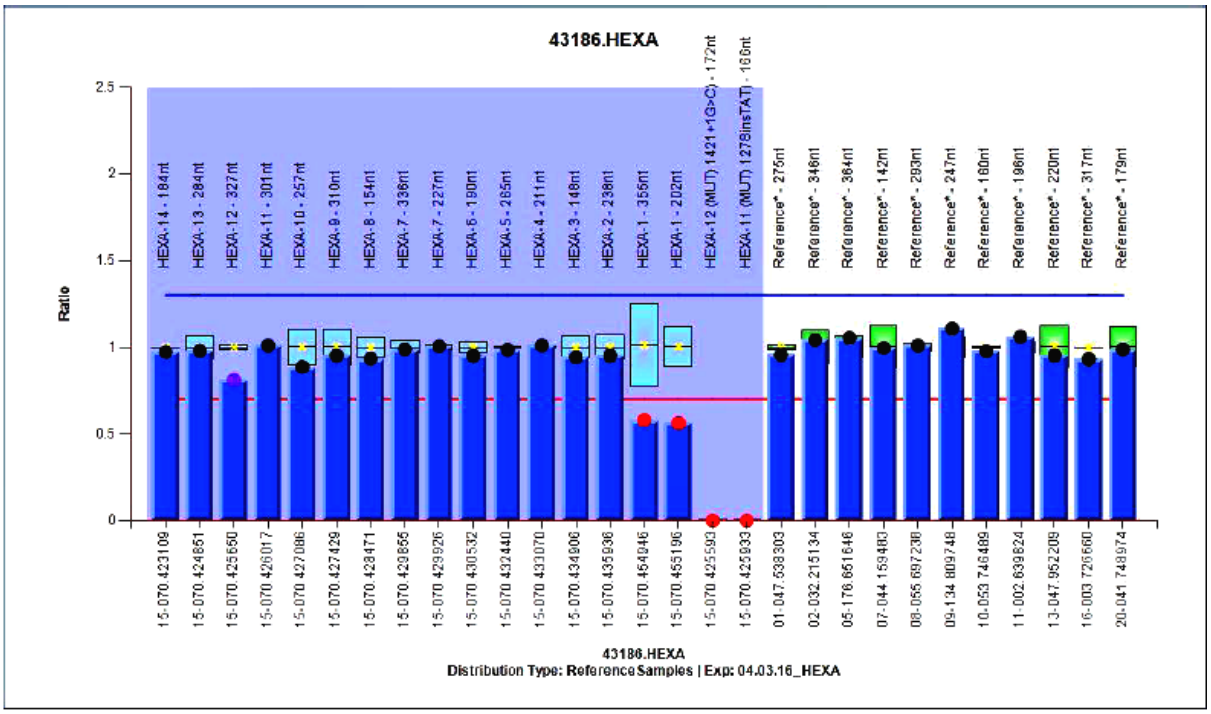


Figure 4.10(c): *MLPA analysis suggests heteroygous deletion of exon 1 in HEXA gene*

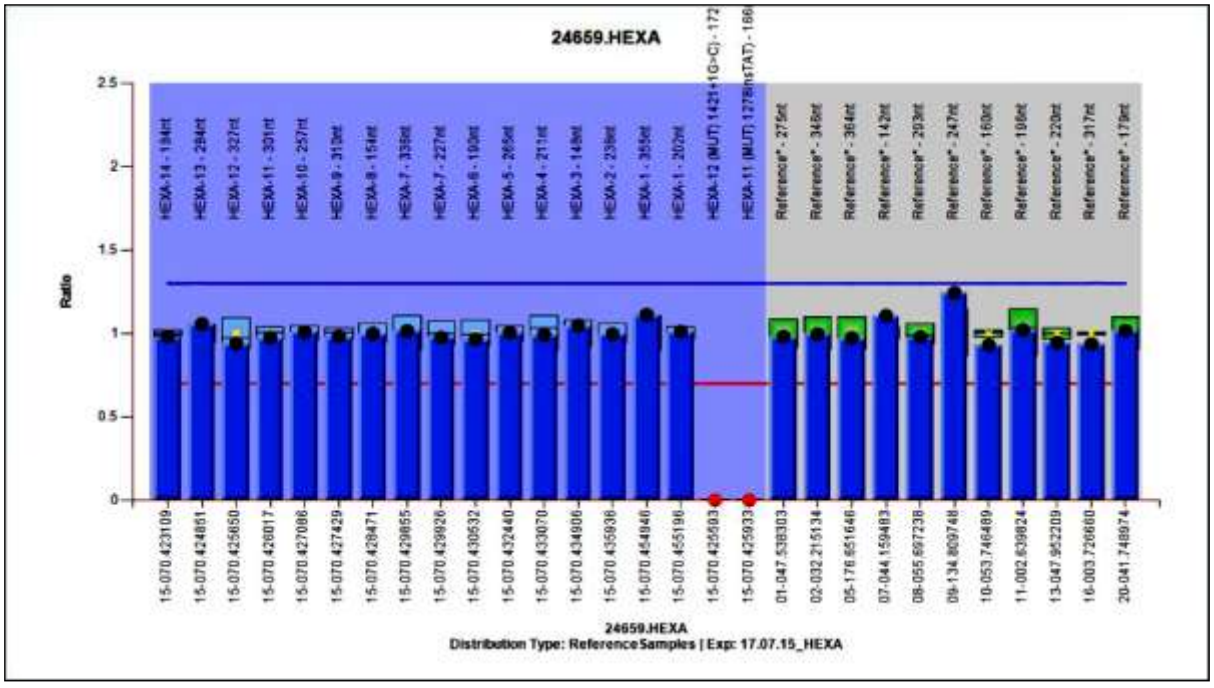


Figure 4.10(d): *MLPA analysis suggests normal pattern in HEXA gene*

4.2.4 Bidirectional sequencing of *GM2A* gene in enzymatically normal patients to rule out AB variant form of TSD (GM2 activator protein deficiency)

Molecular analysis for GM2A gene was carried out using bi-directional exon sequencing in 2 cases. Exon sequencing analysis revealed 2 variants in 2 cases, both were novel, including 1 nonsense variants c.472G>T (p.E158X) and 1 splice site mutation c.243-2 A>T (*Figure 4.11*).

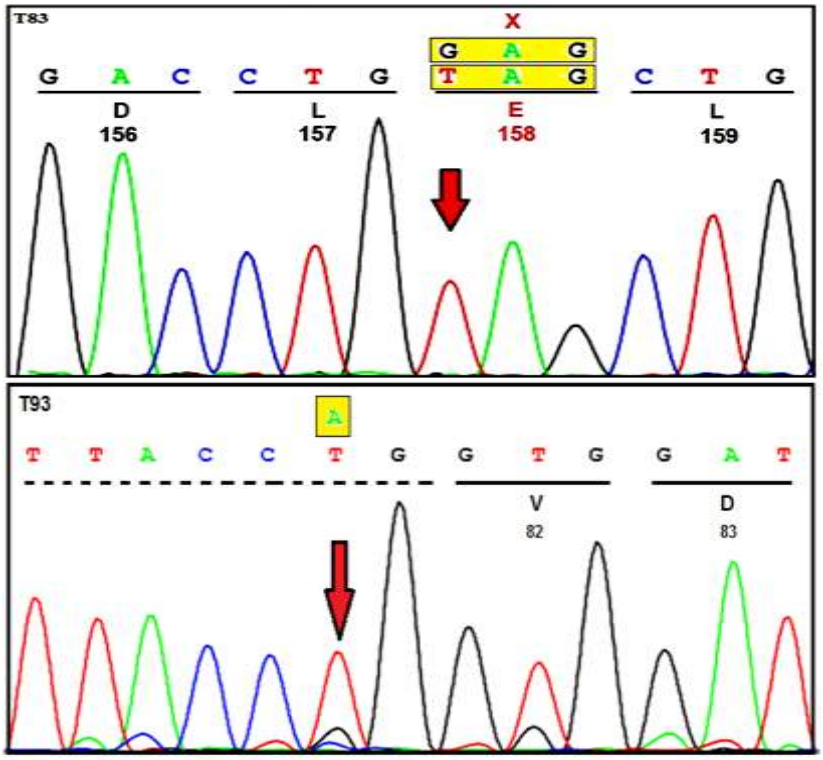


Figure 4.11: *Chromatogram of patients with AB variants TSD (GM2 activator protein deficiency)*

4.2.5 To evolve a cost effective DNA strategy to prevalent mutations in our population through identification of the carrier frequency by mass screening

In present study p.E462V, p.D322Y, and c.1278insTATC are the most common variants of *HEXA* gene identified in 21/51 (41.1%) cases. Considering these data, we have developed a simple cost effective ARMS and RFLP PCR based diagnosis to identify the affected as well as carrier individuals.

Present study revealed that the haplotypes unique to the founder mutation p.E462V which was not found in other patients and any ethnic groups. The founder mutation p.E462V was screened in 500 unaffected individual using ARMS–PCR (amplification refractory mutation

system polymerase chain reaction) and 1 individual was found to be carrier for this mutation leading to an allele frequency of 1/500. We also screened 100 unaffected individuals for c.1278insTATC and p.D322Y mutations using ARMS-PCR and PCR-RFLP respectively and none were found to be carrier for these mutations. However, sample size is too small to make out an allele frequency for c.1278insTATC and p.D322Y mutations.

