Genetic Mapping and Sequence Analysis of Thiamine Responsive Megaloblastic Anemia in Three Pakistani Families

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by

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My parents and siblings have always been my source of inspiration. Their hard work, dedication, encouragement, and values have shaped me into the person I am today. This thesis is dedicated to them as a token of my love and respect.

Declaration

I hereby declare that the work presented in these thesis is my own work. It has been authored and composed by me. No part of this thesis has been published in pursuit of any degree or certification.

Sher Aziz

Table of Contents

ACKNOWLEDGMENTS----------1

LIST OF FIGURES----------3

LIST OF TABLES----------4

LIST OF ABBREVIATIONS----------5

ABSTRACT----------7

CHAPTER 01: INTRODUCTION----------8

Thiamine Responsive Megaloblastic Anemia----------9

Prevalence of TRMA----------10

Diagnosis of TRMA----------11

Treatment of TRMA----------12

Genetics of TRMA----------13

Mutations in *SLC19A2* gene----------14

Spectrum of *SLC19A2* variant in Pakistan----------17

Proteins encoded by *SLC19A2*----------17

Localization of *SLC19A2*----------18

Transportation of thiamine----------18

Thiamine uptake from gastrointestinal tract----------19

Saturable, Low Capacity/high Affinity transporter----------19

Non saturable, high capacity/low affinity transporter----------19

Transmembrane transportation into individual cells----------19

Activation of thiamine by TPK----------19

Binding of TPP with the enzymes----------19

Biochemical function of thiamine in the cell----------20

Cytosolic role of TPP----------20

Mitochondrial role of TPP----------20

Peroxisomal role of TPP----------20

Defects in thiamine transportation cause TRMA----------21

Megaloblastic anemia----------23

Sensorineural deafness----------23

Diabetes Mellitus----------23

Aims and Objectives----------24

CHAPTER 02: MATERIALS AND METHODS----------25

Research endorsement----------26

Mapping of ancestry----------26

Collection of blood samples----------26

Extraction of genomic DNA----------37

Extraction of genomic DNA through the Phenol-Chloroform method----------27

Kit based method for DNA extraction----------29

Agarose gel electrophoresis----------30

Quantification and Dilutions of extracted DNA----------31

Linkage analysis using microsatellite markers----------31

Polymerase Chain Reaction (PCR)----------32

Polyacrylamide Gel Electrophoresis (PAGE)----------35

Variant Screening in *SLC19A2* Gene----------36

Pre sequencing Amplification of *SLC19A2*----------36

Purification of PCR Products----------38

Sequencing PCR----------39

Sanger sequencing----------39

Variant interpretation----------41

CHAPTER 03: RESULTS----------42

Family A----------43

Clinical characteristic of family A----------43

Family B----------45

Clinical characteristic of family B----------45

Family C----------47

Clinical characteristics of family C----------47

Collection of blood and DNA extraction----------49

Linkage analysis by using microsatellite markers----------50

Sequencing of *SLC19A2* gene----------57

CHAPTER 04: DISCUSSION----------60

CHAPTER 05: REFERENCES----------67

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Sher Aziz

LIST OF FIGURES

Fig. No	Title	P. No
1.1	Triade of thiamine responsive megaloblastic anemia (TRMA).	**9**
1.2	Worldwide distribution of TRMA.	**11**
1.3	Bone marrow film showing megaloblastic cells of erythroid lineage.	**12**
1.4	Schematic representation of *SLC19A2* gene.	**13**
1.5	Percentage of different mutations in different exons of *SLC19A2* gene.	**16**
1.6	Structure of *SLC19A2* gene and their reported mutations.	**16**
1.7	Schematic representation of the predicted topology of the *SLC19A2* protein across the cell membrane and the reported mutations.	**17**
1.8	Steps involved in thiamine utilization.	**18**
1.9	Thiamine transport and metabolic pathway.	**22**
3.1	The pedigree of Family A.	**44**
3.2	The pedigree of Family B.	**46**
3.3	The pedigree of Family C.	**48**
3.4	Extracted DNA of family A, B and C.	**50**
3.5	Polyacrylamide electropherogram of family A	**52**
3.6	Polyacrylamide electropherogram of family B	**54**
3.7	Polyacrylamide electropherogram of family C	**56**
3.9	Comparative analysis of exon 2 among affected daughter, normal father and mother in family A	**58**
3.10	Partial sequencing chromatogram of exon 2 in affected member of family B	**59**

LIST OF TABLES

Table No.	Title	Page No.
1.1	Different syndromes caused by thiamine transporter abnormalities.	**14**
1.2	Different mutations in *SLC19A2* and their predicted effects on proteins.	**15**
2.1	Solutions used in DNA extraction and their composition.	**29**
2.2	Composition of chemicals used in agarose gel electrophoresis.	**31**
2.3	Microsatellite markers used for linkage analysis of *SLC19A2*.	**32**
2.4	Chemical components and their roles in PCR	**33**
2.5	Thermo cycling conditions employed during each PCR	**34**
2.6	Composition of 50 ml polyacrylamide gel	**36**
2.7	Sequence of primers used to amplify *SLC19A2* gene	**37**
2.8	Chemical composition of the pre-sequencing PCR mixture	**38**
2.9	Composition of PCR purification kit	**40**
2.10	Ingredients of sequencing PCR	**40**

LIST OF ABBREVIATIONS

TRMA	Thiamine responsive megaloblastic anemia
PDA	Patent ductus arteriosus
Hb	Hemoglobin
MCV	Mean cell volume
CBC	Complete blood count
FBG	Fasting blood glucose
OGTT	Oral glucose tolerance test
TPK	Thiamine phosphokinase
THTR	High affinity thiamine transporter
TPP	Thiamine pyrophosphate
BBB	Blood brain barrier
ThDPK	Thiamine diphosphokinase
HMP	Hexose monophosphate
DNA	Deoxyribonucleic acids
TMP	Thiamine monophosphate
TTP	Thiamine triphosphate
RNA	Ribonucleic acids
DM	Diabetes mellitus
WES	Whole exome sequencing
IRB	Institutional review board
PIMS	Pakistan institute of medical sciences
EDTA	Ethylene diamine tetra acetic acids
RPM	Revolution per minute
SDS	Sodium dodecyl sulphate
TE	Tris EDTA
TBE	Tris borate EDTA
UV	Ultraviolet
OD	Optical density
PB	PCR buffer
DNTPs	Deoxyribonucleosides triphosphate

PCR	Polymerase chain reaction
PAGE	Polyacrylamide gel electrophoresis
APS	Ammonium per sulphate
TEMED	Tetramethylethylenediamine
HCT	Hematocrit
BLAST	Basic Local Alignment Search Tool
MCH	Mean cell hemoglobin
MCHC	Mean cell hemoglobin concentration
RDW	Red cell distribution width
TLC	Total leukocytes count
RBC	Red blood cell
%	Percentage
bp	Base pair
cM	Centimorgan
SLC19A2	Solute carrier family 19 member 2
F	Forward
R	Reverse
kb	Kilo base
fs	Frameshift
kDa	Kilodalton
kg	Kilogram
p	Short arm of chromosome
q	Long arm of chromosome
rpm	Revolution per minute
Taq	Thermus aquaticus

ABSTRACT

Thiamine-responsive megaloblastic anemia (TRMA) is a rare autosomal recessive monogenic disorder characterized by megaloblastic anemia, diabetes mellitus and sensorineural deafness. It is highly prevalent among those families having high ratio of consanguinity. TRMA arises due to molecular defect in *SLC19A2* gene encoding high affinity thiamine transporter across the cell membrane. Intracellular concentration of thiamine plays a crucial role in biochemical reactions. Deficiency of thiamine inside the cell leads to various manifestations of TRMA. This study aimed to characterize TRMA patients in Pakistani population both clinically and genetically. In the current study we have included three Pakistani families underlying TRMA from Punjab province of Pakistan. Whole blood was collected and DNA was extracted by phenol-chloroform method. Genetic screening was performed by homozygosity mapping and Sanger sequencing to identify the cytogenetic position as well as pathogenic variant in the gene linked with TRMA, respectively. Highly mutated exons of *SLC19A2* gene were subjected to Sanger sequencing. Family A established linkage at marker D1S398 and were subjected to Sanger sequencing. We found a novel homozygous variant in exon 2 [NM_006996: exon 2: c.519_519delT, p.(V174Sfs*2)], encoding abnormal thiamine transporter protein. Family B also established linkage at marker D1S210 and Sanger sequencing revealed no pathogenic variant in Exon 2. Family C established linkage at marker D1S370 and all exons have been amplified and ready for Sanger sequencing. In conclusion, this research study provides valuable insight into pathogenesis, clinical manifestations, genotyping and molecular mechanism of TRMA in Pakistani population. Clinical and genetic assessment of TRMA patients will increase our ability for the timed diagnosis and treatment of TRMA patients. The current research also paves the way for gene therapy and genetic counselling of patients suffering from TRMA.

Key words: Thiamine, Anemia, megaloblastosis, sensorineural deafness, diabetes mellitus.

Chapter 01

INTRODUCTION

1. INTRODUCTION

1.1 Thiamine Responsive Megaloblastic Anemia

Thiamine-responsive megaloblastic anemia was first described by Rogers *et al* in 1969 hence also called Rogers syndrome (Rogers *et al.*, 1969). TRMA or Rogers syndrome is an autosomal recessive disorder characterized by a triad of diabetes mellitus, megaloblastic anemia and sensorineural deafness (Figure 1.1). Some other less frequent findings of TRMA include congenital heart anomalies, cardiomyopathy, arrhythmias, retinopathy, optic atrophy, stroke, situs inversus and aminoaciduria (Lorber *et al.*, 2003; Villa *et al.*, 2000). Cardinal findings of the disease can be appeared anytime from infancy to adolescence but all findings may not be present in a single patient (Ozdemir *et al.*, 2002). Thiamine supplements bring improvement in the endocrine and hematological functions while neurological symptoms do not show any improvement hence the name thiamine responsive megaloblastic anemia (Scharfe *et al.*, 2000).

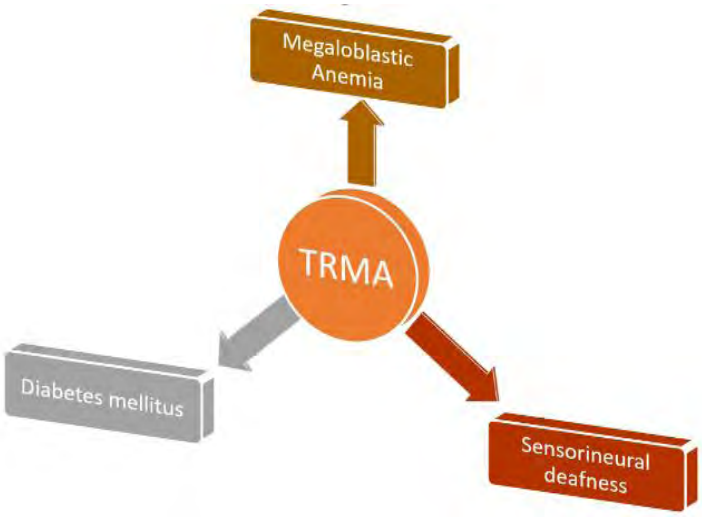


Figure 1.1: Triade of thiamine responsive megaloblastic anemia (TRMA).

1.2 Prevalence of TRMA

TRMA is very rare genetic entity outside consanguineous marriages and isolated populations. It has few well documented cases globally (Ganie *et al.*, 2012). People of different ethnicities have been observed to be affected by TRMA. Few cases of TRMA have been reported from Arab, Alaskan natives, Oman, Kashmiri families in Great Britain, ethnic kurds, Tunisia, Caucasians, African American, Russians, Japanese, Iranian, Israeli, Lebanese, Brazilian, Italian and Pakistani families (Bergmann *et al.*, 2009; Naeem *et al.*, 2008). Turkish population have a high rate of consanguinity which can explain the increased frequency of TRMA in Turkey (Figure 1.2) (Bay *et al.*, 2010).

In 88 papers, the review of the literature found 183 TRMA patients from 138 families. Although the reported cases were rare instances from 33 countries of origin, the Middle East (37.7%), South Asia (21.9%), and the northern Mediterranean (17.0%) accounted for the majority of cases. Sequencing analysis established the diagnosis of TRMA in155 of 183 patients. Out of 157 TRMA patients, 97 (61.8%) were the offspring of consanguineous couples. A total of 151 individuals had clinical data retrieved; 144 (95.4%) of them had anemia, 140 (92.7%) had diabetes, and 140 (92.7%) had hearing loss. The cardinal triad was present in the majority of cases (84.1%). Average age of TRMA children at diagnosis was about 10 months while the age of onset of anemia and diabetes mellitus was 20 months. (Zhang *et al.*, 2021). Due to the rare nature of the disease and unavailability of diagnostic facilities, there is only few published reports on the prevalence of TRMA in Pakistan.