4.3 BIOINFORMATICS ANALYSIS

4.3.1 Prediction of novel and known variants of *HEXA* gene

The SIFT index, Poyphen2 and Mutation Taster scores for the non-synonymaous single nucleotide substitutions are given in *table 4.10*. The predictions of the SIFT, Polyphen2 and Mutation Taster algorithms were in concordance with the observed pathogenicity in case of mutations p.M1T, p.E114K, p.R170W, p.R170Q, p.D175A, p.R178C, p.V206L, p.Y213H, p.T263I, p.G269R, p.D322Y, p.D322N, p.Q374P, p.R393P, p.G454R, p.E462V, p.G478R and p.R499C. Most of the missense variants were predicted as deleterious, that are affecting proteins. There were unmatched results of prediction of variant p.R393P, as it was predicted as tolerated or benign by SIFT software and as disease causing by Polyphen2 and Mutation taster (*Table 4.10*). The intronic variants c.459+4A>C, c.459+*5 G*>A, c.460-1 G>A, c.805+1 G>C, c.1073+1 G>A, frameshift variants p.F142LfsX57, p.F300HfsX21 and p.Y427IfsX5 were evaluated for pathogenicity using Mutation Taster (http://www.mutationtaster.org/) and/or NNSplice 0.9 softwares (http://www.fruitfly.org/seq_tools/splice.html) as shown in *table 4.11*.

Table 4.10: *Details of HEXA gene missense variants detected in infantile TSD patients and Insilico analysis*

Exon No	Codon number	Codon change	Amino acid change	Mutation T@ster score	SIFT Score	Polyphen2 Score (sensitivity, specificity)	RMSD between native and mutant structures	Potential energy after minimization (Kcal/mol) (Native Structure energy -25289.27)	Structural Effect	Amino acid change
1	1	ATG – ACG	Met1Thr	0.0 (DC)	0.00 (IT)	0.002 (0.99,0.30) (B)	0.185 (DC)	25111.12	Causes conformational changes	Non-Polar to Polar
2	114	GAG – AAG	Glu114Lys	3.30 (DC)	0.00 (IT)	0.998 (0.16,0.99) (PD)	0.134 (P)	-25045.67	Causes conformational changes	Acidic to basic
5	170	CGG – TGG	Arg170Trp	2.75 (DC)	0.00 (IT)	1.00 (0,1) (PD)	0.177 (DC)	-25025.01	Disrupts the beta sheet	Basic to nonpolar
5	170	CGG – CAG	Arg170Gln	1.17 (DC)		1.00 (0, 1) (PD)	0.171 (DC)	-25205.23	Disrupts the beta sheet	Basic to Polar
5	175	GAT – GCT	Asp175Ala	3.44 (DC)	0.00 (IT)	1.00 (0, 1) (PD)	0.268 (PD)	-25322.11	Disrupts the beta sheet	Acidic to nonpolar
5	178	GAT – GCT	Arg178Cys	4.91 (DC)	0.00 (IT)	1.00 (0,1) (PD)	0.278 (DC)	-25078.50	Affects active catalytic site	Basic to Uncharged
6	206	GTA – CTA	Val206Leu	0.87 (DC)	0.02 (IT)	0.999 (0.14, 0.99) (PD)	0.385 (DC)	-25031.02	Overpacking of residues and disruption of beta sheet	Uncharged to Non-polar
6	213	TAT – CAT	Tyr213His	2.26 (DC)	0.03 (IT)	1.00 (0,1) (PD)	0.175 (DC)	-25466.12	Over packing of residues and disruption of beta sheet	Cyclic to Non-cyclic
7	263	ACT – ATT	Thr263Ile	2.43 (DC)	0.02 (IT)	0.998 (0.27, 0.99) (PD)	0.198 (DC)	-25411-18	Disrupts the beta sheet	Polar to Non-Polar
7	269	GGT – GCT	Gly269Arg	3.41 (DC)	0.00 (IT)	1.00 (0,1)(PD)	0.225 (PD)	-25045.82	Over packing and backbone distortion	Non-polar to basic
8	322	GAT – AAT	Asp322Asn	0.63 (DC)	0.00 (IT)	1.00 (0,1) (PD)	0.176 (PD)	-25599.05	Affects active catalytic site	Acidic to uncharged
8	322	GAT- TAT	Asp322Tyr	4.36 (DC)	0.00 (IT)	1.00 (0,1) (PD)	0.178 (PD)	-25476.57	Affects active catalytic site	Non-cyclic to cyclic
10	374	CAG – CCG	Glu374Pro	2.0 (DC)	0.04 (IT)	1.00 (0,1) (PD)	0.205 (DC)	-25791.08	Affect the influence of catalytic active site and cause conformational changes	Neutral to nonpolar
11	393	CGA – CCA	Arg393Pro	2.81 (DC)	0.10 (T)	0.872 (0.83, 0.93) (PD)	0.387 (DC)	-24852.34	Causes conformational changes	Basic to non-polar
12	454	GGT – CGT	Gly454Arg	3.4 (DC)	0.00 (IT)	1.00 (0,1) (PD)	0.377 (DC)	-24998.07	Over packing, backbone strain and	Non-polar to basic

									disruption of beta sheet	
12	462	GAA – GTA	Glu462Val	3.3 (DC)	0.00 (IT)	1.00 (0,1) (PD)	0.182 (PD)	-25043.90	Affects the active site and the dimerization of alpha-beta subunits	Acidic to non-polar
13	478	GGG – AGG	Gly478Arg	3.41 (DC)	0.04 (IT)	0.946 (0.80, 0.95) (PD)	0.453 (PD)	-24590.66	Disrupts the alpha helix	Non-polar to basic
14	499	CGT – TGT	Arg499Cys	4.9 (DC)	0.00 (IT)	1.00 (0, 1) (PD)	0.171 (DC)	-25171.25	Causes conformational changes, salt bridge lost	Basic to polar

DC = Disease causing, P = Polymorphism, IT = Intolerant, PD= Probably damaging

The Mutations Taster score is taken from an amino acid substitution matrix (Grantham Matrix) which takes into account the physico-chemical characteristics of amino acids and scores substitutions according to the degree of difference between the original and the new amino acid scores may range from 0.0 to 6.0.

The SIFT score is the normalized the probability that the amino acid change is tolerated and ranges from 0 to 1. The amino acid substitution is predicted damaging is the score id <=0.05, and tolerated if the score is >0.05.

The Polyphen2 score is the naïve Bayes posterior to probability that this mutation is damaging and thus ranges from 0 to 215

Table 4.11: *Details of HEXA gene Intronic variants/deletion/insertion predicted by NNSplice 0.9 and Mutation Taster*

Mutations	Mutation Taster	MaxEntScan								NNSplice 0.9	
		Natural splice-site				Mutant splice-site				Natural splice site	Mutant splice site
		MAXENT	MDD	MM	WMM	MAXENT	MDD	MM	WMM		
c.459+4A>C	Disease causing	-	-	-	-	-	-	-	-	0.98	0.42
c.459+5 G>A	Disease causing	-	-	-	-	-	-	-	-	0.98	0.00
c.460-1 G>A	Disease causing	7.12	-	8.18	7.08	-1.63	-	-0.57	-1.67	0.89	0.00
c.805+1 G>C	Disease causing	10.77	14.98	11.11	11.73	2.49	6.70	2.84	3.46	1.0	0.00
c.1073+1 G>A	Disease causing	4.68	9.78	6.26	6.70	-3.50	1.60	-1.92	-1.48	0.71	0.00
p.F142LfsX57	Disease causing	-	-	-	-	-	-	-	-	-	-
p.F300HfsX21	Disease causing	-	-	-	-	-	-	-	-	-	-
p.Y427IfsX5	Disease causing	-	-	-	-	-	-	-	-	-	-

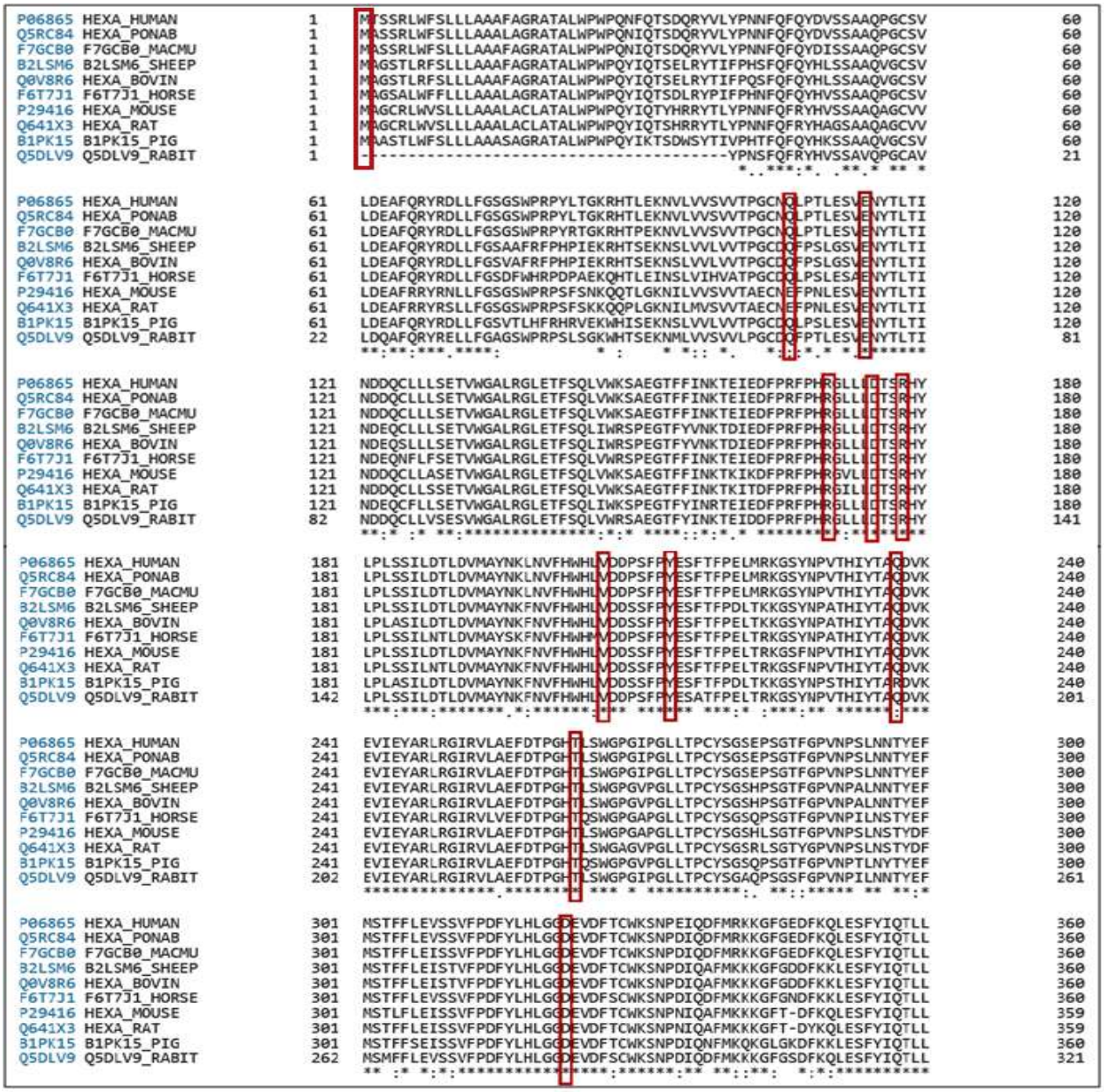
MEMENT: Maximum Entropy Model to score
MDM: Maximum Dependence Decomposition Model to score
MM: Markov Model to score
WMM: Weight Matrix Model to score