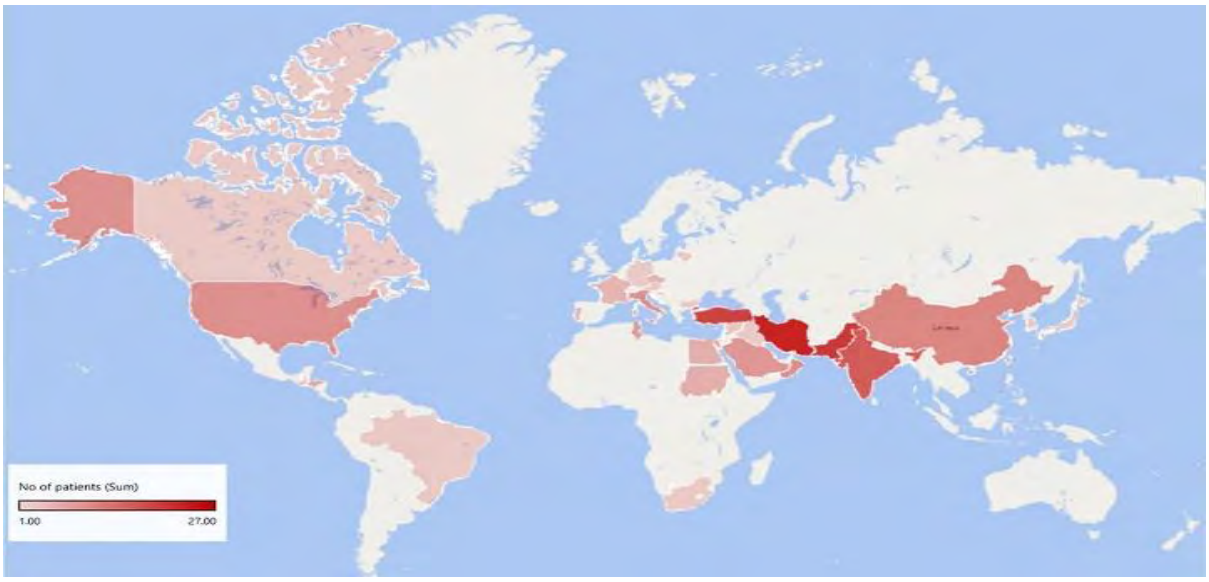


Figure 1.2: Worldwide distribution of TRMA (Zhang *et al*., 2021).

1.3 Clinical features and complications associated with TRMA

Most common clinical features of TRMA include headache, tiredness, anorexia, polydipsia, polyuria, lethargy, irregular heartbeat, fatigue, pale skin, cold hands, loss of stamina, shortness of breath and feelings of weakness (Bhadra and Deb, 2020). In addition to the clinical triade of TRMA (Megaloblastic anemia, diabetes mellitus, sensorineural deafness), complications associated include patent ductus arteriosus (PDA), congenital heart anomalies, cardiomyopathy, arrhythmias, hepatomegaly, retinopathy, cone-rod dystrophy, optic atrophy, speech impairment, stroke, situs inversus, hypothyroidism, mild thrombocytopenia, leukopenia, sideroblastic anemia, and aminoaciduria (Lorber *et al.*, 2003; Ozdemir *et al.*, 2002; Villa *et al.*, 2000).

1.4 Diagnosis of TRMA

Diagnosis of TRMA is performed on the basis of clinical characteristics including diabetes mellitus, megaloblastic anemia and sensorineural deafness. Bone marrow of TRMA patients show megaloblastic cells of all lineages with prominent erythroblasts having iron filled mitochondria (Figure 1.3) (Patil *et al.*, 2020). Peripheral blood smear shows macrocytosis with low hemoglobin and high mean cell volume (MCV). Serum folic acid, vitamin B_{12} and thiamine level is normal in TRMA patients. TRMA patients also have thrombocytopenia and neutropenia. Pancytopenia in TRMA patients can be treated with pharmacologic doses (50-100 mg/day) of thiamine (B1). As TRMA patients are suffering from diabetes mellitus, hence

hyperglycemia, glycosuria and ketoacidosis are evident. Another diagnostic feature is that patients are initially responsive to oral hypoglycemic agents but eventually become dependent on insulin. Diagnosis of TRMA can be confirmed by the sequencing analysis of *SLC19A2* gene (Sako *et al.*, 2022).

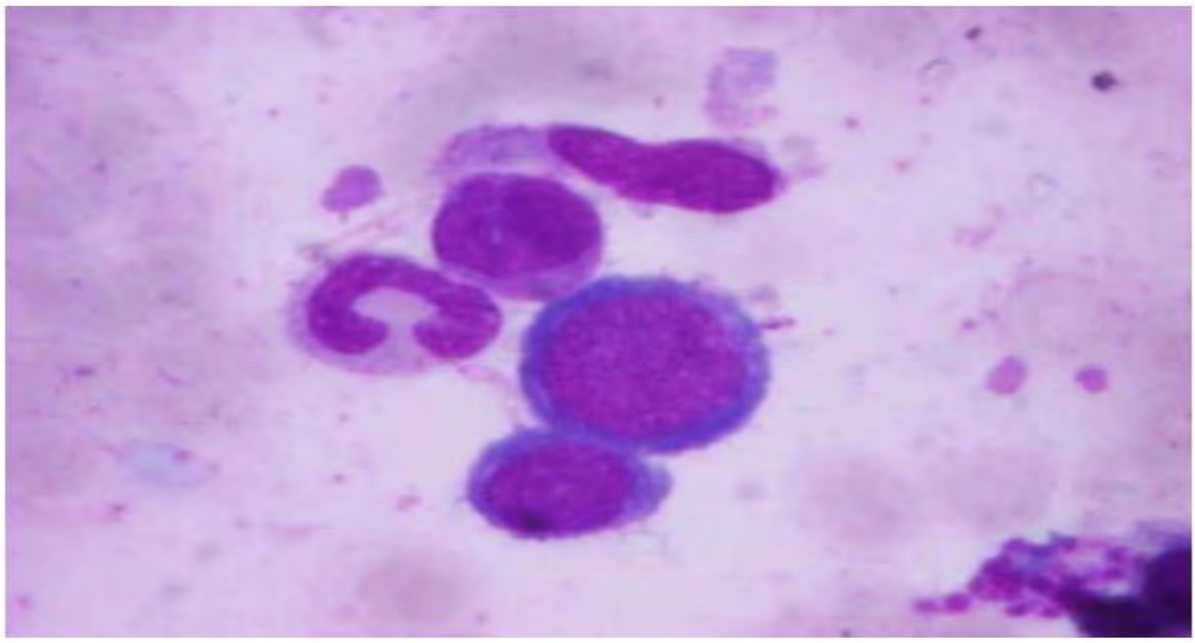


Figure 1.3: Bone marrow film showing megaloblastic cells of erythroid lineage (Bay *et al.*, 2010).

1.5 Treatment of TRMA

Symptomatic treatment of TRMA can be provided by lifelong use of oral thiamine (50-100 mg/day) to the affected patients regardless of their age. In case of severe anemia, red blood cell transfusion is done for the patients. Standard treatment is provided for diabetes mellitus, sensorineural deafness, ophthalmologic, neurological and cardiovascular manifestations. Efficacy of thiamine therapy and disease progression can be monitored annually by performing various tests e.g. CBC, reticulocytes count, FBG, OGTT, and urinalysis as well as evaluations of cardiac, ophthalmologic, neurologic and hearing defects. Diabetes mellitus must be controlled before and during pregnancy (Sako *et al.*, 2022).

1.6 Genetics of TRMA

Thiamine responsive megaloblastic anemia is a rare monogenic disorder affecting thiamine transporter across the cell membranes. It is an autosomal recessive disorder which arises due to the loss of function mutation in *SLC19A2* gene (Rogers *et al.*, 1969). *SLC19A2* consists of six exons and is localized to a 1.4 cM region on the long arm of chromosome 1(1q23.2–23) (Figure 1.4). *SLC19A3* also encode thiamine transporter across the cell membranes but it work under high thiamine concentration in the body. Hence, *SLC19A3* does not play any role in causing TRMA. Genes, other than *SLC19A2* which encode thiamine transporters include *SLC19A1, SLC22A1, SLC35F3, SLC44A44, SLC25A19* (Marcé-Grau *et al.,* 2019). Although these genes does not play any significant role in causing TRMA their abnormalities cause other conditions (Table 1.1).

Figure 1.4: Schematic representation of normal *SLC19A2* gene (*Biorender.com*).

Table 1.1: Different syndromes caused by thiamine transporter abnormalities (Marcé-Grau *et al.*, 2019).

Gene	Dysfunction syndrome	Disorder
SLC19A2	Abnormal thiamine metabolism syndrome 1	TRMA syndrome
SLC19A3	Abnormal thiamine metabolism syndrome 2	Thiamine responsive encephalopathy like syndrome
SLC25A19	Abnormal thiamine metabolism syndrome 3 Abnormal thiamine metabolism syndrome 4	Microcephaly Amish type Bilateral neurodegeneration and polyneuropathy syndrome type
TPK1	Abnormal thiamine metabolism syndrome 5	Episodic encephalopathy type

1.7 Mutations in *SLC19A2* gene

Approximately, 60 different mutations have been identified in six exons of *SLC19A2* gene in different countries, 48.3% of them are in exon 2 (Table 1.2). Sequence analysis of this gene shows considerable heterogeneity of mutations, although majority of them are nonsense, missense, frameshift, single amino acids changes or small insertions and deletions (Manimaran *et al.*, 2016). Percentage of different mutations in different exons of *SLC19A2* gene is given in the figure 1.5. Some of these mutations are silent while some cause the production of abnormally truncated and nonfunctional thiamine transporters (THTR-1) e.g. frameshift mutations, nonsense mutations and deletions produces truncated proteins which are more common than missense and indels.

Table 1.2: Different mutations in *SLC19A2* and their predicted effects on proteins (Labay *et al.*, 1999).

Family	Exon	Mutation	Amino acid	Predicted effect on protein
Israel 3	2	724delC	Del242fs/ter259	Frameshift and creation of stop codon
Israel 4	2	724delC	Del242fs/ter259	Frameshift and creation of stop codon
Italy 2	2	515G→A	G1720	Glycine→Aspartate
India 5	2	750G→A	W250X	Tryptophan→Stop
Japan 10	2	484C→T	R162X	Arginine→Stop
Pakistan 6	2	484C→T	R162X	Arginine→Stop

Few mutations in splicing site have also been reported (Marcé-Grau *et al.*, 2019). Other mutations cause abnormal folding of THTR-1 preventing it from reaching to the cell membrane. However, all these different mutations disrupt the normal activity of THTR-1 to transfer thiamine into the cell (Labay *et al.*, 1999). Structure of *SLC19A2* gene and their reported variants are given (Figure 1.6).

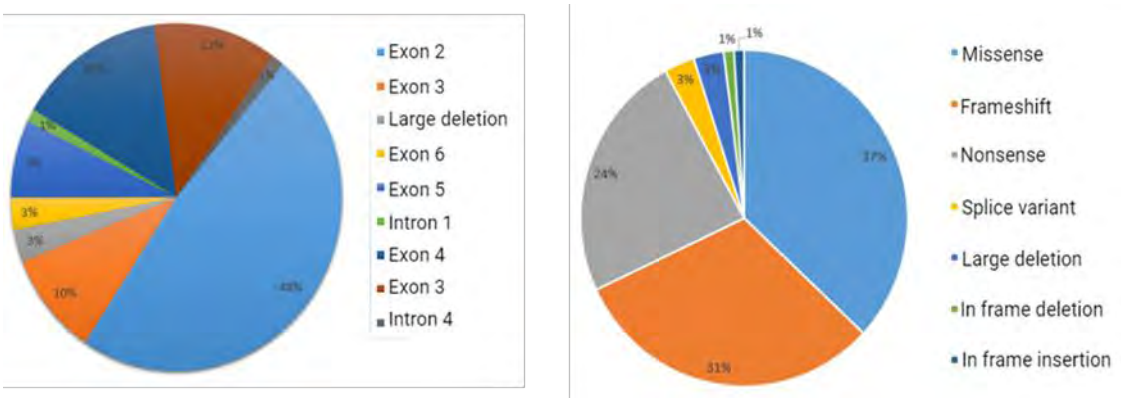


Figure 1.5: Percentage of different mutations in different exons of *SLC19A2* gene. Edited and modified (Zhang *et al.*, 2021).

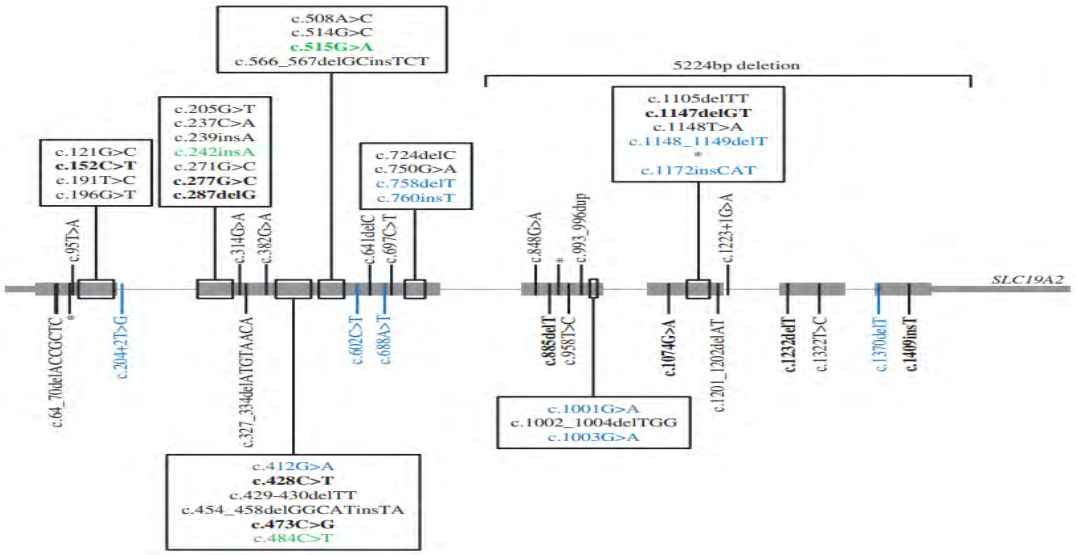


Figure 1.6: Structure of *SLC19A2* gene and their reported mutations. Black colored variants are homozygous, blue colored are compound heterozygous and green colored variants are both homozygous as well as compound heterozygous (Marcé-Grau *et al.*, 2019).

1.8 Spectrum of *SLC19A2* variants in Pakistan

In January 2020, homozygous pathogenic frameshift variant was detected in exon 2 of *SLC19A2* in a baby boy of 2.5 years in Karachi (Askari *et al.*, 2020). In 1982, 93 and 97, nonsense substitution (196G>T) in exon 1 and exon 2 (484C>T) was detected in Pakistani families (Barrett *et al.*, 1997; Haworth *et al.*, 1982; Vora *et al.*, 1993). In 1999, insertion (242insA) in exon 2 was detected in another family of Pakistan (Freisinger *et al.*, 1999).

1.9 Proteins encoded by *SLC19A2*

SLC19A2 gene encodes one of the two known high-affinity thiamine transporters (THTR), i.e., THTR-1 encoded by *SLC19A2* and THTR-2 encoded by *SLC19A3*. THTR-1 is a high affinity transporter of thiamine across the cell membranes having 12 transmembrane domains, two N-glycosylation sites and consist of 497 amino acids (Patil *et al.*, 2020). Both the NH2 and COO termini of THTR-1 is located in the cytoplasm of the cell (Ganapathy *et al.*, 2004). Predicted topology of *SLC19A2* protein across the cell membrane and their reported mutations (Figure 1.7).

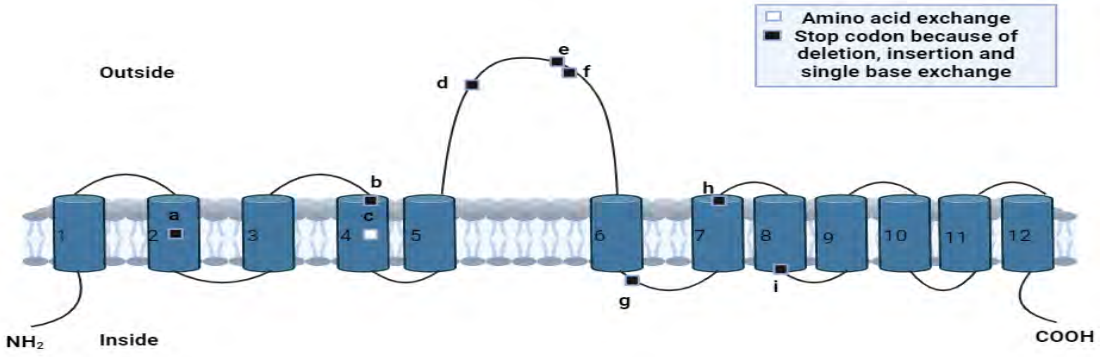


Figure 1.7: Schematic representation of the predicted topology of the *SLC19A2* protein across the cell membrane and the reported mutations (*Biorender.com*).

1.10 Localization of *SLC19A2*

This gene is expressed in many tissues of human body including bone marrow, brain, retina, pancreas, colon, skeletal muscle, liver, lung, placenta, heart, kidney, small intestine, fibroblasts and lymphocytes. Bone marrow and pancreatic beta cells also express *SLC19A3* gene, encoding low affinity thiamine transporter. Hence bone marrow and pancreatic beta cells of TRMA patients show response to high thiamine concentration during therapy. Neuronal and hairy cells of the cochlea have no *SLC19A3* expression which explains no response to thiamine therapy (Diaz *et al.*, 1999).

1.11 Transportation of thiamine

Thiamine is necessary for the normal functions of all tissues and is highly concentrated in brain, heart, skeletal muscles, liver, brain and kidneys (Martin, 2001). Steps involved in transport and homeostasis of thiamine include: (i) Thiamine uptake from gastrointestinal tract; (ii) transmembrane transportation into individual cells; (iii) activation of thiamine by thiamine pyro phosphokinase (TPK) enzyme to convert it into cofactor thiamine pyrophosphate (TPP); (iv) TPP binding with the concern enzyme (Stagg *et al.*, 1999). Steps involved in transportation of thiamine (Figure 1.8).

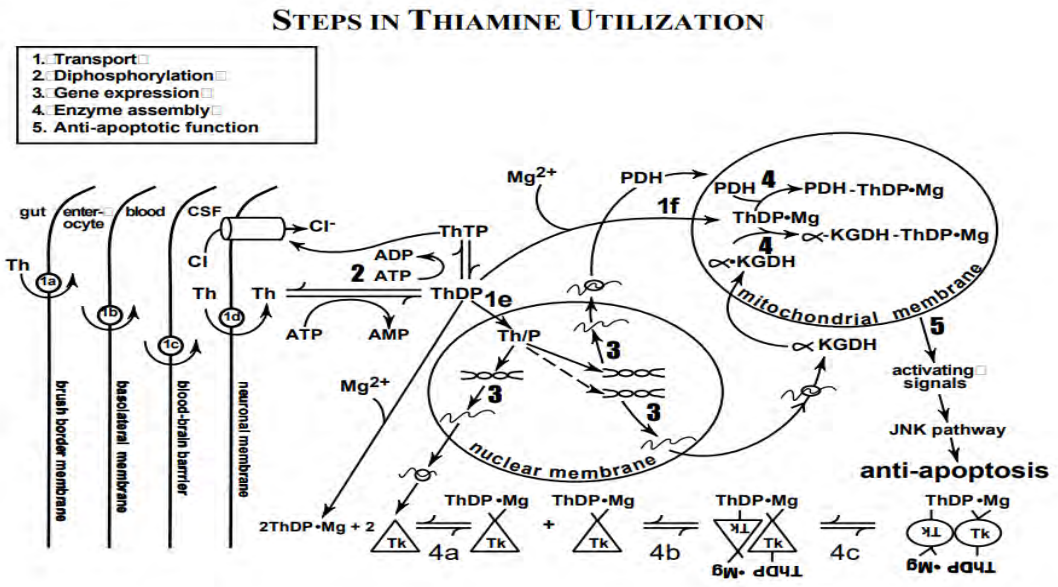


Figure 1.8: Steps involved in thiamine utilization (Martin, 2001).

1.11.1 Thiamine uptake from gastrointestinal tract

Intestinal absorption of thiamine indicates that there are two different systems for thiamine transportation. One is for low while other is for high thiamine concentration.

1.11.1.1 Saturable, Low Capacity/high Affinity transporter

When concentration is low (<2mmol/L), thiamine is transported by saturable, low capacity/high affinity carrier which is an energy requiring process. Due to limited amount of thiamine in the diet, its concentration in the intestine is low, hence its absorption occurs through highy affinity transporters (Laforenza *et al.*, 1997). Defect in this high affinity thiamine transporter leads to TRMA (Stagg *et al.*, 1999).

1.11.1.2 Non saturable, high capacity/low affinity transporter

Passive diffusion of thiamine by non-saturable, high capacity/low affinity carriers occurs at high concentration (>2mmol/L). This route of transportation is present in TRMA patients but cannot transport thiamine at low concentration in the diet (physiological concentration). Duodenal mucosa has the high rate of thiamine absorption (Hoyumpa *et al.*, 1982).

1.11.2 Transmembrane transportation into individual cells

Mechanism of thiamine transportation is same across all type of tissues and cells. High and low affinity transporters are present on the membranes of all tissues and cells including blood brain barrier (BBB). These transporters are responsible for the transportation of thiamine into individual cells of the body (Meire *et al.*, 2000).

1.11.3 Activation of thiamine by TPK

Upon entering into the cell, thiamine is di-phosphorylated rapidly by thiamine diphosphokinase (ThDPK) enzyme to produce thiamine diphosphate (ThDP). ThDP is an active cofactor that assists in the catalytic activity of several enzymes, including the enzymes of citric acid cycle and hexose monophosphate shunt (HMP-shunt) (Martin, 2001).

1.11.4 Binding of TPP with the enzymes

Thiamine pyrophosphate binds many enzymes including transketolase of hexose monophosphate shunt (HMP shunt). Enzymes of oxidative decarboxylation (citric acid cycle), including ketoacid dehydrogenase, alpha ketoglutarate dehydrogenase and pyruvate dehydrogenase also use TPP as coenzymes (Stagg *et al.*, 1999). Enzymes of HMP shunt are important for the biosynthesis of neurotransmitters and reducing equivalent ($NADPH_2$), used as reducing agent in biosynthetic reactions as well as for the reduction of oxidized glutathione

(GSSG). These enzymes are also responsible for biosynthesis of nucleic acids precursors like pentoses which are necessary ingredients during DNA replication and transcription (Martin, 2001).

1.11.5 Biochemical function of thiamine in the cell

Thiamine, also called vitamin B1 is water soluble vitamin which is present in different form inside the human body. Depending upon the functional activity of different tissues, thiamine is present either in the form of free-thiamine, thiamine monophosphate (TMP), thiamine pyrophosphate (TPP), and thiamine triphosphate (TTP). Thiamine pyrophosphate (TPP) is the active form of thiamine which make 80 % of the total thiamine in the tissues. TPP play very crucial role in cytosolic, Peroxisomal and mitochondrial metabolic reactions (Losa *et al.*, 2005).

1.11.5.1 Cytosolic role of TPP

Cytosolic TPP act as cofactor of transketolase and transaldolase which are involved in pentose phosphate pathway (Ortigoza-Escobar, Molero-Luis, Arias, Martí-Sánchez*, et al.*, 2016). Enzymes of pentose phosphate pathway are important for the biosynthesis of neurotransmitters and reducing equivalent ($NADPH_2$). $NADPH_2$ is used as reducing agent in biosynthetic reactions as well as for the reduction of oxidized glutathione (GSSG). These enzymes are also responsible for biosynthesis of nucleic acids precursors like pentoses which are necessary ingredients during DNA replication and transcription (Martin, 2001).

1.11.5.2 Mitochondrial role of TPP

Mitochondrial TPP act as cofactor of several enzyme complexes including pyruvate dehydrogenase complex responsible for the conversion of pyruvate into Acetyl-CoA, oxoglutarate dehydrogenase complex, responsible for oxidation of alpha-ketoglutarate in the Krebs cycle, branched-chain alpha-ketoacid dehydrogenase complex responsible for the decarboxylation of branched, short-chain alpha-ketoacid (Ortigoza-Escobar *et al.*, 2017).

1.11.5.3 Peroxisomal role of TPP

Peroxisomal TPP act as cofactor of several enzymes including 2-hydroxyacyl-CoA lyase responsible for the fatty acid degradation and Oxoglutarate dehydrogenase, responsible for the production of succinyl-coenzyme A (Ortigoza-Escobar, Molero-Luis, Arias, Oyarzabal*, et al.*, 2016). Succinyl Co-A act as substrate of aminolevulinate synthase 2, responsible for heme biosynthesis. The role of TPP in biosynthesis of Succinyl Co-A and heme may explain

megaloblastic anemia, which is one of the main character of TRMA (Bergmann *et al.*, 2009). Role of thiamine in different metabolic reactions (Figure 1.9).

1.12 Defects in thiamine transportation cause TRMA

SLC19A2 and *SLC19A3* are high affinity thiamine transporters, expressed on most tissues of human body including brain, liver, heart, intestines and skeletal muscles. If one is absent or defective, other can compensate. However, bone marrow, cochlear cells and pancreatic beta cells have only *SLC19A2* for thiamine transportation. Therefore, nuclear genetic defects in thiamine transportation by *SLC19A2* or activation cause thiamine deficiency in these tissues. Deficiency of active thiamine leads to multisystem disorders including megaloblastic anemia, deafness and diabetes which are the manifestations of TRMA (Liberman *et al.*, 2006).

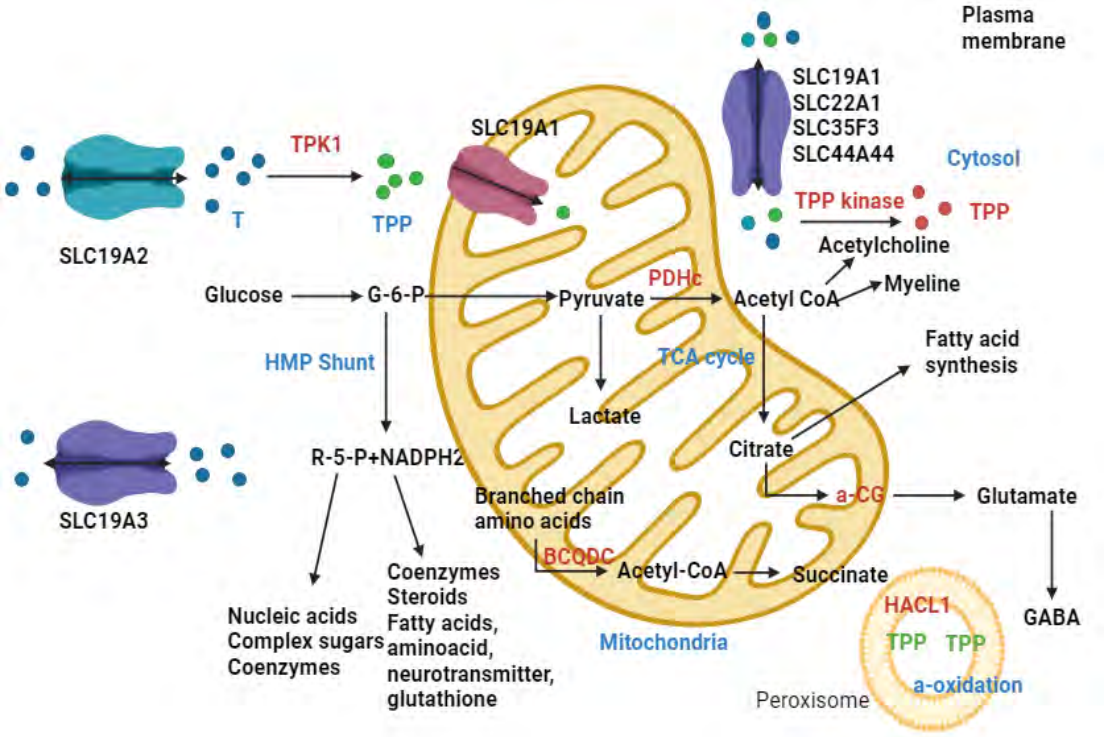


Figure 1.9: Thiamine transport and its involvement in different metabolic pathways (*Biorender.com).*

1.12.1 Megaloblastic anemia

Megaloblastic anemias are mainly caused by mitochondrial disorders. However abnormal DNA/RNA biosynthesis as occurred in folate and vitamin B12 deficiency can also cause megaloblastic anemia. Deficiency of thiamine in various tissues can also be the cause of reduced DNA/RNA biosynthesis. Thiamine pyrophosphate is an active cofactor of transketolases of HMP shunt which are responsible for de novo biosynthesis of nucleic acids precursors like riboses. Hence deficiency of thiamine in bone marrow parenchyma cause poor production of riboses, leads to impaired DNA/RNA biosynthesis. Impaired DNA replication cause reduce proliferation of hematopoietic stem cells in the bone marrow which leads to megaloblastic anemia (Boros *et al.*, 1998).