MaxEntScan is based on the approach for modeling the sequences of short sequence motifs such as those involved in RNA splicing which simultaneously accounts for non-adjacent as well as adjacent dependencies between positions. This method is based on the 'maximum entropy principle' and generalizes most previous probabilistic models of sequence motifs such as weight matrix models and inhomogeneous Markov models.

NNSplice 0.9 is an improved splice site predictor for the gene finding program Genie. The training and test sets of human and Drosophila melanogaster splice sites are available to the community for testing splice site predictors. They can be obtained from a collection of representative, standardized data sets of human and D. melanogaster genes.

4.3.2 Multiple sequence alignment

Using ClustalW, the sequence of Human *HEXA* gene was aligned with different species as shown in *figure 4.12*. We found that most of the missense and truncating variants identified in present study were affecting the conserved residues of the protein, so predicted to be affecting the functional status of the protein.



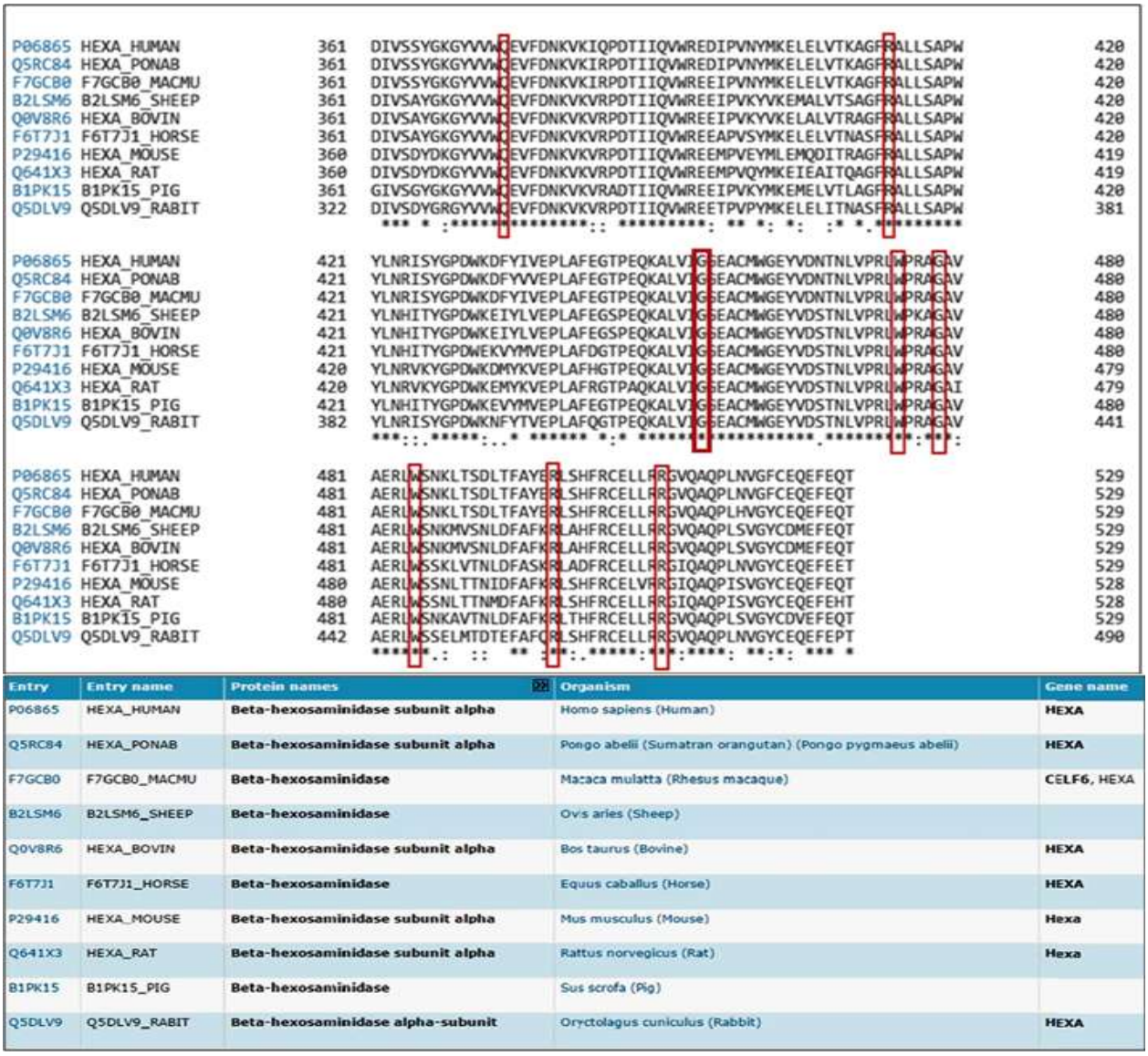
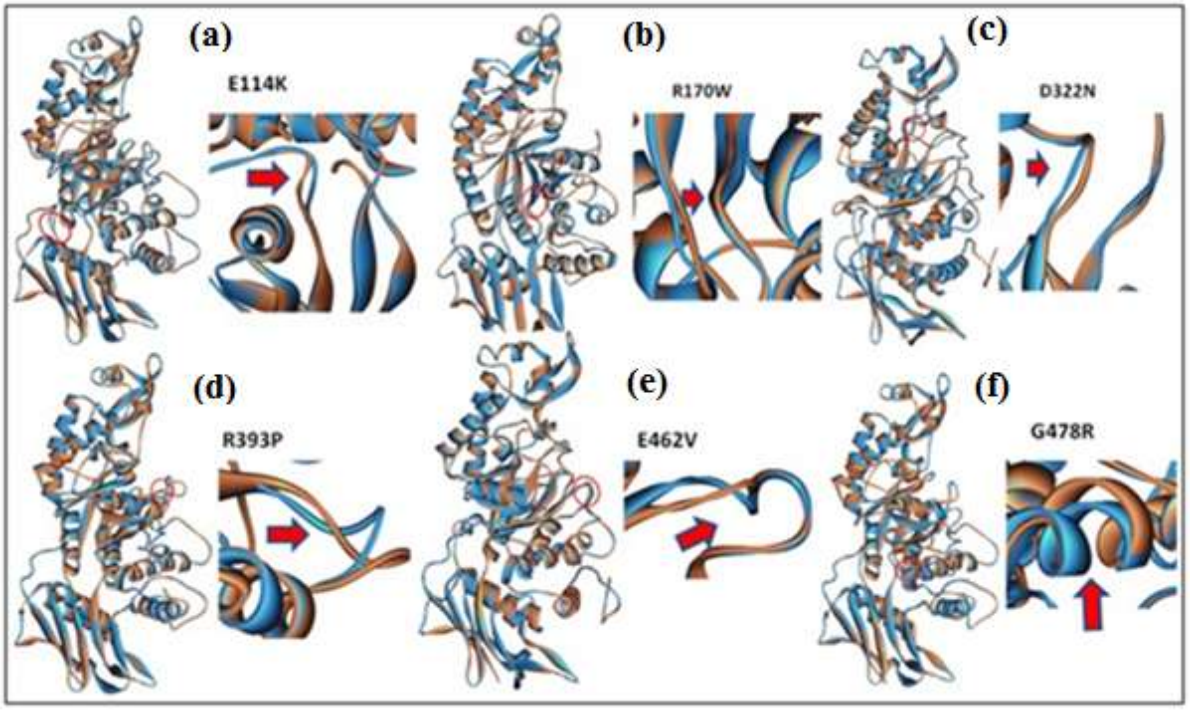
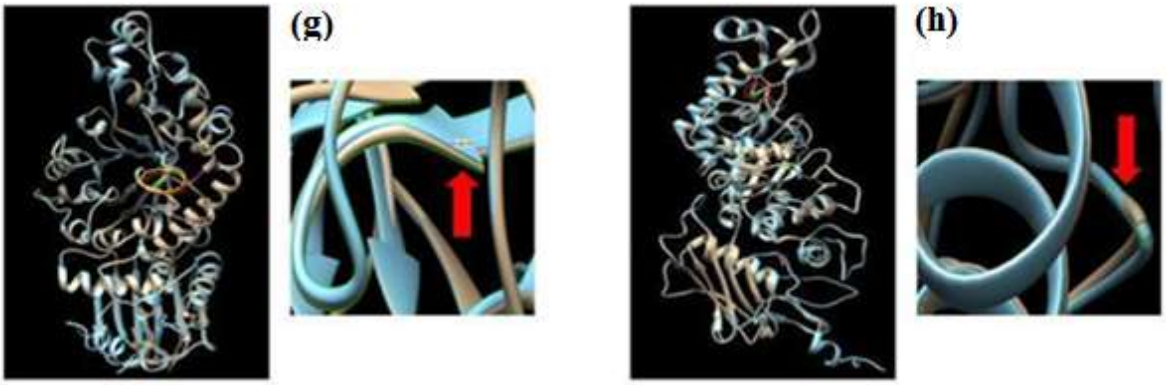


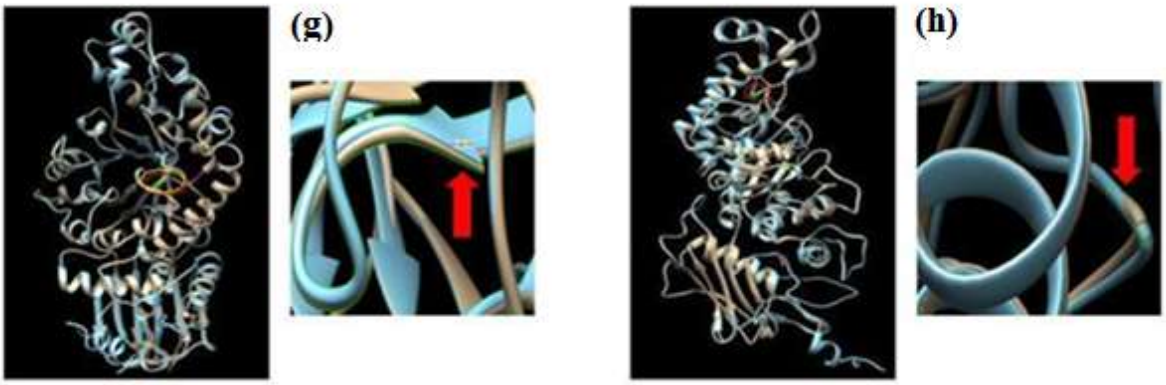
Figure 4.12: *Multiple protein sequence alignment using ClustalW shows evolutionary conservation of amino acid residues*

4.3.3 Protein Modeling and docking study

Structural study of missense mutant alleles were built using build mutant protocol of Accelrys Discovery studio 2.0 and/or UCFC Chimera software using the crystallographic structure of Hex-A (PDB ID: 2GJX) as the template. The RMSD (Root mean square deviation) values for the modeled mutants were found to be significant for pathogenicity for all missense mutations except p.E114K (*Table 4.10 and Figure 4.13(a)*). This mutation was determined pathogenic by SIFT and Polyphen analysis. The docking studies revealed that the residues αAsp322, αTyr421, αArg178, αGlu462, play a crucial role in the binding and orientation of GalNAc [*Figure 4.14(a)-(c) and Table 4.12 & 4.13*].


(a)
E114K
(b)
R170W
(c)
D322N
(d)
R393P
(e)
E462V
(f)
G478R


(g)
(h)


(g)
(h)

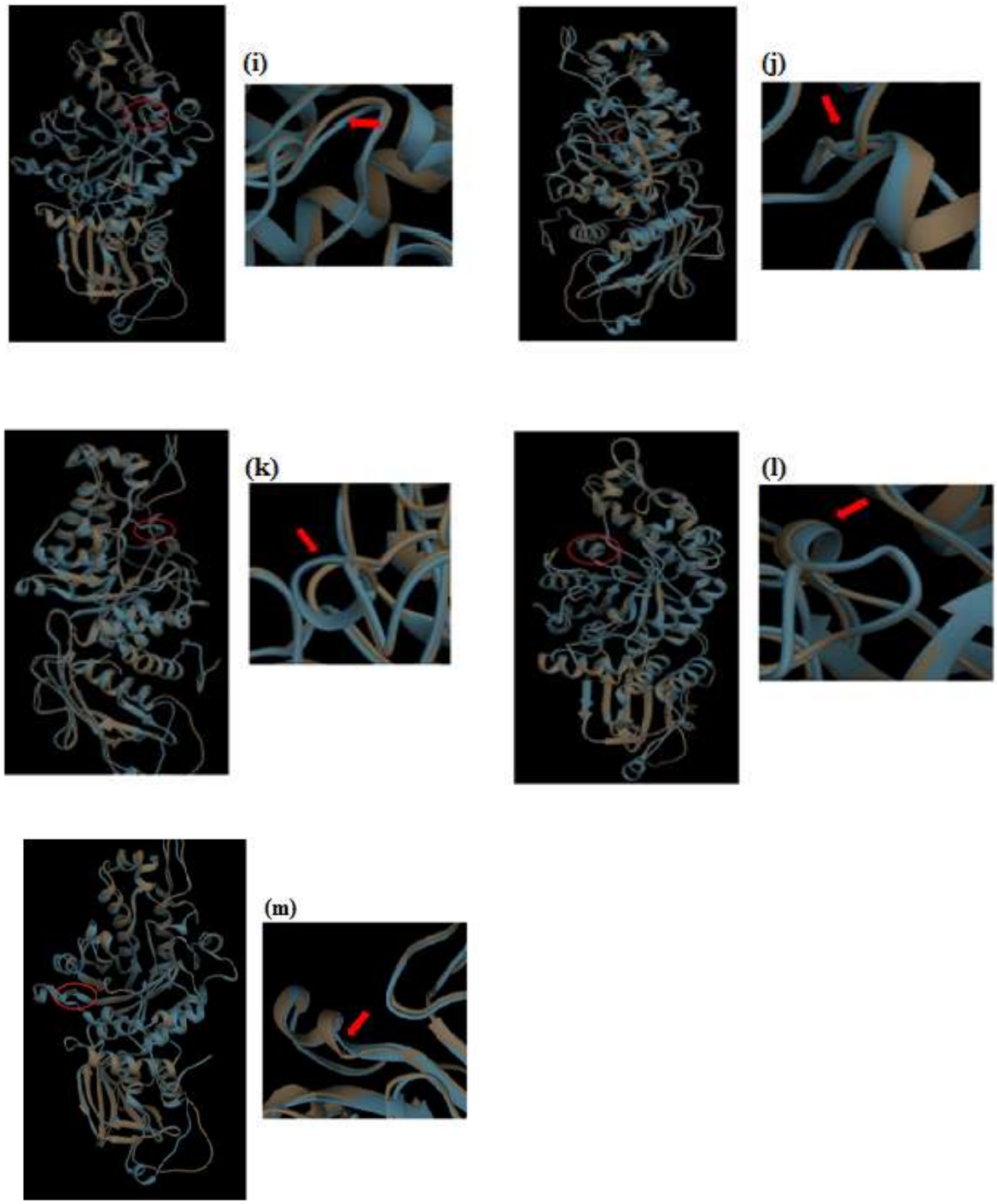


Figure 4.13: Superimposed native structures (blue) and mutant structure (brown) of the α subunit produced using Accelrys Discovery Studio software from top left clockwise: *(a) mutation p.E114K causes conformational change; (b) p.R170W disrupts the beta sheet; (c) p.D322N affects the active catalytic site, (d) p.R393P causes conformational change; (e) p.E462V affects the active site and the dimerization of alpha-beta subunits; (f) p.G478R disrupts the alpha helix; (g) mutation p.D175A disrupts the β-sheet; (h) p.G269R disrupts the beta sheet; (i) p.V206L creates Overpacking of residues and disruption of β-sheet; (j) p.Y213H creates overpacking of residues and disruption of β-sheet; (k) p.T263I creates overpacking and backbone disruption; (l) p.Q374P affect the influence of catalytic active site and cause conformational changes and (m) p.G454R creates overpacking, backbone and β-sheet disruption.*