1.12.2 Sensorineural deafness

Exact mechanism of sensorineural deafness in TRMA patients is not fully understood as it is unknown whether the cause of sensorineural deafness is abnormal thiamine depended metabolism of auditory nervous system or cochlea. It has been determined that TPK activity is compromised in TRMA patients reducing the conversion of thymidine into thiamine which is necessary for DNA synthesis and DNA repair. Also accumulation of thymidine and its related compounds in the cochlea, the auditory portion of the inner ear, may lead to damage to the hair cells and neural structure responsible for transmitting sounds signals to the brain, causing sensorineural deafness (Fleming and Neufeld, 2003; Meire *et al.*, 2000).

1.12.3 Diabetes Mellitus

Diabetes mellitus (DM) in TRMA patients appear during childhood. It is a new type of diabetes which is non type I in nature. Intracellular thiamine deficiency may cause impairment of beta cells functions, lead to insulin deficiency hence cause diabetes mellitus (Gianguido Rindi *et al.*, 1992). Some TRMA children suffering from diabetes, require reduced amount of insulin during thiamine treatment probably because either beta cells of the pancrease or target tissues have intermediate sensitivity to cellular thiamine deficiency. Target tissues of insulin have some sensitivity to thiamine deficiency. Irrespective of long term thiamine therapy, TRMA patients still develop pancreatic insufficiency and become insulin dependent (Borgna-Pignatti *et al.*, 1989; Gianguido Rindi *et al.*, 1992). Anti-islet beta cells and anti-insulin autoantibodies are absent in TRMA patients suffering from DM (G Rindi *et al.*, 1994).

1.13 Aims and Objectives

The present study aims to characterize three Pakistani families exhibiting features of TRMA both clinically and genetically, by screening of the selected candidate genes responsible for TRMA through homozygosity mapping using microsatellite markers. Furthermore, we have opted Sanger sequencing to identify a pathogenic variant in candidate gene and correlated the variant with their respective phenotypes.

Chapter 02

MATERIALS AND METHODS

2. Materials and Methods

2.1 Research endorsement

The current research study was established on the inclusion of three Pakistani families (Family A, Family B, Family C), each having single member suffering from TRMA. The research study was granted permission to proceed by the Institutional Review Board (IRB) of Quaid-i-Azam University, Islamabad. Affected members of all three families were diagnosed with TRMA in PIMS Islamabad and blood samples were collected for genetic analysis. Furthermore, informed consent was obtained from the affected individuals for the acquisition of medical reports.

2.2 Mapping of ancestry

During the hospital visit, all three families underwent thorough questioning regarding their family background and medical records. Pedigrees were created to visually represent the families, adhering to Mendelian inheritance patterns. The determination of inheritance patterns relied on clinical phenotypes and family history. Following the pedigree construction guidelines outlined by Bennett *et al.* (1995), marriage was represented by a single horizontal line, and consanguineous relationships were denoted by double horizontal lines. Different generations were marked out using vertical lines. Affected individuals were represented by filled squares and circles, while non-affected members were shown as empty squares and circles, respectively. Family members who have passed away were represented as squares and circles divided by a diagonal line. Arabic numbers were used to identify individual members of each generation, while Roman numerals were used to mark each generation in ascending order.

2.3 Collection of blood samples

Using sterile 10 ml syringes (Franklin Lakes, USA; BD 0.8mm x 38mm 21G x 11 2 TW), blood samples were drawn from both healthy and afflicted family members. Every person gave about three to five milliliters of venous blood. The blood samples were quickly placed in vacutainers containing the anticoagulant ethylene diamine tetra acetate (EDTA) (BD Vacutainer® Franklin Lakes, New Jersey, USA, K3 EDTA) in order to prevent blood clotting. After that, all of the samples were kept in storage at 4°C until the DNA was isolated and purified.

2.4 Extraction of genomic DNA

Genomic DNA extraction from blood samples of both healthy and affected family members was carried out using both commercially available kits and manual phenol-chloroform procedures.

2.4.1 Extraction of genomic DNA through the Phenol-Chloroform method

After incubating for two hours, blood samples were used to extract DNA, which was then kept cold (4°C). Whole blood was used to extract nuclear DNA using standard phenol-chloroform techniques (Sambrook and Russell, 2001).

- Approximately 750 microliters of blood were collected in a 1.5-milliliter Eppendorf tube, combined with equal amount of Solution A, and gently mixed by inversion. Subsequently, the mixture was allowed to incubate at room temperature for a duration of 30-45 minutes. Different solutions were utilized in the phenol-chloroform method (Table 2.1).
- The centrifuge was used to spin the Eppendorf tube at a speed of 13,000 revolutions per minute (rpm) for a duration of 60 seconds. Afterward, the liquid supernatant was removed, and 500µl of Solution A was once again added to dissolve the gathered pellet sediments.
- The centrifugation step at a speed of 13,000 revolutions per minute (rpm) for a duration of 60 seconds was performed again.
- A solution comprising 500µl of Solution B, 15µl of 20% SDS or 30µl of 10% SDS, and 12µl of Proteinase K enzymes was added to dissolve the pellet that formed after centrifugation. This was followed by an overnight incubation at 40°C.
- A freshly prepared mixture of Solution C and D, 500µl of it, was then added to the sample mixture, and the resulting mixture was centrifuged at 13,000rpm for 10 minutes.
- The centrifugation led to the formation of three visible layers, with the uppermost layer containing DNA transferred to an autoclaved Eppendorf tube.
- The DNA-containing tube had 500µl of Solution D added and underwent centrifugation at 13,000rpm for 10 minutes, resulting in three layers once again, with the upper layer being collected for DNA.
- For DNA precipitation, 500µl chilled isopropanol and 55µl of 3M sodium acetate were added to the tube, followed by observation of DNA thread formation after inverting the tubes.

- The supernatant was disposed of after a centrifugation at 13,000 rpm for 10 minutes. After adding 200µl of cold 70% ethanol to the tube holding the DNA pellet, the tube was spun at 13,000 rpm for seven minutes.
- After centrifugation, the DNA pellet formed, and the solution was carefully removed. The pellet was allowed to dry in a vacuum concentrator at 45°C for about 10 minutes.
- To guarantee total suspension of the dry DNA pellet and defense against DNases and RNases enzymes, the pellet was dissolved in 100–120µl of TE buffer and incubated for an entire night at 37°C.

Table 2.1: Solutions used in DNA extraction and their composition.

Solutions	Composition of solutions
Solution A	Sucrose [0.32M] + MgCl2 [5mM] + Tris [10mM: 7.5pH] + 1% (v/v) TritonX-100
Solution B	EDTA [2mM: 8.8pH] + NaCl [400mM] + Tris [10mM: 7.5pH]
Solution C	Phenol
Solution D	Chloroform + Isoamyl-alcohol [24:1]
70% ethanol	Absolute Ethanol [70ml] + Distilled water [30ml]
20% SDS	

2.4.2 Kit based method for DNA extraction

- The Gene-Elute Blood Genomic DNA kit, supplied by Sigma-Aldrich, was employed to extract genomic DNA.
- A mixture comprising around 200µl of whole blood sample, 200µl of lysis buffer, and 20µl of proteinase K enzyme was prepared in a 1.5ml tube and homogenized.
- The homogenized mixture underwent incubation in a water bath at 55°C for 10 minutes.
- Approximately 200µl of cold, 100% ethanol was added to the mixture, vortexed for 15 seconds, briefly spun at 3,000rpm, and transferred to a Sigma Aldrich mini prep column.
- The columns were centrifuged at 8,000rpm and then moved to separate tubes.
- A washing buffer (500µl) was added to the column, centrifuged at 13,000rpm, and this washing step was repeated twice.
- An elution buffer (approximately 240µl) was introduced into the middle of the column for thorough rinsing after an empty spin. Subsequently, the samples were incubated for 10 minutes at 25°C.

- Finally, the DNA was obtained from the column using high-speed centrifugation and then preserved in tubes.

2.5 Agarose gel electrophoresis

By dissolving 1g of agarose powder in 100ml of 1X TBE buffer (prepared by mixing 10ml of 10X TBE buffer with 90ml of distilled water), agarose Gel Electrophoresis allowed for the evaluation of the DNA's integrity and purity. A 1% agarose gel (Table 2.2) was used for the experiment. The agarose-buffer mixture was heated in a microwave until clear, allowed to cool to touch, and then 5µl of ethidium bromide was added. Subsequently, the gel mixture was poured into a casting tray with combs for pore creation. The gel solidified in approximately 20 minutes at room temperature, was removed from the casting tray, and placed in the running tank filled with 1X running buffer. For sample loading, a DNA sample and loading dye (Bromophenol blue) were mixed in a 1:1 ratio and loaded into the gel wells. The apparatus was connected to a power supply and run at 80V for 20-30 minutes. Finally, DNA bands were visualized using a UV-trans illuminator (Biometra, Gottigen, Germany).

Table 2.2 Chemicals and their composition used in agarose gel electrophoresis.

Chemicals	Composition of chemicals
Agarose	0.5g
TBE (10X) buffer	EDTA [0.032M: 8.3pH] + Boric ac id [0.025M] + Tris [0.89M]
Ethidium Bromide	4µg/ml
Gel Loading Dye	Bromophenol blue dye [0.25%] + Sucrose [40%]

2.6 Quantification and Dilutions of extracted DNA

DNA sample quantification was carried out using the Colibri micro-volume spectrophotometer (Titertek Berthold, Germany) at an optical wavelength density (OD) of 260nm. Prior to sample loading, the instrument was blanked using 2µl of Tris-EDTA (TE) buffer. The quantification, expressed in ng/µl units, involved loading 2µl samples onto the spectrophotometer. DNA dilutions were created by adding nuclease-free water to 20-30µl DNA samples.

2.7 Linkage analysis using microsatellite markers

To establish genetic connections within all three families, homozygosity mapping was employed using microsatellite markers. Based on the observed homozygous allele patterns in affected individuals, it was assumed that a given marker was linked to a particular gene or locus; similarly, heterozygous allele patterns in non-affected individuals were also thought to be suggestive of linkage. Conversely, if both affected and unaffected individuals exhibited heterozygous allele patterns, it was concluded that the family was not linked to that particular gene or locus. Various microsatellite were markers used for homozygosity mapping of *SLC19A2* gene (Table 2.3).

Table 2.3: Microsatellite markers used for linkage analysis of *SLC19A2*.

No.	Gene name	Cytogenetic Location	Markers	Distance (cM)
1	*SLC19A2*	1q23.2–2	D1S1165	186.5
			D1S2814	188.09
			D1S210	185.82
			D1S398	165.94
			D1S370	187.13

2.8 Polymerase Chain Reaction (PCR)

The amplification of polymorphic microsatellite markers for the identified loci associated with thiamine-responsive megaloblastic anemia was conducted using 200 µl PCR tubes (Axygen, USA) obtained from Invitrogen Genelink (USA). The PCR reaction mixture, sourced from Invitrogen Genelink, was prepared with a total volume of 25 µl. Different chemicals were used in PCR (Table 2.3). The reaction mixture underwent gentle vortexing and centrifugation until it was thoroughly mixed. Make sure that the liquid in the PCR tube collects at the tube's base. Then, for DNA amplification, every PCR tube was combined and put inside a Biometra T3 PCR machine (T3000) from Göttingen, Germany.

Table 2.4: Chemical components and their roles in PCR.

Chemicals	Composition	Functions	Volume per reaction
Mg	(25 mM)	Cofactor for DNA polymerase enzyme	1.5 µl
PB	(200) mM (NH4)2SO4, (750) mM Tris HCl (pH 8.5), (0.1%) Tween 20	Optimum conditions for DNA polymerase	2.5 µl
Taq polymerase	1 unit (PerkinElmercetus, Fermentas, Burlington, Canada)(	production of a new strand of DNA	0.5 µl
dNTPs	(10nM)	monomers for fresh strands of DNA	0.5 µl
Forward primer		Give the DNA synthesis a jump start by providing a "free 3/" hydroxyl group.	0.3 µl
Reverse primer			0.3 µl
Sample DNA	Template DNA	It has specific gene sequence that need to be amplified	2 µl
PCR water	Deionized water	Providing a relevant backdrop for the response	17.4 µl
Total volume			25 µl

Table 2.5: Thermo cycling conditions employed during each PCR.