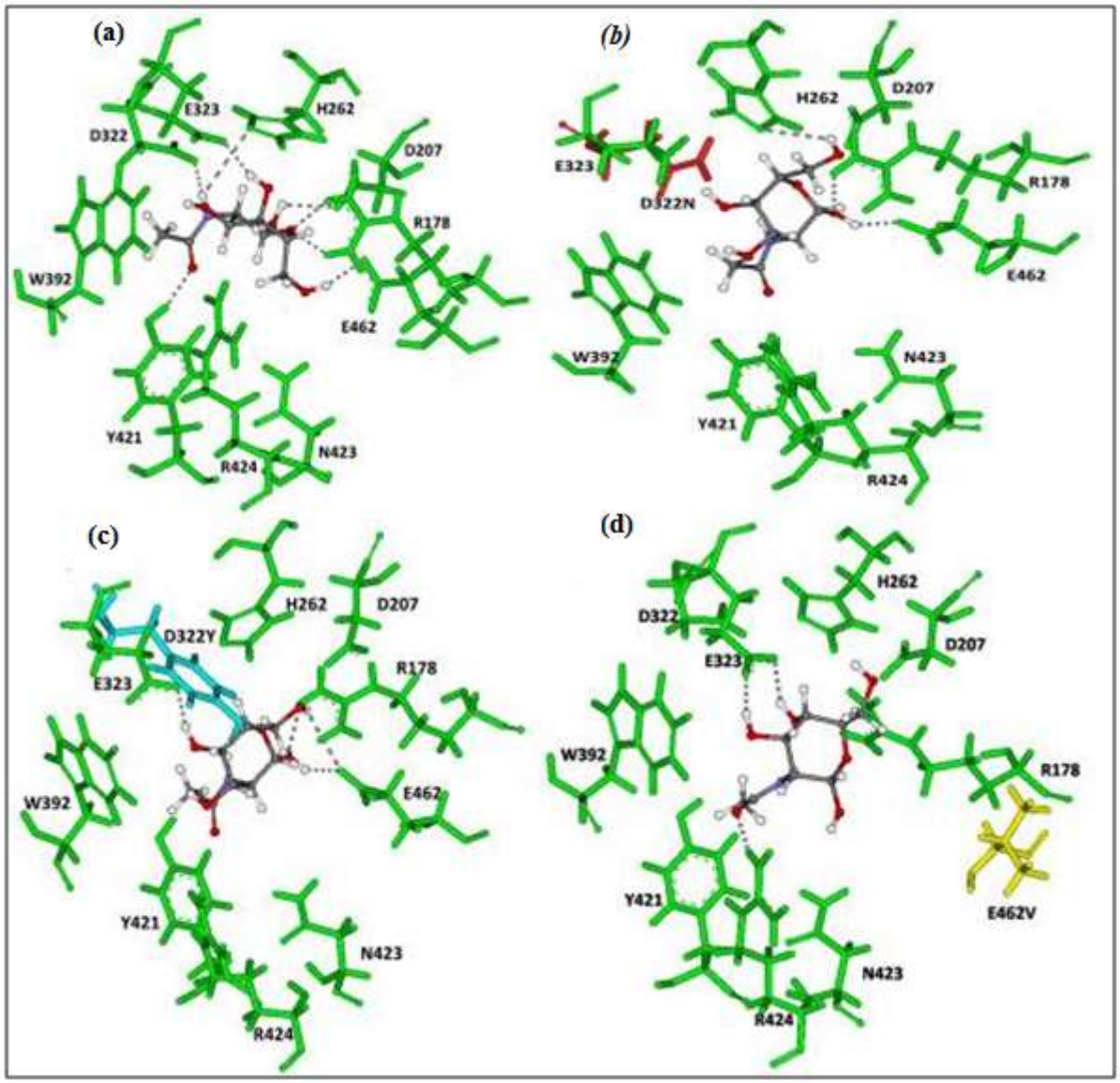


Figure 4.14: Stereoscopic view of the docking experiments. *Green sticks indicate C^{α} trace of amino acids involved in the active site and CPK (Corey-Pauling-Koltun) coloring scheme represents ligand GalNAc (N acetyl galactosamine) portion of GM2 ganglioside. (a) Wild type (αHex-A-GalNAc complex); (b) Mutant (αD322N-GalNAc), Red shows mutation p.D322N; (c) Mutant (αD322Y-GalNAc), Cyan shows mutation p.D322Y; (d) Mutant (α E462V-GalNAc), yellow shows mutation p.E462Y.*

Table 4.12: *Ligand binding energy details for wild type and mutant α-subunits*

Complex	**Binding Energy (kcal/mol)**
Wild Type-GalNAc	-115.94
αD322N-GalNAc	-38.87
αD322Y-GalNAc	-52.22
αE462V-GalNAc	-80.88

Table 4.13: *Hydrogen bond interaction for the wild type and Mutant complex*

Complex	**Protein (Residue: Atom)**	**Ligand Atom**
Wild Type-GalNAc	ARG178:HH12	O2
	ARG178:HH22	O2
	TYR421:HH	O6
	TRP460:HE1	O6
	ASP207:OD1	H26
	GLU323:OE1	H28
	GLU462:OE2	H29
	HIS262:NE2	H30
	ASP322:OD2	H20
αD322N-GalNAc	ARG178:HH22	O1
	GLU462:OE2	H26
	HIS262:NE2	H29
αD322Y-GalNAc	ARG178:HH22	O1
	GLU462:OE2	H26
	GLU323:OE2	H28
	GLU462:OE2	H29
αE462V-GalNAc	ARG424:HH21	O6
	GLU323:OE2	H27
	GLU323:OE1	H28

4.3.4 Haplotype analysis

The mutation c.1385A>T (p.E462V) was found in the homozygous and compound heterozygous state in 8 and 3 infantile form of TSD patients respectively, belonging to unrelated families with common ethnicity (from Gujarat) indicating a founder effect Haplotype analysis was performed by sequencing of the intron 1, 5, 12, 13 and a 417 base pair region 3' to *HEXA* gene which contained several polymorphic markers. In all TSD patients and different ethnic controls (*Table 4.14*) revealed the haplotypes, unique to the founder mutation and not found in other patients and any ethnic controls.

Table 4.14: *Haplotype analysis of founder mutation E462V in Gujarati patients with Tay-Sachs disease*

3' to *HEXA* gene (a)	**Allele**	**Intron 13 (b)**	**Allele**	**Intron 12 (c)**	**Allele**	**Intron 5 (d)**	**Allele**	**Intron 1 (e)**	**Allele**
rs35949555	T	rs12904378	C	rs74020947	G	rs191809305	A	rs191094610	A
rs11629508	C	rs113387077	A	rs57733983	T	rs188570040	T	rs188410016	C
rs76075374	C	rs2912217	G	rs189856670	A	rs60920713	C	rs186683578	A
rs60288568	G	rs190224431	G	rs140288703	G	rs59427837	C	rs184065715	T
rs112626309	G	rs145393752	T	rs185764548	C	rs12910617	C	rs78629973	C
rs3087652	T	rs112806142	G	rs111680766	A	rs12593333	A	rs4470105	C
		rs890313	G	rs147324677	C	rs12592727	T	rs78278321	G
		rs111827252	C	rs113941121	A	rs4776594	G	rs76530364	T
		rs75015614	T	rs34085965	A			rs75756977	C
		rs62022857	C	rs2303448	C			rs75981720	C
		rs118002327	C					rs76941148	C
		rs2912218	T					rs80039124	T
		rs113665670	CTCT					rs78970750	A
		rs149948017	T					rs76950885	C
		rs4777502	C					rs74738827	A
		rs147502219	T					rs80238386	G
		rs140091006	G					rs77154656	C
		rs145012038	C					rs78097627	C
		rs149092488	G					rs7511366	A
								rs74325922	G
								rs74787391	C

Region (a)= 417 bp region 3' to HEXA gene (chromosome 15 plus strand: 72635626 to 72636042); region (b) = 405 bp region in intron 13 of HEXA gene (chromosome 15 plus strand: 72637428 to 72637994; region (c): 489 bp region in intron 12 of HEXA gene (chromosome 15 plus strand: 72638306 to 72638794); region (d): 424 bp region in intron 5 of HEXA gene (chromosome 15 plus strand: 72639456 to 72639879); region (e): 405 bp region in intron 1 of HEXA gene (chromosome 15 plus strand: 72656546 to 72656950 and 461 bp region in intron 1 of HEXA gene chromoseome 15 plus strand: 72659310 to 72659590). The chromosome coordinates are as per Human Genome Assembly Feb 2009 (GRCh37/hg

4.4 GENOTYPE-PHENOTYPE CORRELATION OF TSD PATIENTS

In present study, the age of onset (AO) and the age at diagnosis (AD) are almost similar for all types of variants, there is no patients with TSD showing decreased level of residual activity of the Hex-A enzymes in early age correlating with infantile form of the disease (*Table 4.15 and 4.16*). Though, adult onset TSD has been shown in the literature, none of our subjects were in the adult age group. This could be due to reference bias as all patients referred in our study were by a pediatrician or pediatric neurologist.

When different types of sequence variations were compared with the phenotypes of TSD patients, it was observed that patients carrying early truncating variants were showing little severe type as compared to the patients carrying point variants. However, study required more number of patients, to prove this statement. Further comparison between missense, nonsense and splice variants and individual clinical features of patients showed that the patients carrying missense variants had more severed phenotypes (*Table 4.17*). Moreover, the study observed all missense, nonsense, splice site mutations, small/large deletions and insertions to be associated with infantile form of TSD.

Present study demonstrates that TSD patients are more or less of infantile type in India with severe phenotype irrespective of the genotype. None of our patients were juvenile or late onset TSD. Though, it could be due to failure of clinical identification as well.

Table 4.15: *Overall clinical, biochemical and molecular profile of Indian TSD patients*

Patient ID	Age at diagnosis (Months/ Sex	Age at onset (Months)	Native State	Cosan-guinity	Hex-A activity (MUGS) (nmol/hr/mg) = (x)	Total Hex activity (MUG) = (y)	Hex A % =(x/y) X 100	Genotypes		Phenotypes
								Nucleotide level (Allele from Father/ Allele from Mother)	Protein level (Allele from Father/ Allele from Mother)	
T-1	11/M	6	Gujarat	No	4.5	ND	-	c.1385A>T/ c.1385A>T	p.E462V/ p.E462V	Regression of milestones, cherry red spot, abnormal muscle tone, seizures, hyperacusis, abnormal MRI study, abnormal EEG
T-5	17/M	6	Gujarat	No	4.8	1827.6	0.2	c.1385A>T/ c.1385A>T	p.E462V/p.E462V	Regression of miletones, cherry red spot, hyperaccusis, hypotonia, normal MRI study
T-11	12/M	6	Gujarat	No	5.9	2208.9	0.26	c.1385A>T/ c.1385A>T	p.E462V/p.E462V	Regression of milestones, GTC convulsion at 6 month of age, neuroregression, unable to neck holding, cherry red spot, vision loss, hearing loss, hypotonia, brisk reflexes, Power>3, constipation since birth, abnormal MRI study
T-12	17/M	7	Gujarat	No	10.3	2009	0.51	c.1385A>T/ c.1385A>T	p.E462V/p.E462V	Regression of milestones, cherry red spot, neuroregression, paracardiac pneumonia, hypotonia
T-13	17/M	4	Gujarat	No	5.8	2288	0.25	c.1385A>T/ c.1385A>T	p.E462V/p.E462V	Regression of milestone, cherry red spot, abnormal muscle tone, seizures, hyperacusis, abnormal MRI study, abnormal EEG
T-29	13/M	6	Gujarat	Yes	0.0 Father -60%	2131.0 731.7	0.0 -	c.1385A>T/ c.1385A>T	p.E462V/p.E462V	Regression of milestone noticed after 6 month of age,

					(HLA) Mother– 53% (HLA)	528.3	-			social smile started at 3 month and stopped since last 2 month, seizures, hyperacusis, DTR brisk, hypotonia, cherry red spot
T-76	18/F	6	Gujarat	No	6.1	2451	0.25	c.1385A>T/ c.1385A>T	p.E462V/p.E462V	Regression of milestones, cherry red spot, abnormal muscle tone, seizures, hyperacusis, abnormal MRI study, abnormal EEG
T-85	12/M	4	Gujarat	No	4.7	2049.7	0.23	c.1385A>T/ c.1385A>T	p.E462V/p.E462V	Regression of milestones, cherry red spot, abnormal muscle tone, seizures, hyperacusis, abnormal MRI study
T-86	17/M	5	New Delhi	Yes	1.39	ND	-	c.1385A>T/ c.1385A>T	p.E462V/p.E462V	Regression of milestones, seizures, hypotonia, deep and superficial reflexes, hyperaccusis, cherry red spot, mild hepatomegaly present, abnormal MRI study
T-58	12/F	6	Gujarat	No	7.9	2185.7	0.36	c.1385A>T/ Exon-1 deletion	p.E462V/ Not identified	Regression of milestone, cherry red spot, abnormal muscle tone, seizures, hyperacusis, hearing impairment
T-80	12/F	6	Gujarat	No	2.5	1635	0.15	c.1385A>T/ Exon-1 deletion	p.E462V/ Not identified	Regression of milestone, hypotonia, hyperacusis, cherry red spot, abnormal MRI
T-4	15/M	8	Gujarat	No	0.00	761.2	0.00	c.964G>A/ c.964G>A	p.D322Y/p.D322Y	Regression of milestones, cherry red spot, hypotonia, seizures, brisk reflexes, hyperacusis, abnormal EEG
T-44	24/M	4	Gujarat	No	7.1	1999.9	0.35	c.964G>A/ c.964G>A	p.D322Y/p.D322Y	Regression of milestones, DTR++, poor vision, seizures, hyperacusis, hearing problem,

										abnormal EEG
T-56	16/F	8	Gujarat	No	8.3	1612.9	0.5	c.964G>A/ c.964G>A	p.D322Y/p.D322Y	Regression of milestone since 8 month of age, cherry red spot, DTR+++, hypotonia, irritability, abnormal MRI
T-59	1.5/M	1.5	Gujarat	No	8.9	2254.3	0.39	c.964G>A/ c.964G>A	p.D322Y/p.D322Y	Mild regression of milestone, sluggishness, hypotonia
T-30*	16/F	6	Gujarat	No	2.1	ND	-	NA Father – (c.1277_1278insTATC/-) Mother- (c.1277_1278insTATC/-)	NA Father – [p.Y427IfsX5/-] Mother- [p.Y427IfsX5/-]	Regression of milestones, cherry red spot, abnormal muscle tone, seizures, hepatomegaly, normal MRI
T-48	16/M	6	Maharashtra	No	6.2	2585.3	0.24	c.1277_1278insTATC/ c.1277_1278insTATC	p.Y427IfsX5/ p.Y427IfsX5	Regression of milestones, cherry red spot, abnormal muscle tone, seizures, hyperacusis, hepatomegaly, normal MRI, abnormal EEG
T-50	24/M	6	Maharashtra	Yes	0.0	833.0	0.0	(c.1277_1278insTATC/ c.1277_1278insTATC)	p.Y427IfsX5/ p.Y427IfsX5	Regression of milestones. cherry red spot, hypotonia
T-60	15/M	7	Maharashtra	Yes	9.3	2254.3	0.41	c.1277_1278insTATC/ c.1277_1278insTATC	p.Y427IfsX5/ p.Y427IfsX5	Regression of milestones, cherry red spot, hypotonia, hyperacusis
T-73	24/M	4	Maharashtra	No	2.5	862	0.29	c.1277_1278insTATC/ c.1277_1278insTATC	p.Y427IfsX5/ p.Y427IfsX5	Regression of milestones, cherry red spot, abnormal muscle tone, seizures, abnormal MRI, abnormal EEG
T-38*	14/F	7	Gujarat	No	2.5	1792	0.14	NA Father – (c.1277_1278insTATC/-) Mother- [c.964G>A/-)	NA Father - [p.Y427IfsX5/-] Mother- [p.D322N/-]	Regression of milestones, cherry red spot, abnormal muscle tone, seizures
T-8	30/M	8	Uttar Pradesh	No	4.08	1453.0	0.28	Father-[c.964G>T/-] Mother-[c.964G>T/-]	Father - [p.D322N/-] Mother -[p.D322N/-]	Regression of milestones, cherry red spot, seizures, poor

										vision Café-au-lait spot on back
T-84	12/F	6	Gujarat	No	6.7	1525	0.44	c.964G>T/ c.964G>T	p.D322N/ p.D322N	Regression of milestones, cherry red spot, abnormal muscle tone, hyperacusis, seizures
T-6*	12/M	4	Andhra Pradesh	Yes	Father- 59.3 % (HLA) Mother - 59.1 % (HLA)	ND	-	Father -[c.805 +1G>C/-] Mother-[c.805 +1G>C/-]	Father – Not identified Mother – Not identified	Regression of motor milestone cherry red spot, poor vision hypotonia
T-32	17/M	9	Maharashtra	No	9.6	1589.0	0.6	c.805+1G>C/ c.805+1G>C	Not identified/ Not identified	Regression of miletone, cherry red spot, abnormal muscle tone, recurrent seizures, normal MRI, abnormal EEG
T-45	13/F	6	Maharashtra	No	7.0	2602.7	0.27	c.524A>C/c.805G>C	p.D175A/p.G269R	Regression of miletone, cherry red spot, abnormal muscle tone, hyperacusis, abnormal MRI study
T-28*	17/M	5	Maharashtra	Yes	NA Mother –62% (HLA) Father – 60% (HLA)	-	-	NA Father-[c.1454G>A/-] Mother-[c.1454G>A/-]	NA Father – [p.W485X/-] Mother- [p.W485X/-]	Regression of milestones, seizures, abnormal muscle tone, abnormal MRI study
T-39*	12/M	7	Maharashtra	No	10.7	1785.0	0.59	NA Father – (c.1454G>A/-) Mother- c.1454G>A	NA Father - [p.W485X/-] Mother -[p.W485X/-]	Regression of milestones, seizures, hypotonia
T-7	20/M	10	Tamil Nadu	Yes	17.2	2054.5	0.83	c.1178G>C/ c.1178G>C	p.R393P/ p.R393P	Regression of milestones, cherry red spot, loss of eye contact, hypotonia, seizures nystagmus
T-9	17/M	8	Maharashtra		6.2	2090	0.30	c.426delT/c.426delT	p.F142LfsX57/ p.F142LfsX57	Regression of milestones, cherry red spot, hypotonia, seizures, hyperacusis, abnormal MRI, abnormal EEG

T-15	11 /F	6	Gujarat		10.8	1273.2	0.84	c.1528 C>T/c.1528 C>T	p.R510X/ p.R510X	Regression of milestones, cherry red spot, abnormal muscle tone, seizures, hyperacusis, hearing loss, abnormal EEG
T-16	17/F	8	Iraq	Yes	18.4	1776.9	1.03	c.508C>T/c.508C>T	p.R170W/p.R170W	Regression of milestones, vision loss, deep and superficial reflexes, seizures, abnormal MRI study, abnormal EEG
T-17	10 /M	5	Maharashtra	No	23.8	1840.2	1.29	c. 340G>A /c.340G>A	p.E114K/p.E114K	Regression of milestones, cherry red spot, seizures, mild hepatomegaly
T-26	24/M	10	Maharashtra	No	6.2	1721.0	0.36	c.788C>T/c.788C>T	p.T263I/p.T263I	Regression of milestone, cherry red spot, spasticity, hypotonia, seizures, abnormal MRI study
T-27	12/M	6	Maharashtra	Yes	7.2	2100	0.3	c.672+30T>G/ c.1432G>A	Not identified/ p.G478R	Regression of milestone, cherry red spot, hypotonia, seizures, abnormal MRI study
T-33*	-		Maharashtra	No	NA	NA	-	NA Father –(c.709C>T/-) Mother-(c.709C>T/-)	NA Father -[p.R237X/-] Mother -[p.R237X/-]	Regression of milestone, cherry red spot, poor vision, abnormal muscle tone, abnormal EEG study
T-37	12/M	6	Maharashtra	No	12.4	2129.0	0.58	c.1121A>C/ c.1121A>C	p.Q374P/p.Q374P	Regression of milestones, cherry red spot, abnormal muscle tone, hyperacusis
T-40	12/M	6	Maharashtra	No	0.0	2318.7	0.0	c.1421G>A/ c.1421G>A	p.W474X/p.W474X	Mild regression of milestone, cherry red spot, seizures
T-43	12/M	5	Kerala	Yes	0.9	761	0.12	c.532 C>T/ c.459 +4A>C	p.R178C/ Not identified	Regression of milestone, cherry red spot, abnormal muscle tone, seizures,