Steps		**Temperature (°C)**	**Time**	**Cycles**	**Mechanism**
Initial Denaturation		96° C	7 min	1	Initial breaking down of the DNA molecule
Amplification	a. Denaturation	96° C	30 sec	1-35	Unwinding of double stranded DNA into single strands.
	b. Annealing	50-64° C	30 sec		Priming of each strand of the target sequence in relation to its complementary sequence
	c. Extension	72° C	30 sec		Extension of complementary DNA strand from each respective primer
Final extension		72° C	7 min	35th cycle	Taq DNA polymerase completes the synthesis of un-extended remaining strands

2.9 Polyacrylamide Gel Electrophoresis (PAGE)

After the amplification of the PCR product, the reaction mixture was properly mixed in a cone-shaped flask to prepare an 8% non-denaturing polyacrylamide gel (Table 3.5). After that, the material was transferred between two glass plates that were 1.5 mm apart, and twenty wells were made using a comb. The gel was left at room temperature for 40–50 minutes to solidify. After carefully mixing each PCR result with four to six microliters of bromophenol blue dye, the wells were assigned. For the electrophoresis procedure, a vertical gel tank (Whatman Biometra, Germany) full of 1XTBE buffer was utilised. The electrophoresis conditions required three hours of application of a current of 120 volts (50 mA). Following the PCR result, ethidium bromide dye was applied.

Table 2.6: Composition of 50 ml polyacrylamide gel.

Chemical	Composition	Volume	Function
30% Acrylamide	In a 29:1 ratio, 1g of NN'-Methylenebisacrylamide and 29g of polyacrylamide	13.5 ml	Helps the polymer to create cross-links, which speeds up the polymerization process.
(10x) TrisBorate (EDTA)	0.02 M (EDTA), 0.89 M Tris, and 0.89 M Borate	5 ml	Offers optimum background
(10%) APS	(NH4)2SO4	0.35 ml	Supplies free radicals, which act as a catalyst to start polymerization.
(TEMED)	Tetra-Methyl-Ethylene Diamine	25 µl	Free radicals' stability
(Distilled water)		31.1 ml	Volume adjustment
(Total volume)		50 ml	

2.10 SLC19A2 Gene Variant Screening

2.10.1 Pre sequencing Amplification of *SLC19A2*

In this current research investigation, sequencing was conducted on all three families (Family1, Family2, Family3). The genomic DNA from the single affected member of each family was subjected to PCR amplification for all six exons of *SLC19A2* gene. Specific primers for each exon were designed using various online tools such as primer blast, primer3 (Rozen &

Skaletsky, 2000), and BLAST to ensure specificity. Different primers were used for amplification of *SLC19A2* (Table 2.7).

Table 2.7: Sequence of primers used to amplify *SLC19A2* gene.

Exon	Forward primer (F) (Nucleotide Sequence 5'-3')	Reverse primer (R) (Nucleotide Sequence 5'-3')
1	TGACCCACGACCTTGGAAG	TCGTCCTACAGAGCCGAAGA
2A	GTTATCTGAACTGCTGTTGTCAAGG	CCATTCACTCTCTGGCAGGTA
2B	ACTGCTGGCCATTCAATTTCTA	TGTATCAGTCTGACTTTGGAGGTC
3	ACAGTACATCTTTGCTGGGTTGA	CCAGTTTGTTAGTCATAGTCCTG
4	ACTGTGGGTAACATTTGGGTG	AGGAAGGAGGGTATGCTTTAC
5	GTCTTGGCCCTCAGAAAACACT	AATACAATGCTTCCTCCCATTTGC
6A	GCTGTGATGCTGCTTTGTGT	ATTTTGTGGCCTCTGTGGA
6B	AGTCAGAGCAATCAAAGC	CCTAGTTCCTGTCCCATTG
6C	GCATTATTCATATGCTTCCCAGAG	TCTTATCCCCATCCATTTGC
6D	GCAAAGTCGACATACTCATTTGGT	CAAGTTAGACCAGGGGAAGACT

A reaction mixture of 25 µl (Table 2.5) was prepared in a 200 µl PCR tube for each exon-specific primer, and amplification was carried out. The primers were designed from different intronic regions of each exon. The specificity of the primers was verified using the BLAST tool. Following successful amplification, purification of all the products was performed. The thermocycling conditions remained the same (Table 2.3), with the annealing temperature ranging from 60°C to 64°C.

Subsequently, the sequencing PCR products were run on a 2% Agarose gel along with 1000 base pair DNA ladder. During gel loading, 3 µl of PCR product with 3 µl bromophenol blue was loaded into the wells, and the gel was run horizontally for 20 minutes at 110 volts. Following 20 minutes of gel electrophoresis, the photo was taken using a UV trans illuminator

that was connected to a digital system (DC EDAS, Kodak, Digital Sciences New York, USA). By contrasting the product sizes with the ladder, they were assessed. The PCR products were purified once the particular bands were chosen.

Table 2.8: Chemical composition of the pre-sequencing PCR mixture.

Chemicals	Composition	Volume
Genomic DNA	Dilution (20:80 ratio)	2 µl
Reverse Primer	Dilution (10:40)	1 µl
Forward Primer	Dilution (10:40)	1 µl
Taq Polymerase	One unit (Burlington, Canada; Perkin-Elmercetus, Fermentas)	0.5 µl
MgCl2	(25 Mm)	2 µl
dNTPs	Fermentas, UK; 10 mM)	0.5 µl
10x buffer, or PB buffer	KCl 500 mM, Tris-HCl (pH 8.3) 100 mM	2.5 µl
PCR water		15.5 µl
Total volume		25 µl

2.10.2 Purification of PCR Products

All amplified products underwent purification using the "Axygen Biosciences PCR Cleanup Kit" as outlined in Table 2.6. The following steps were executed:

1. Each PCR tube holding the amplified product received 120 µL of binding buffer solution, which was pipetted in and thoroughly mixed.
2. The mixtures were moved to membrane spin cartridges made of silica that were put together in 2 ml collecting tubes with double-stranded DNA adsorption capabilities for the purification procedure. After centrifuging the collecting tube containing the column for one minute at 13,000 rpm, the flow-through was disposed of. The column that was left empty was saved for later use.
3. After removing proteins, dNTPs, DNA polymerase, and unbound primers with two washes using 500 µL ethanol-added washing solution, the column was centrifuged for one minute at 13,000 rpm. After discarding the waste from the collection tube, an empty spin was performed at the same speed.

4. The columns were put into Eppendorf tubes that had already been labelled, and they were then treated with 18–25 µL of heated elution buffer (70°C) and allowed to incubate for six minutes.
5. The final collection of purified products in the Eppendorf tube was obtained by centrifuging the column constructed in the tube for two minutes at 13,000 rpm after incubation.
6. Following purification, the result was examined by loading 3 µL of DNA along with 3 µL of loading dye onto a 2 percent agarose gel.

2.10.3 Sequencing PCR

After successful purification of amplicons, each exon was subjected to sequencing PCR and sanger sequencing (Table 2.7).

2.10.4 Sanger sequencing

The ABIM Prism 3310L, an automated DNA sequencer from Applied Biosystems in the USA, was employed to perform dideoxy chain termination analysis on the PCR-purified product. This analysis necessitated the use of the Ready Reaction Kit v3.1 from PE Applied Biosystems, also based in the USA. All six exons of *SLC19A2* gene were sequenced and the data were obtained.

Table 2.9: Composition of PCR purification kit.

Solution	Chemical composition
Solution of binding buffer	Concentrated Guanidine-HCl Isopropanol Tris HCl EDTA
Solution of binding buffer	NaCl, EDTA, and Tris HCl
Elution buffer	10 mM Tris HCl and 0.1 mM EDTA

Table 2.10: Ingredients of sequencing PCR.

Ingredients	Volume
DNA blueprint	1.0 µl (25 ng)
Primer (Reverse/Forward)	1.0 µl (10 pmol)
5x sequencing-buffer	1.0 µl
Ready reaction mix (RR)	1.0 µl
PCR Water	6.0 µl
Total amount	10.0 µl

2.10.5 Variant interpretation

With the help of BioEdit alignment editor version 7.2 (https://bioedit.software.informer.com/7.2/), the sequencing data from Sanger Sequencing was examined. To find any nucleotide alterations, the chromatogram of the afflicted person was compared to the matching reference (normal) gene sequence that could be found in the Ensemble GenomeBrowser Database (https://asia.ensembl.org/index.html). Software such as MutationTaster (https://www.mutationtaster.org/), Franklin genoox, Varsome, and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) was used to determine the pathogenicity of any variations found in the affected sequence.

Chapter 03

RESULTS

3. RESULTS

3.1 Family A

Family A, suffering from thiamine responsive megaloblastic anemia, was sampled from District Rawalpindi, Punjab province, Pakistan. and underwent both clinical and genetic analyses. The pedigree for four generations was constructed using information provided by the family. The pedigree composed of four generations, encompassing fifteen members, including one affected member, i.e., daughter (Figure 3.1). In the current pedigree we have sampled the normal parents and affected daughter. After a written consent, blood samples were collected in EDTA-vacutioner tube from affected daughter (**IV-2**) and normal parents (**III-3, II-4**). The affected daughter is 4.5 years old female exhibiting clinical characteristics of TRMA.

3.1.1 Clinical Characteristic of Family A

Affected member of family A is 4.5 years old female having clinical characteristics of TRMA. It includes megaloblastic anemia (Hb 6.4g/dl, HCT 19 %, MCV 87.2fl, MCH 29.5pg, MCHC 33.9g/dl, RDW-CV 15.8%, reticulocytes count 0.07%, uric acid 3.12 mg/dl), Pancytopenia (platelet count 100000/ul, TLC 3.35 10^9/L, RBC count 2.18 10^9/L, sensorineural deafness and diabetes mellitus. Other signs and symptoms of the patient include sore throat, fever, petechiae, bruises all over the body, swollen lips and bleeding from different body sites.

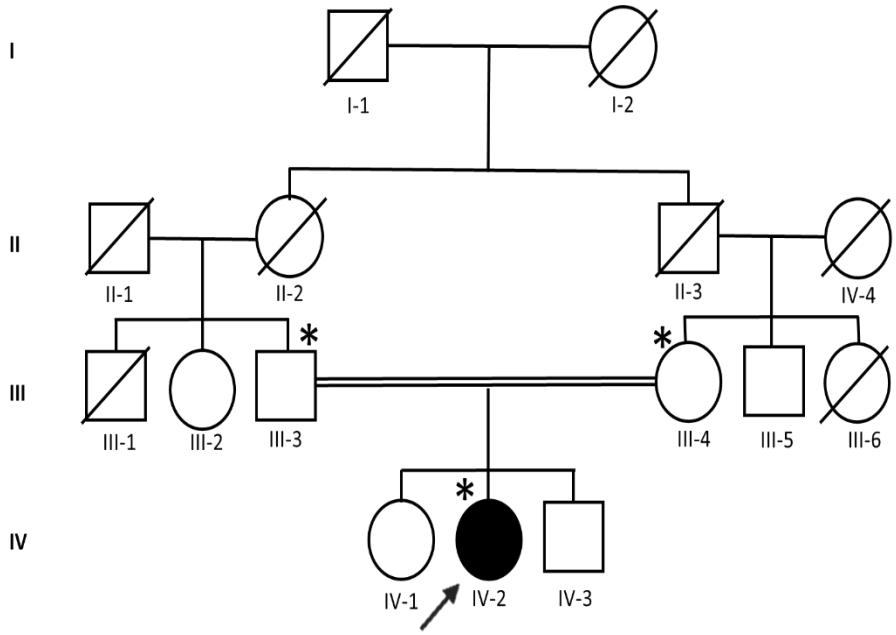


Figure 3.1: The pedigree of Family A having TRMA: Males and females are represented by squares and circles, respectively. Double line show consanguinity while filled squares and circles represent affected individuals. In pedigree, generations are represented by Roman numerals, while members in generations are indicated by Arabic numerals. Asterik symbol (*) shows blood donors while diagonal arrow shows the proband.

3.2 Family B

Family B, was also sampled from District Rawalpindi, Punjab province, Pakistan and underwent both clinical and genetic analyses. The pedigree composed of three generations, encompassing eleven members, including one affected member, i.e., daughter (Figure 3.2). After a written consent, blood samples were collected in EDTA-vacutioner tube from affected daughter (**III-3**), normal parents (**II-2, II-3**) and normal sons (**III-1, III-2**). The affected daughter is an 8-month-old child exhibiting clinical characteristics of TRMA.

3.2.1 Clinical Characteristic of Family B

The affected individual in family B is an 8-month-old girl exhibiting clinical features of TRMA syndrome. These manifestations encompass megaloblastic anemia (with hemoglobin at 4.3g/dl, hematocrit at 13.8%, mean corpuscular volume at 100 fl, mean corpuscular hemoglobin at 31.2 pg., mean corpuscular hemoglobin concentration at 30.9 g/dl, and a red cell distribution width coefficient of variation at 24.0%), pancytopenia (platelet count of 57000/ul, total leukocyte count of 3.35 x 10^9/L, and red blood cell count of 1.37 x 10^9/L), sensorineural deafness, and diabetes mellitus. Additionally, the patient experiences symptoms such as a sore throat, fever, petechiae, widespread bruises, swollen lips, and bleeding from various body sites. Uric acid levels are noted to be 3.12 mg/dl.