										abnormal MRI
T-69	12/M	6	Kerala		8.1	1301.5	0.62	c.459+4A>C/ c.459+4A>C	Not identified/ Not identified	Regression of milestone, cherry red spot, abnormal muscle tone, seizures, hyperacusis, abnormal muscle tone, abnormal MRI, abnormal EEG
T-46	14/M	8	Gujarat	No	1.05	-	-	Exon-2-3del/ Exon-2-3del	Not identified/ Not identified	Regression of milestone, cherry red spot, poor vision, abnormal muscle tone, hyperacusis, seizures, abnormal MRI, abnormal EEG
T-47	14/M	5	Gujarat	Yes	12.5	2050.0	0.6	c.898_905delTTCATGAG/ c.898_905delTTCATGAG	p.F300HfsX21/ p.F300HfsX21	Regression of milestone, cherry red spot, convulsion, spasticity, poor vision
T-49	17/F	5	Maharashtra	No	0.0	-	0.0	c.459+5G>A/c.459+5G>A	Not identified/ Not identified	Regression of milestone, convulsion, abnormal MRI
T-65	24/F	8	Kerala	Yes	9.0	1807	0.50	c.460-1G>A/c.460-1G>A	Not identified/ Not identified	Regression of milestone, hearing impairments, cherry red spot, abnormal muscle tone, abnormal MRI, abnormal EEG
T-70	36/F	11	Gujarat	No	12.5	2499.9	0.50	c.1173+1G>A/c.316C>T	Not identified /p.Q106X	Regression of milestone, cherry red spot, seizures, hypotonia, hyperacusis, abnormal MRI
T-74	24/F	9	Karnataka	No	5.2	1000	0.52	c.1495C>T/c.1495C>T	p.R499C/p.R499C	Regression of milestone, cherry red spot, seizures, hypotonia, hyperacusis, abnormal MRI, abnormal EEG
T-75	24/F	9	Gujarat	No	9.6	1682.2	0.57	c.616G>C/c.616G>C	p.V206M/p.V206M	Regression of milestone, cherry red spot, abnormal

										muscle tone, hyperacusis
T-81	16/M	5	Karnataka	No	7.5	1958.8	0.38	c.637T>C/c.637T>C	p.Y213H/p.Y213H	Regression of milestone, cherry red spot, abnormal muscle tone, seizures, hearing impairment, abnormal MRI
T-87	17/M	8	Gujarat	Yes	5.6	1280.1	0.44	c.2T.>C/c.2T.>C	p.M1T/p.M1T	Regression of milestone, cherry red spot, hypotonia, seizures, hyperacusis, hearing impairment, abnormal EEG
T-88	12/F	6	Gujarat	No	1.2	-	-	NA Father –(c.509G>A/-) Mother-(c.509G>A/-)	NA Father –(p.R170Q/-) Mother-(p.R170Q/-)	Regression of milestones, cherry red spot, abnormal muscle tone, seizures, mongolion spot, abnormal EEG
T-90	17/M	8	New delhi	No		-	-	c.1360G>C/ c.1360G>C	p.G454R/p.G454R	Regression of milestone, cherry red spot, hypotonia, hyperacusis, abnormal MRI

*DNA of index case is not available, HLA-heat labile activity in carrier parents, NA-Not available

Table 4.16: *Genotype-phenotype correlation of classical and AB variants (GM2A) TSD patients based on type of sequence variant*

Clinical features		MS mutation (n=31)	Intronic variants (n=9)	NS mutation (n=7)	FS mutation (n=8)	Large deletion (n=2)
Age at Diagnosis (Months)	***Mean±SD***	15.5±6.1	17.8±8.5	16.6±9.7	17.5±4.1	13.0±1.4
	Median (IQR)	15.5	14.5	17	16	13
Age of Onset (Months)	***Mean±SD***	6.2±1.9	6.8±2.4	6.8±2.1	6.1±1.2	6.1±1.2
	Median (IQR)	6.0	6.0	6.0	6.0	6.0
Phenotypes	***Infantile***	31 (100%)	9 (100%)	7 (100%)	8 (100%)	2 (100%)
	Juvenile	0	0	0	0	0
	Adult onset	0	0	0	0	0
Loss of motor milestone (Cognitive and physical)		32 (100%)	9 (100%)	7 (100%)	8 (100%)	2 (100%)
Ophthalmic impairment	***Cherry red spot***	29 (93.5%)	8 (88.8%)	7 (100%)	8 (100%)	2 (100%)
	Poor vision/ vision loss	4 (12.9%)	1(12.5%)	1 (16.6%)	0	1 (50%)
Abnormal muscle tone		29 (93.5%)	6 (75%)	5 (83.3%)	8 (100%)	2 (100%)
Seizures/ convulsion		26 (83.8%)	6 (75%)	3 (50%)	6 (75%)	2 (100%)
Hyperacusis		12 (38.8%)	2 (25%)	2 (33.3%)	3 (37.5%)	2 (100%)
Hearing impairment		5 (16.1%)	2 (25%)	1 (16.6%)	0	0
Mild hepatomegaly		3 (9.6%)	0	0	0	0
Mongolian spots		0	1 (12.5%)	1 (12.5%)	0	0
Abnormal Imaging study (n=24)		14 (45.1%)	5 (62.5%)	2 (25%)	2 (25%)	1 (50%)
Abnormal EEG/EMG (n=17)		10 (32.2%)	2 (25%)	1 (12.5%)	3 (37.5%)	1 (50%)

Table 4.17 *Genotype-phenotype correlations of classical and AB variants (GM2A) TSD patients individually*

Phenotypes / Genotypes	No of Pts. (n)	Loss of motor milestone (Cognitive and physical)	Cherry red spot	Poor vision/ vision loss	Abnormal muscle tone	Seizures/ Convulsion	Hyperac usis	Hearing impair-ment	Mongolian spot	Mild Hepato-megaly	Imaging study (n=30)	Abnormal EEG/EMG (n=17)
HEXA gene												
M1T	1	+	+	-	+	+	+	+	-	-	NA	Abnormal
Exon 1 delition/ p.E462V	2	+ (n=2)	+ (n=2)	-	+	+ (n=1)	+	-	-	-	Abnormal (n=1) NA (n=1)	NA
Exon 2-3 delition/ Exon 2-3 delition	1	+	+	+	+	+	+	-	-	-	Abnormal	Abnormal
p.Q106X/c.1173+1G>A	1	+	+	-	+	+	+	-	-	-	Abnormal	NA
p.E114K/p.E114K	1	+	+	-	-	+	-	-	-	+	NA	NA
p.F142LfsX57/ p.F142LfsX57	1	+	+	-	+	+	+	-	-	-	Abnormal	Abnormal
c.459+4A>C/c.459+4A>C	1	+	+	-	+	+	-	+	-	-	Abnormal	Abnormal
c.459+5G>A/c.459+5G>A	1	+	-	-	-	+	-	-	-	-	Abnormal	NA
c.460-1G>A/ c.460-1G>A	1	+	+	-	+	-	-	+	-	-	Abnormal	Abnormal
p.R170W/ p.R170W	1	+	-	-	+	+	-	-	-	-	Abnormal	Abnormal
p.R170Q/ p.R170Q	1	+	+	-	+	+	-	-	+	-	NA	Abnormal
p.D175A/ p.G269R	1	+	+	-	+	-	+	-	-	-	Abnormal	NA
p.R178C/ c.459 +4A>C	1	+	+	-	+	+	-	-	-	-	Abnormal	NA
p.V206M/ p.V206M	1	+	+	-	+	-	+	-	-	-	NA	NA
p.Y213H/ p.Y213H	1	+	+	-	+	+	-	+	-	-	Normal	NA
p.Q237X/p.Q237X	1	+	+	+	+	-	-	-	-	-	Normal	Abnormal
p.G478R/c.672+30T>G	1	+	+	-	+	+	-	-	-	-	Abnormal	NA
p.T263I/ p.T263I	1	+	+	+	+	+	-	-	-	-	Abnormal	NA
c.805+1G>C/ c.805+1G>C	2	+ (n=2)	+ (n=2)	+ (n=1)	+ (n=1)	+ (n=1)	+ (n=1)	-	-	-	Normal (n=1) NA (n=1)	Abnormal (n=1)
p.F300HfsX21/ p.F300HfsX21	1	+	+	+	+	+	-	-	-	-	NA	NA
p.D322N/ p.D322N	2	+ (n=2)	+ (n=2)	+ (n=1)	+ (n=1)	-	+ (n=1)	-	-	-	NA	NA
p.D322Y/ p.D322Y	4	+ (n=4)	+ (n=2)	+ (n=1)	+ (n=3)	+ (n=2)	+ (n=1)	+ (n=1)	-	-	Abnormal (n=1)	Abnormal (n=2)

p.Q374P/ p.Q374P	1	+	+	-	+	-	+	-	-	-	Abnormal	NA
p.R393P/ p.R393P	1	+	+	-	+	+	-	-	-	-	NA	NA
p.Y427IfsX5/ p.Y427IfsX5	5	+ (n=5)	+ (n=5)	-	+ (n=5)	+ (n=4)	+ (n=1)	-	-	+ (n=1)	Abnormal (n=1) Normal (n=2) NA (n=2)	Abnormal (n=1)
p.Y427IfsX5/ p.D322N	1	+	+	-	+	-	-	-	-	-	NA	NA
p.G454R/p.G454R	1	+	+	-	+	+	+	-	-	-	Abnormal	NA
p.E462V/ p.E462V	9	+ (n=9)	+ (n=8)	+ (n=1)	+ (n=9)	+ (n=7)	+ (n=4)	+ (n=1)	-	+ (n=1)	Abnormal (n=6) Normal (n=1) NA (n=2)	Abnormal (n=3) NA (n=6)
P.E462V/?	1	+	+	-	+	-	+	-	-	-	Abnormal	NA
p.W474X/ p.W474X	1	+	+	-	-	+	-	-	-	-	NA	NA
p.W485X/ p.W485X	2	+ (n=2)	+ (n=2)	-	+ (n=2)	+ (n=2)	-	-	+	-	Abnormal (1) NA (1)	NA
p.R499C/ p.R499C	1	+	+	-	+	+	+	+	-	-	NA	Abnormal
p.R510X/ p.R510X	1	+	+	-	+	-	+	+	-	-	Normal	NA
GM2A gene												
p.E158X/ p.E158X	1	+	+	-	-	+	-	-	-	-	Abnormal	NA
c.243-2 A>T/ c.243-2 A>T	1	+	+	-	+	-	-	-	-	-	NA	NA
Correlations	**53**	**53/53 (100%)**	**51/53 (96.2%)**	**8/53 (15.1%)**	**49/53 (92.4%)**	**39/53 (74.5%)**	**21/53 (39.6%)**	**8/53 (15.1%)**	**2/53 (3.8%)**	**3/53 (5.7%)**	**24/31 (77.41%)**	**17/17 (100%)**