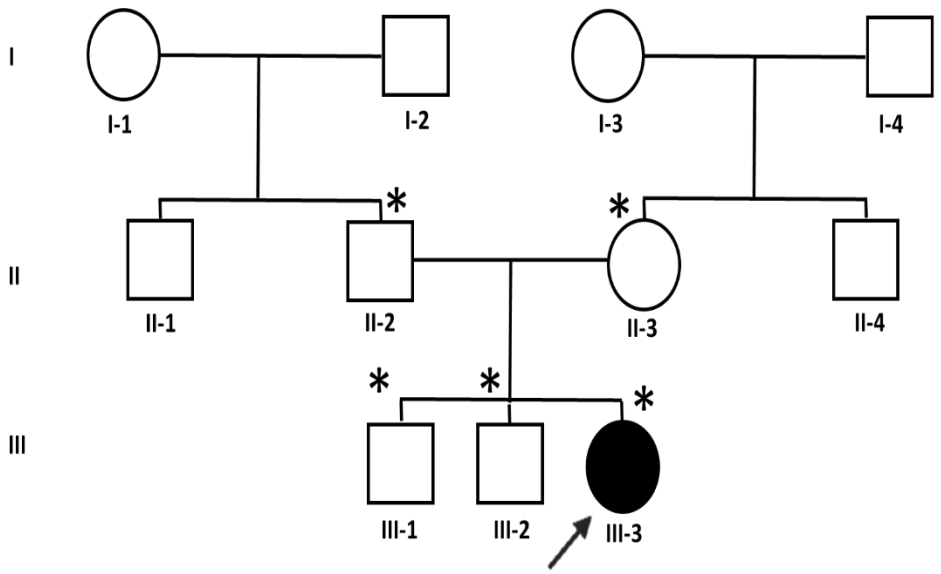


Figure 3.2: Pedigree of Family B having TRMA: Males and females are represented by squares and circles, respectively. Filled squares and circles represent affected individuals while unaffected individuals are represented by empty shapes. In pedigree, generations are represented by Roman numerals, while members in a generation are indicated by Arabic numerals. Asterik symbol (*) shows blood donors while diagonal arrow shows the proband.

3.3 Family C

Family C, was also sampled from District Rawalpindi, Punjab province, Pakistan. and underwent both clinical and genetic analyses. The pedigree composed of four generations, encompassing ten members, including one affected member, i.e., daughter (Figure 3.3). After a written consent, blood samples were collected in EDTA-vacutioner tube from affected daughter (**IV-1**) and normal parents (**III-1, III-2**). The affected daughter is an 11-month-old child exhibiting clinical characteristics of TRMA.

3.3.1 Clinical Characteristics of Family C

The affected individual in family C is a 10-month-old girl exhibiting clinical features of TRMA syndrome. These manifestations encompass megaloblastic anemia (with hemoglobin at 4g/dl, hematocrit at 11.8%, mean corpuscular volume at 102 fl, mean corpuscular hemoglobin at29.2 pg., mean corpuscular hemoglobin concentration at 28.9 g/dl, and a red cell distribution width coefficient of variation at 26.0%), pancytopenia (platelet count of 67000/ul, total leukocyte count of 3.15 x 10^9/L, and red blood cell count of 1.27 x 10^9/L), sensorineural deafness, and diabetes mellitus. Additionally, the patient experiences symptoms such as a sore throat, fever, petechiae, widespread bruises, swollen lips, and bleeding from various body sites. Uric acid levels are noted to be 4.12 mg/dl.

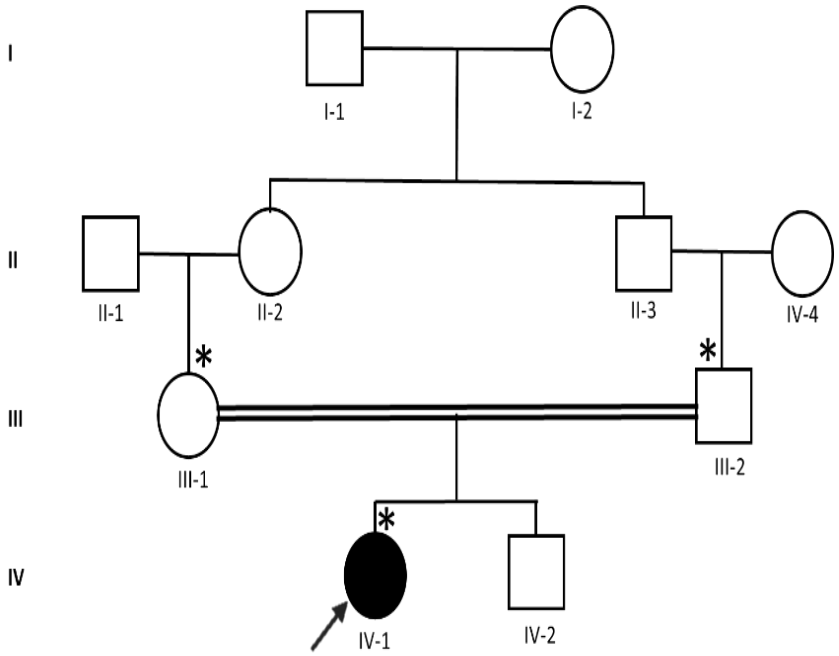


Figure 3.3: The pedigree of Family C having TRMA: Males and females are represented by squares and circles, respectively. Double line show consanguinity while filled squares and circles represent affected individuals. In pedigree, generations are represented by Roman numerals, while members in generations are indicated by Arabic numerals. Asterik symbol (∗) shows blood donors while diagonal arrow shows the proband.

3.4 Collection of Blood and DNA Extraction

Blood were collected from the normal father (**I-1**), mother (**I-2**) and affected daughter (**II-2**) of family A. For family B, samples were collected from normal mother (**I-1),** father (**I-2**), sons (**II-1**, **II-3**) and affected daughter **(II-2).** Family C, samples were collected from normal mother (**III-1**), father (**III-2**) and affected daughter (**IV-I**). DNA was extracted from all blood samples through phenol chloroform method and its integrity was estimated by 1% agarose gel (Figure 3.4).

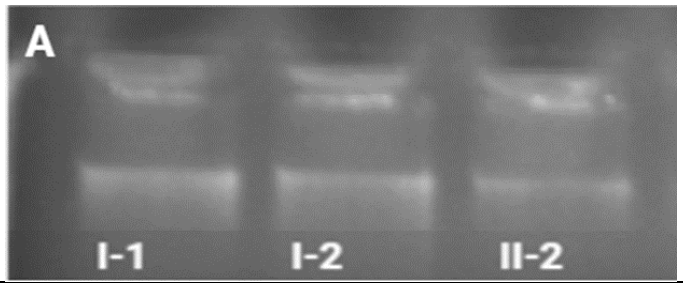
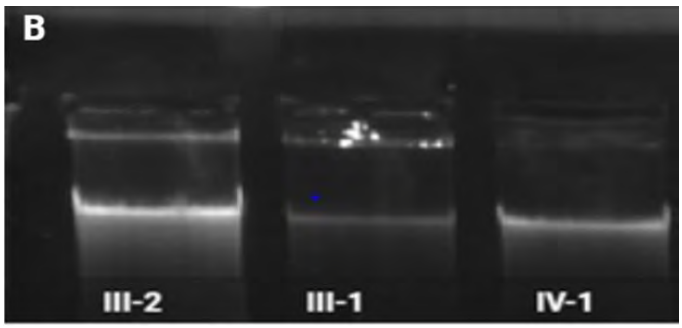
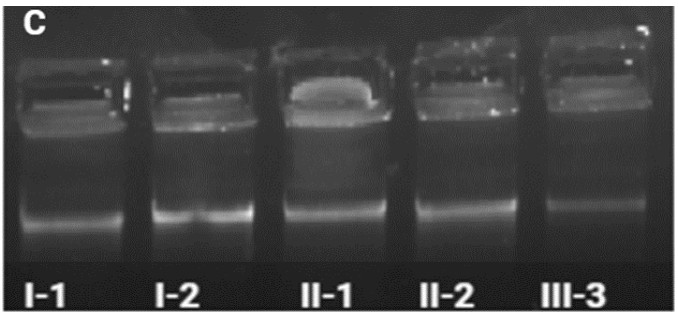
Family ID	DNA Quality analysis on 1% Agarose gel
Family A	
Family B	
Family C	

Figure 3.4: Extracted DNA of family A, B and C.

3.5 Linkage Analysis by Using Microsatellite Markers

For the genomic localization of genes involved in TRMA, homozygosity mapping was performed by using polymorphic microsatellite markers linked to the previously known genes for TRMA. In all three families, *SLC19A2* gene was tested for linkage with the microsatellite markers (Table 2.3). As described in the preceding section, PCR was carried out using the conventional procedure, followed by resolving the products on an 8% polyacrylamide gel and staining with ethidium bromide. The validation of the association of the tested gene with

TRMA in all three families relied on the observation that unaffected individuals displayed a heterozygous allele pattern, while affected individuals exhibited a homozygous allele pattern. In family A, the DNA of three individuals, including the father (**I-1**), mother (**I-2**), and the affected daughter **(II-2**), was analyzed using microsatellite markers (D1S1165, D1S2814, D1S210, D1S398, D1S370) closely associated with known causative genes for thiamine-responsive megaloblastic anemia. The markers examined were related to the *SLC19A2* gene, located at cytogenetic position 1q23.2–23. The genotyping analysis revealed a connection between the family and the *SLC19A2* gene, with the linked markers appearing in a homozygous state in the affected daughter and in a heterozygous state in the unaffected family members. Members of the family were identified as heterozygous for markers unrelated to the *SLC19A2* gene. Marker **D1S398** was found to be linked with *SLC19A2* gene (Figure 3.5).

Family A

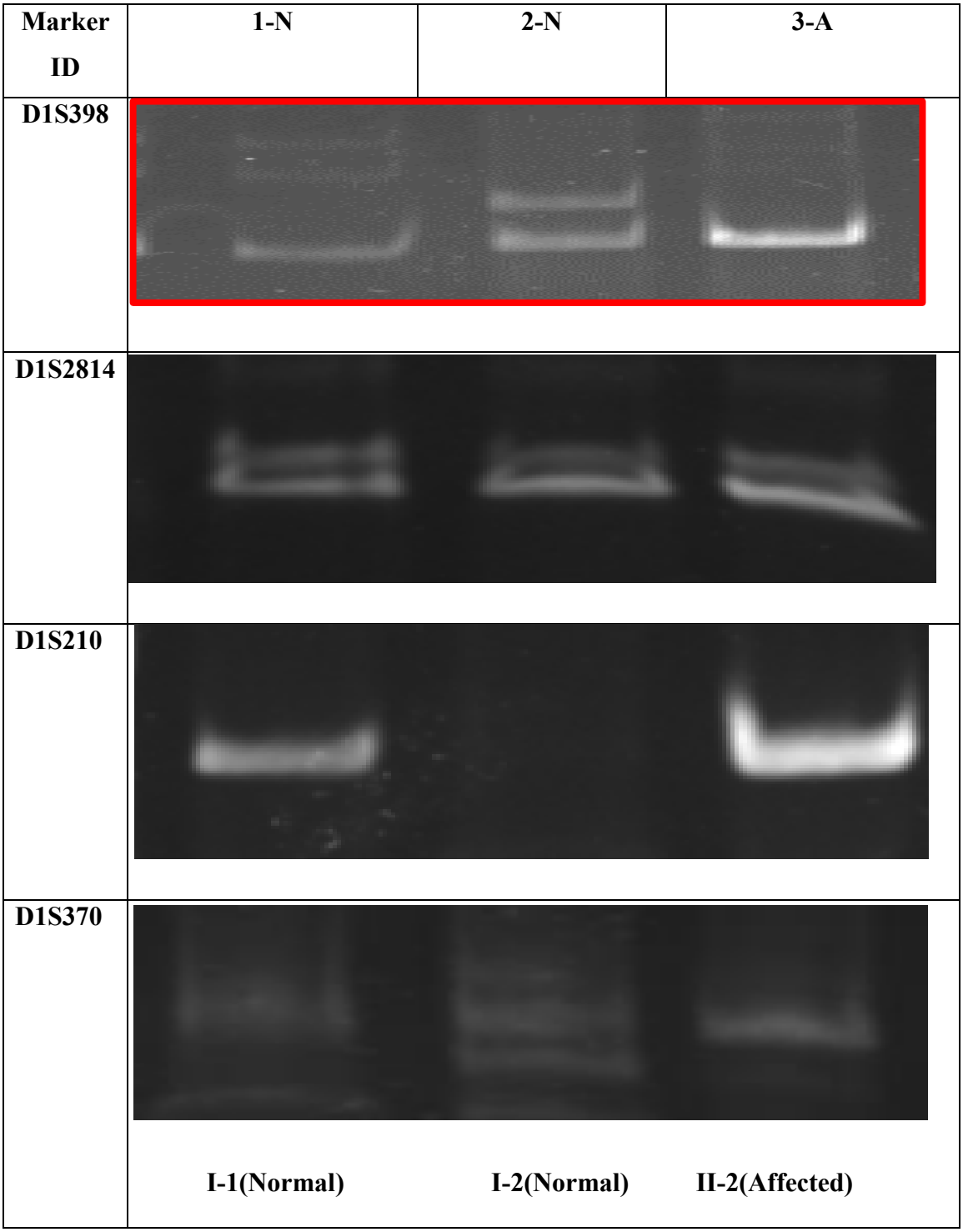
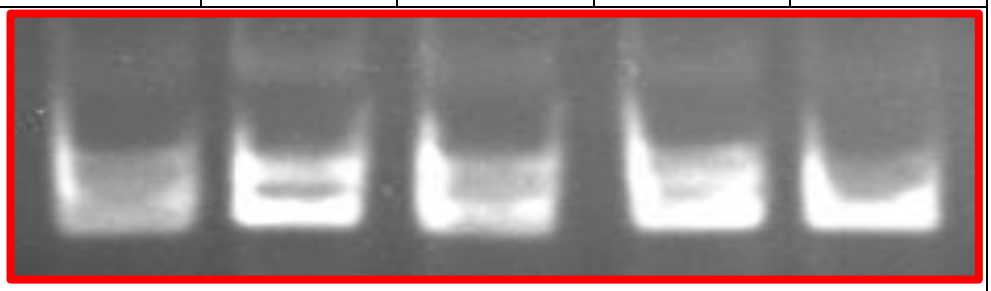
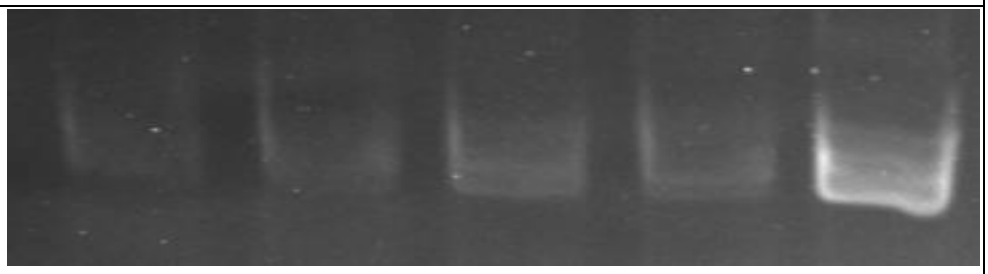
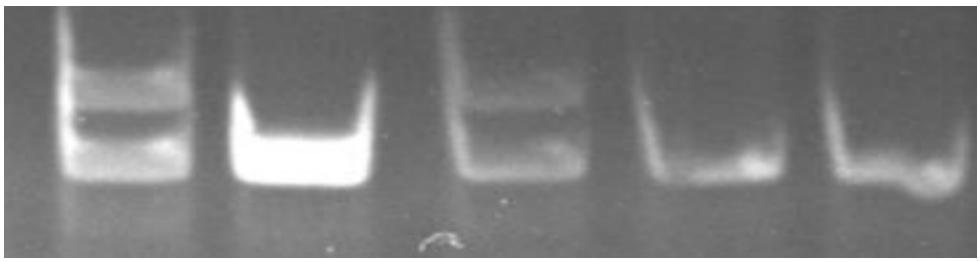
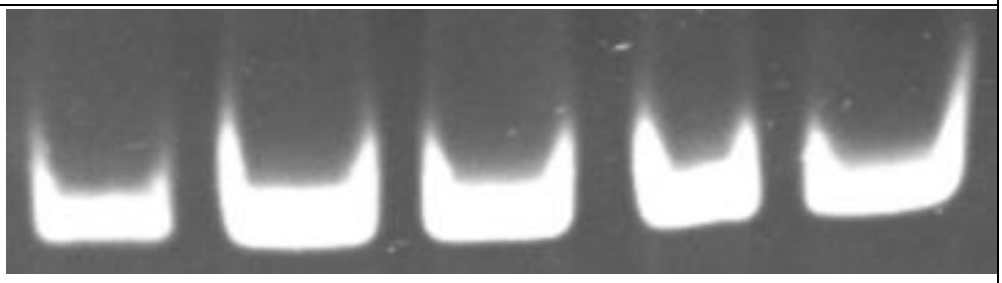


Figure 3.5: Polyacrylamide electropherogram of family A illustrating homozygosity in the affected member (II-2) for microsatellite markers D1S398 flanking the*SLC19A2* gene on chromosome 1q23.3. Roman and Arabic number define the generation position and number of the member in the family pedigree.

In family B, the DNA of five individuals, including father (**I-1**), mother (**I-2**), two sons (**II-1, II-2**) and affected daughter (**III-3**) was also analyzed using the same microsatellite markers (Table 2.3). Linked markers appeared in a homozygous state in the affected daughter and in a heterozygous state in the unaffected family members. Both affected and non-affected members were identified as heterozygous for markers unrelated to the *SLC19A2* gene. Marker **D1S210** was found to be linked with *SLC19A2* gene (Figure 3.6).

Family B

Marker ID	1-N	2-N	3-N	4-N	5-A
D1S210					
D1S2814					
D1S398					
D1S370					

I-1(Normal) I-2(Normal) II-1(Normal) II-2(Normal) III-3(Affected)

Figure 3.6: Polyacrylamide electropherogram of family B illustrating homozygosity in the affected member (III-3) for microsatellite markers D1S210 flanking the *SLC19A2* gene on chromosome 1q23.3. Roman and Arabic number define the generation position and number of the member in the family pedigree.

In family C, the DNA of three individuals, including father (**III-2**), mother (**III-1**) and affected daughter (**IV-1**) was also analyzed using the same microsatellite markers given in table 2. Linked markers appeared in a homozygous state in the affected daughter and in a heterozygous state in the unaffected family members. Both affected and non-affected members were identified as heterozygous for markers unrelated to the *SLC19A2* gene. Marker **D1S370** was found to be linked with *SLC19A2* gene (Figure 3.7).

Family C

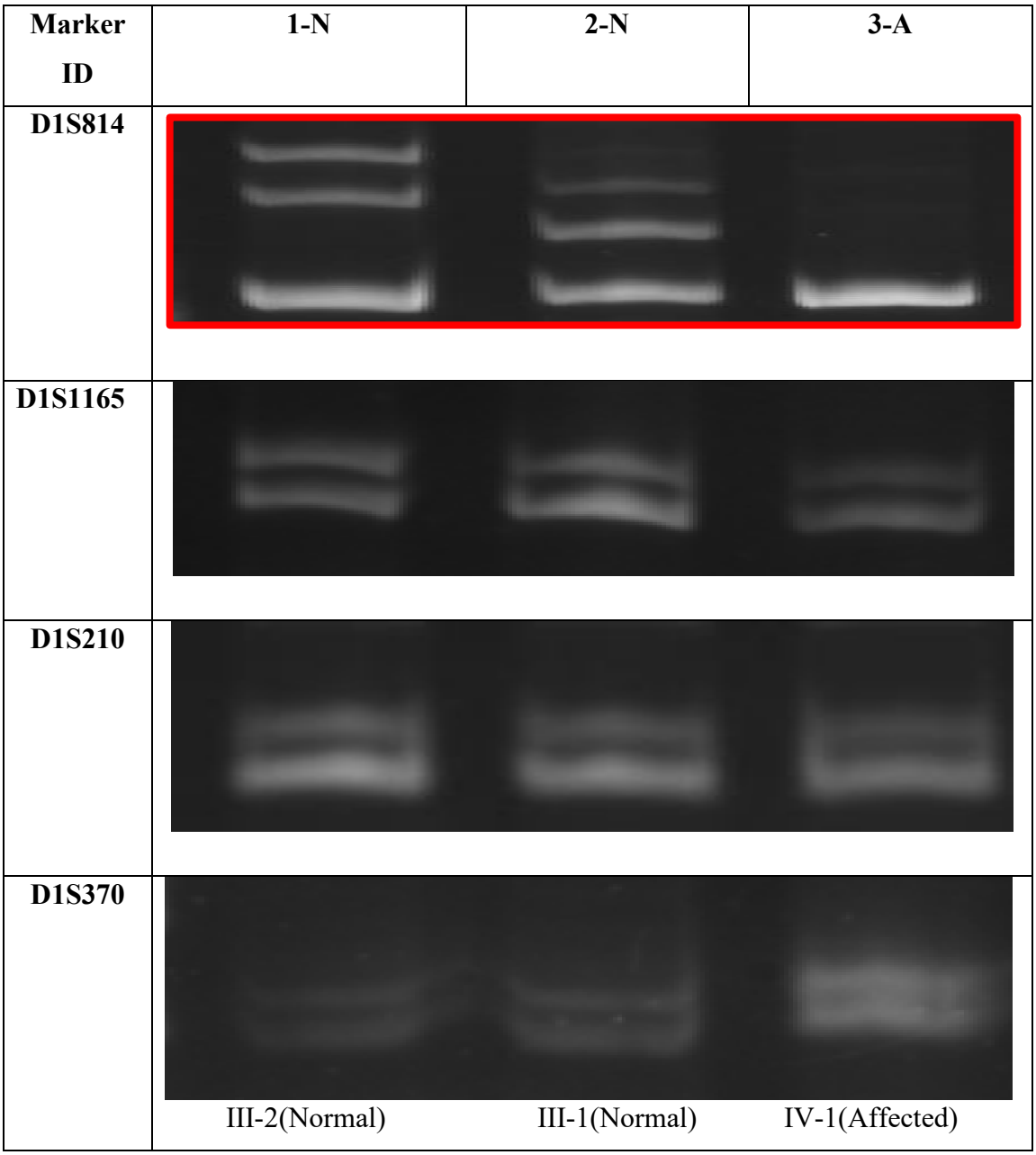


Figure 3.7: Polyacrylamide electropherogram of family C illustrating homozygosity in the affected member (IV-1) for microsatellite markers D1S814 flanking the *SLC19A2* gene on chromosome 1q23.3. Roman and Arabic number define the generation position and number of the member in the family pedigree.

3.6 Sequencing of *SLC19A2* gene

The genotyping outcomes for all three families reveal a connection to the *SLC19A2* gene situated on chromosome 1q23.2–23. To detect a potentially harmful genetic variation within the coding regions or intron and exon boundaries, primers for each of the six coding exons were designed utilizing Primer3 software (https://primer3.ut.ee/) (Table 3.6).

In Family A, highly mutant exon 2 of the affected daughter was sequenced followed by exon 3, 4 and 5 (Figure 3.8). Sanger Sequence analysis of the exon 2, identified a homozygous novel variant [NM_006996: exon 2: c.519_519delT, p.(V174Sfs*2)], confirmed by bioinformatics software i.e. Mutation taster, Polyphen2 and Franklin genoox as pathogenic. Exon 2 of normal father (**I-I**) and mother (**I-2**) was also subjected to Sanger sequencing for expected heterozygous variant but no variant was found in normal members of family 1 (Figure 3.8).

Exon 2 of family B was also subjected to Sanger sequencing through their respective primers; however, we did not find any variant in exon 2 (Figure 5). The remaining exons of family B were not sequenced due to time constraints.

All exons of family C have been amplified successfully. Purification products were compared with 1000bp ladder. Fragments of suitable size were selected for Sanger sequencing. However, exons of family C were not sequenced due to time constraints.

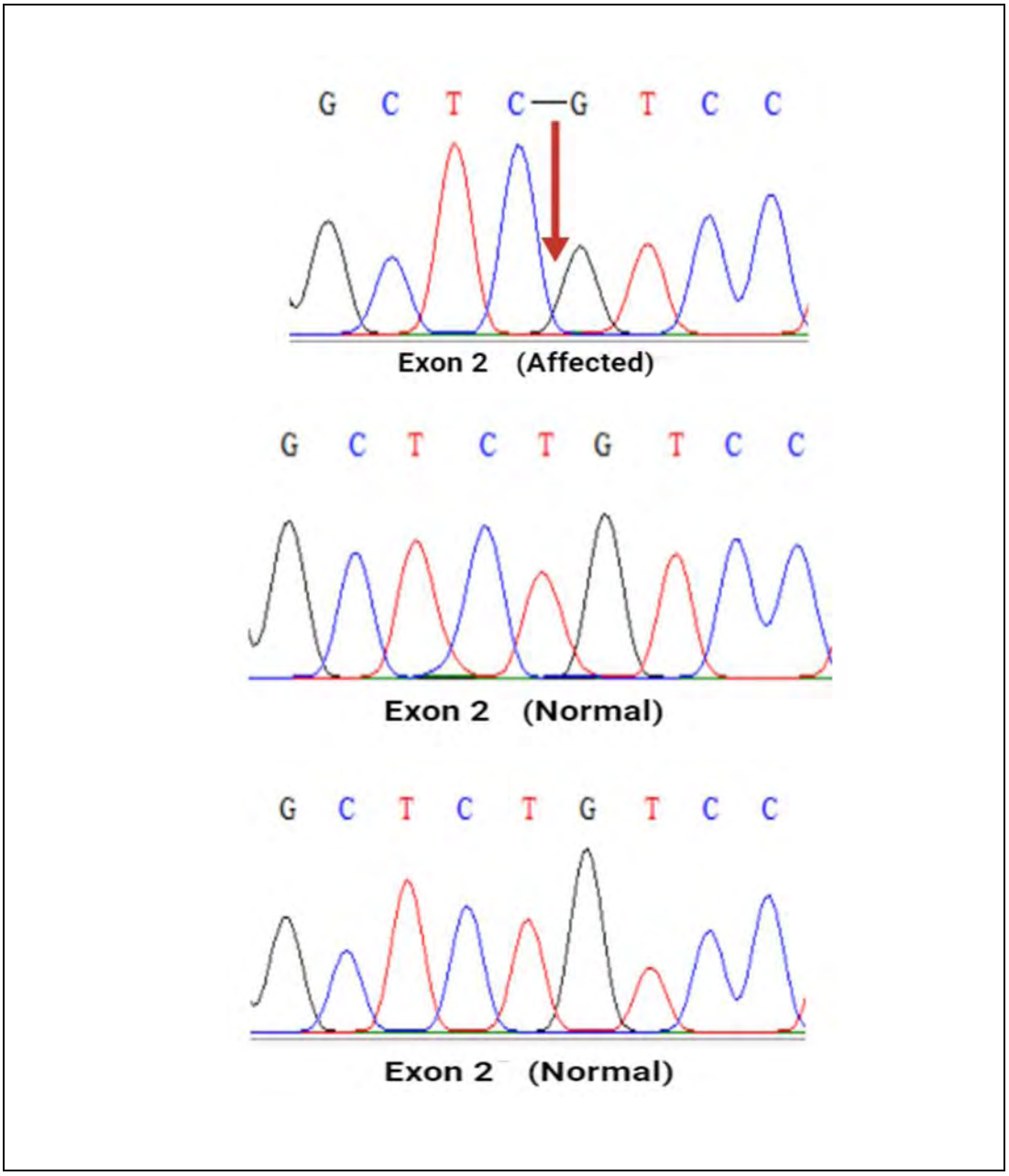


Figure 3.9: Comparative analysis of exon 2 among affected daughter, normal father and mother in family A.