Mutation was not identified in three patients hence genotype-phenotype correlation was carried out in 53/56 cases

4.6 PRENATAL DIAGNOSIS

J. Fetal Med. (March 2014) 1:17–24
DOI 10.1007/s40556-014-0001-3

ORIGINAL ARTICLE

Prenatal Diagnosis of Lysosomal Storage Disorders by Enzymes Study Using Chorionic Villus and Amniotic Fluid

Jayesh Sheth · Mehul Mistri · Frenny Sheth · Chaitanya Datar · Koumudi Godbole · Mahesh Kamate · Kamal Patil

Received: 17 April 2014 / Accepted: 20 May 2014 / Published online: 3 July 2014

Abstract The reported prevalence of lysosomal storage disorder (LSD) is 1:5,000–7,000 live births and with the limited availability of therapeutic option; prenatal diagnosis (PD) remains the only preventable cure for storage disorders. One hundred forty pregnancies having confirmed diagnosis of LSDs in index case were selected for enzymes study during PD from uncultured and/or cultured chorionic villus (CV/CCV) and cultured amniotic fluid (CAF) cells. In seven pregnancies, molecular analysis was additionally carried out where mutation was known in an index case. Of 140 pregnancies, 60 (42.9 %) were diagnosed as affected, 13 (9.3 %) had an intermediate enzyme activity and 67 (47.8 %) had normal enzyme activity. Results were confirmed in 83 cases whereas 57 cases were lost from the follow-up. In one case, enzyme β-galactose-6 sulphate sulphatase specific for Morquio-A disorder [mucopolysaccharidosis-IVA (MPS-IVA)] had shown 30 % reduced activity in CV cells and the case was diagnosed as carrier for MPS-IVA while it delivered an affected child. Further molecular analysis in seven cases that included six with Tay-Sachs and one with Gaucher disease, confirmed the results obtained by enzymatic study during PD. PD of LSDs can be carried out by enzymes study from CV/CCV and CAF with an accuracy of molecular method. However, in cases of MPS and mucolipidosis, Amniotic fluid (AF) is preferred over CV/CCV. In addition, special care is needed while interpreting enzyme results encompassing carrier status and they need to be further evaluated by molecular studies.

Keywords Lysosomal storage disorders · Prenatal diagnosis · Enzyme assays · Chorionic villus sampling · Amniotic fluid studies

4.5.1 Measurement of Hexosaminidase-A (Hex-A) and Hexosaminidase-total (total-Hex) activity in CT/AF cells for prenatal diagnosis

Prenatal diagnosis was provided to 8 families where the affected child was detected by enzyme study with known variants in the *HEXA* gene. Deficiency of Hex-A enzyme activity was detected in two cases, intermediate enzyme activity was found in one case which is suggestive of carrier, while remaining five cases had normal enzyme activity as shown in *table 4.18.*

Based on available biochemical and molecular study genetic counseling and prenatal diagnosis was offered to 8 families. In 2 of these homozygous mutation 4bp insertion at c.1278insTATC (p.Y427IfsX5) and c.340G>A (p.E114K) one each was detected in enzymatically confirmed affected fetus same as seen in the index case. Enzymatically proven carrier fetus (n=1) showed only 1 copy of mutant allele c.964G >A (p.D322N) while second copy of mutant allele c.1278insTATC (p.Y427IfsX5) was absent. In remaining 5 families, absence of mutation [c.964 G>A (p.D322N), c.964 G>A (p.D322N)/c.1278insTATC (p.Y427IfsX5), c.1385A>T (p.E462V), c.1454 G>A (p.W485X) and c.1528 C>T (p.R510X)] in *HEXA* gene further supports the enzymatic results from direct prenatal tissue (CT/AF cells) (*Table 4.19 and Figure 4.15(a)-(c)*).

***Table 4.18**: Enzyme activity in AF/CT cells for prenatal diagnosis*

Pt ID	Enzyme activity		Status
	AF cells	CT cells	
P-1	-	552.8	Normal
P-2	-	21.7	Affected
P-3	23.4	-	Affected
P-4	-	432.1	Normal
P-5	670.5	-	Normal
P-6	247.8	-	Carrier
P-7	534.5	-	Normal
P-8	435.8	-	Normal

***Table 4.19**: List of all HEXA sequence variants identified in prenatal study.*

Patients	**Study in index case**		**Prenatal study**		
	Mutation	Status	Sample types	Enzyme status	Mutations status
P-1*	c.964 G>A (p.D322N)	Homozygous	CT	Normal	Wild type
P-2	c.1278insTATC (p.Y427IfsX5)	Homozygous	CT	Affected	Homozygous
P-3	c.340G>A (p.E114K)	Homozygous	AF	Affected	Homozygous
P-4	c.1278insTATC (p.Y427IfsX5)/ c.964 G>A (p.D322N)	Compound heterozygous	CT	Normal	Wild type
P-5	c.1454 G>A (p.W485X)	Homozygous	AF	Normal	Wild type
P-6*	c.964 G>A (p.D322N)	Homozygous	AF	Carrier	Heterozygous
P-7	c.1385A>T (p.E462V)	Homozygous	AF	Normal	Wild type
P-8	c.1528 C>T (p.R510X)	Homozygous	AF	Normal	Wild type

* Prenatal study was carried out in two different pregnancies from single individual

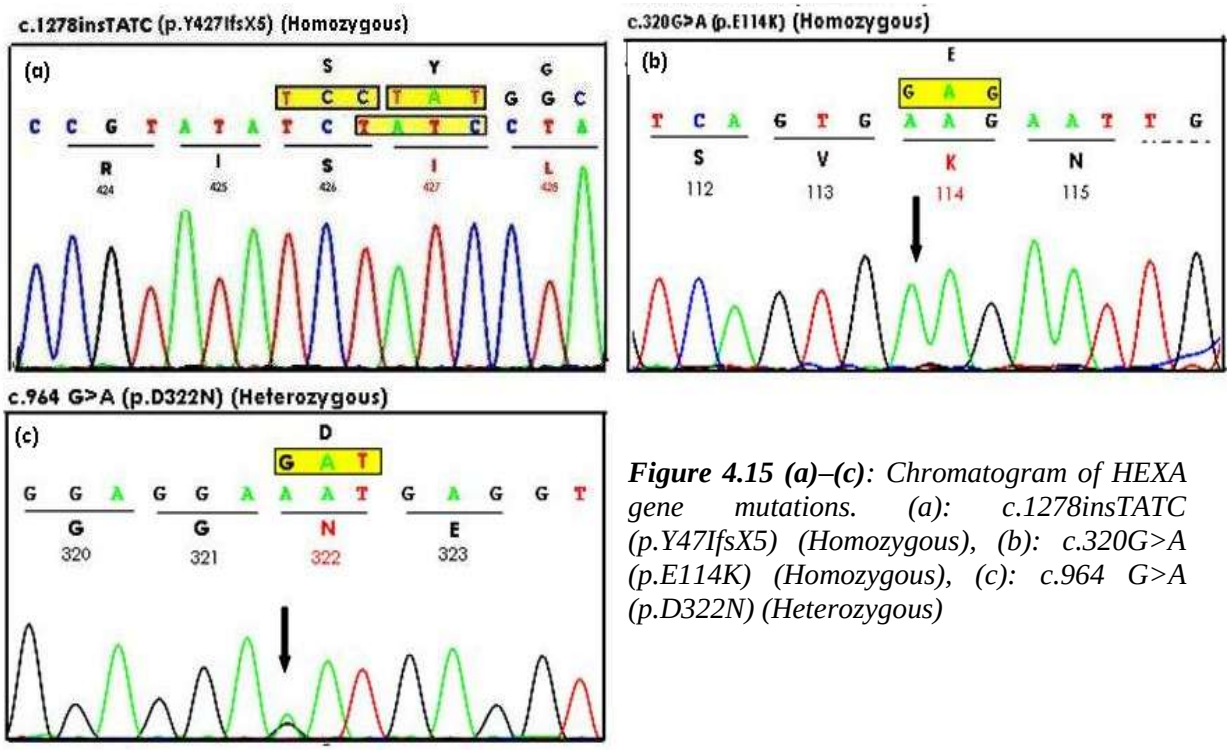


***Figure 4.15 (a)–(c)**: Chromatogram of HEXA gene mutations. (a): c.1278insTATC (p.Y47IfsX5) (Homozygous), (b): c.320G>A (p.E114K) (Homozygous), (c): c.964 G>A (p.D322N) (Heterozygous)*