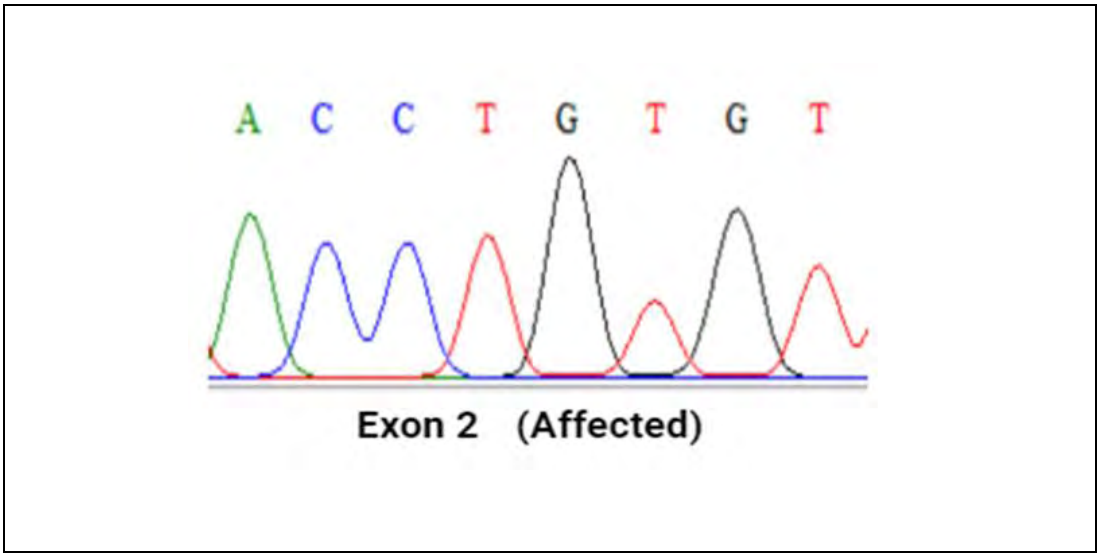


Figure 3.10: Partial sequencing chromatogram of exon 2 in affected member of family B.

Chapter 04

DISCUSSION

4. Discussion

Various genetic disorders of autosomal dominant, recessive and X-linked inheritance patterns in Pakistani population are identified which cover both non-syndromic and syndromic forms. Due to social, religious, economic and cultural factors 82.5% parents in various geographical locations of Pakistan are involved in consanguineous marriages (Iqbal *et al*., 2020). In all four provinces of Pakistan TRMA cases is present. But due to limited resources of genetic screening rare genetic anomalies like TRMA are not getting enough attention. Although rare diseases are affecting small portion of the population but its impact is very large on the patients as well as on the society. Urgent efforts and increased resources are essential to address this issue and provide necessary support to affected individuals. To find out the solution more resources and efforts are necessary for the support of those suffering from rare disease (Umair *et al.,* 2018).

TRMA or Rogers syndrome is an autosomal recessive monogenic disorder characterized by megaloblastic anemia, type I diabetes mellitus and hearing loss. It is inherited from parents into their offsprings and having various degrees of severity from pale skin to deafness and megaloblastosis. It mainly affects the developmental and biochemical pathways in the human body hence it is transmitted in syndromic forms (Kaur *et al*, 2023). In the current study three families of TRMA were samples from various regions of Punjab province and were analyzed both clinically and genetically. Whole blood was collected from affected and normal members of all families for extraction of genomic DNA. Microsatellite markers specifically designed for the previously reported genes were used for homozygosity mapping. When linkage was established, the gene was subjected to Sanger sequencing for the identification of pathogenic variant. Hence, the current research study consists of two phases for the genetic characterization of TRMA families in Pakistan. First phase is homozygosity mapping while the second one is Sanger sequencing.

The causative gene of TRMA is *SLC19A2* which is located on chromosome 1 having cytogenetic location of 1q23.3. It consists of six exons having 22.5kb length (Anna Marcé-Grau *et al.,* 2019). The gene encodes high affinity thiamine transporter across the cell membrane composed of 497 amino acids and 12 transmembrane domains having both COO and NH_2 ends towards the cytoplasm and also possess two N terminal glycosylation sites (Ramaswamy Ganesh *et al.,* 2009). Most of the cells in the body have low concentration of thiamine due to abnormal thiamine transporter in the cell membrane (Violeta Mikstiene *et al.,* 2015). Thiamine depleted cells are unable to produce sufficient amount of energy as well as nucleotides which are necessary for the normal survival of the cells hence cells cannot

reproduce and become died (Ramaswamy Ganesh *et al.,* 2009). TRMA syndrome is caused by a varieties of mutations ranging from point mutation of single nucleotides to macro deletions (Sako *et al.,* 2022). *SLC19A2* gene is expressed in varieties of tissues including brain, heart, liver, kidney, retina, pancrease, intestines, placenta, lungs, lymphocytes and fibroblasts. Its expression in the sensorineural cells of the ear, retina of eyes and beta cells of the pancrease is very important as these tissues have no other high affinity thiamine transporters for sufficient transportation of thiamine. As thiamine act as cofactor in various mitochondrial pathways including hexose monophosphate shunt for biosynthesis of riboses, Kreb cycle for the production of energy and heme for biosynthesis of hemoglobin. Therefore, alteration in *SLC19A2* gene and reduction of thiamine leads to cardinal triade of TRMA including megaloblastic anemia, diabetes mellitus and sensorineural deafness (Rai *et al.*, 2023).

This research study was conducted in three Pakistani families suffering from TRMA. The aim was to assess TRMA families both clinically as well as genetically. All of our three families of TRMA were presented with clinical features of megaloblastic anemia, sensorineural deafness, and diabetes mellitus. In family A, a female baby with the age of 4.5 years was diagnosed with the clinical features of megaloblastic anemia, diabetes mellitus and sensorineural deafness. The specific features observed in the patient were sore throat, fever, petechiae, bruises all over the body, swollen lips and bleeding from different body sites. The clinical features of family A were in lined with the previously reported clinicals of TRMA with few exceptions. A case study of 21 month old female baby suffering from TRMA was diagnosed with megaloblastic anemia, sensorineural deafness, and diabetes mellitus but there was no history of bleeding, fever, sore throat, and jaundice (Patil *et al.*, 2020). Another case study of four TRMA patients suffering from severe anemia and diabetes mellitus was reported. Hearing was normal while bleeding, ecchymosis, astigmatism, and visual impairment was also observed (Kang *et al*., 2021). Similar studies of 16 years old Indian male and 1year female baby was also reported in which all the cardinal findings of TRMA including megaloblastic anemia, diabetes and deafness were present. Additionally, pulmonary hypertension and tricuspid regurgitation was also observed (Kaur *et al.*, 2023; Patil *et al.*, 2020).

In family B, 2.5 years old girl was diagnosed with clinical features of megaloblastic anemia, deafness and diabetes mellitus. Sore throat, fever, petechiae, bruises, and bleeding were not observed. Clinical features of family B were also in lined with the previously reported data of TRMA with few exceptions. Similar study of TRMA patients was conducted involving three patients of TRMA. Patient 1 was 5-month old female having cardinal triade of TRMA. In

Addition, cardiac manifestations and supraventricular tachycardia was also observed. Patient 2 was 13 years old male having diabetes mellitus, megaloblastic anemia and mild sensorineural deafness. Hepatomegaly, abdominal distention and cardiac manifestations were also observed. Patient 3 was 19 years old female having cardinal triade of TRMA as well as atrial tachycardia, atrial fibrillation and chronic heart failure (Lorber *et al.*, 2003).

In family C, 10-month-old girl was diagnosed with clinical features of megaloblastic anemia, diabetes mellitus and mild sensorineural deafness. Petechiae, bruises, and bleeding were also observed from different body sites. Clinical features of family C were parallel with the previously reported data however, no cardiac manifestations were observed. Similar study was conducted in Tunisian family having clinical features of megaloblastic anemia, diabetes mellitus and bilateral sensorineural deafness. Petechiae, bruises and bleeding was also observed. Additionally, atrial septal defect, atrial dysrhythmia, cardiomyopathy and congestive heart failure were also present (Gritli *et al.*, 2001). Another similar study was conducted in 30-month-old Austrian boy having clinical features of megaloblastic anemia, diabetes mellitus and hepatomegaly. Petechiae, bruises and bleeding were also observed but sensorineural deafness was not present (Pichler *et al.*, 2012).

After clinical assessment, we have recruited all the three families for genetic screening. Exon 2, 3 and 4 of affected member of family 1 was subjected to Sanger sequencing. A novel homozygous variant [NM_006996: exon 2: c.519_519delT, p.(V174Sfs*2)] was identified in exon 2 and was confirmed as pathogenic by Mutation taster, Polyphen2 and Franklin genoox. Exon 2 is highly mutant among the six exons of *SLC19A2* gene therefore it was at priority during sequencing. Similar study of TRMA was conducted in different families of different ethnicities including Indian, Italian, Caucasian, Portuguese, Floridian, Canadian and Korean in which compound heterozygous variants was identified in the first four exons of *SLC19A2* gene. Most of these variants are in exon 2 (Bergmann *et al.*, 2009). Another similar study was conducted in four Iranian families with TRMA in which 2 frameshift variants were identified in exon 2 of *SLC19A2* gene. Among them one variant is 1-bp insertion while other is 2-bp deletion (Diaz *et al.*, 1999). Exon 2 of both normal parents in family A was also subjected to Sanger sequencing. Interestingly, no heterozygous variant was identified in both parents. However, the autosomal recessive pattern of inheritance must require a carrier gene for inheriting the trait. Therefore, we can conclude that the mutation is denovo mutation as the parents of affected individual have normal alleles. The pathogenicity of the variant was confirmed by different bioinformatics tools. Additionally, the variant was classified as

pathogenic and protein damaging by ACMG classification. The franklin result interpretation showed that the variant was classified as pathogenic by the following criteria as PVS12, P4, BP2.

Exon 2 of family B was also subjected to Sanger sequencing through their respective primers. However, no variant was found in exon 2 of family B. Hence, it is recommended to sequence the remaining exons of *SLC19A2* gene. Our next strategy is to sequence exon 3 and 4 as they also have high prevalence of variants. A study revealing prevalence of variants in different exons of *SLC19A2* gene, highlighted that after exon 2, exon 3 and 4 have high prevalence of 12% and 14% of variants respectively (Zhang *et al.*, 2021). Another study also revealed the presence of novel variant in exon 4 of *SLC19A2* gene (Habeb *et al.*, 2018). In another similar study a novel nonsense variant was identified in exon 4 which introduces a premature stop codon in *SLC19A2* gene (Tahir *et al.*, 2015). Missense variant in exon 3 of *SLC19A2* gene has also been reported (Pomahačová *et al.*, 2017).

For genetic screening, all exons of *SLC19A2* gene in family C was amplified and purified but no one was subjected to Sanger sequencing due to time constraints. For the identification of pathogenic variant, it is recommended to sequence exon 2 on the first priority followed by exon 3, 4 and 5.

This research study has broadened the range of variants in *SLC19A2* gene and increased the possibility of detecting variants in areas of high consanguinity through genetic screening. It also provided important information about the clinical features of TRMA. The frequency of TRMA in our families is similar to that seen worldwide. Therefore, it is needed to assess TRMA both clinically and genetically in different populations. Various clinical feature like megaloblastosis, consistent hyperglycemia and deafness indicate that TRMA affects various body organs. Earlier findings of thiamine deficiency lead to hematological abnormalities are parallel with our findings. TRMA is one of the important health issue in Pakistan and all health care provider must be well informed about it. Our research study also highlighted the need of thiamine level estimation regularly in patients having macrocytosis and are involved in consanguineous marriages. Genetic screening of TRMA revealed different variants in *SLC19A2* gene indicating its crucial role in pathogenesis of various diseases in Pakistan. Also detection of novel and prevalent mutation in *SLC19A2* gene provides important genetic marker for the diagnosis of TRMA.

Genetic screening of our families support the heterogeneous nature of TRMA highlighting the need of performing genetic testing to determine various mutations associated with TRMA.

Certain variants in *SLC19A2* gene cause more severe clinical features in the patients which make the relationship between genotype and phenotype more complicated. Hence, studying the functional consequences of such variant at molecular level is necessary for determining disease pathogenesis and specific therapeutic interventions. After comparing our findings with the available dataset, we find that our variant is different from the available spectrum of *SLC19A2* gene variants. This highlight the need of specific genetic database and diagnostic technique for Pakistani population. Our research also adds the knowledge to the currently available information about TRMA genetics and tells about the unique genetic features of TRMA patients in Pakistani population during diagnosis. Our research not only help during diagnosis but it also gives new directions for the treatment of the disease. As instead of simple thiamine supplements, we might treat TRMA patients on a better way. Our study can also help those who are working on gene therapy for the treatment of inherited blood disorders.

It is important to acknowledge few of our study's limitations even in spite of its considerable contributions. The limited generalization of our findings comes from the relatively small number of family members and the geographical focus. Working together across geographical boundaries and studying larger populations is essential for confirming the reliability of our findings and to shed light on the genetics of TRMA. To understand TRMA at molecular level, further in-depth functional studies are needed. Also long term study of TRMA pathogenesis would provide important informations about the natural history of the malady and give information about the personalized treatment strategies.

In conclusion, homozygosity mapping was performed to establish linkage of the candidate gene with the microsatellite markers. Linked genes in family A and B were subjected to Sanger sequencing. Exon 2 of family A publicized a novel homozygous and pathogenic variant [NM_006996: exon 2: c.519_519delT, p.(V174Sfs*2)] however, no variant was found in Exon 2 of family B. All exons of family C were amplified and yet to be sequenced. The current study "Genetic Mapping and Sequence Analysis of Thiamine Responsive Megaloblastic Anemia in Three Pakistani Families" give a comprehensive understanding of TRMA pathogenesis in their unique geographical location. Combining both clinical and genetic characteristics of TRMA patients increases our ability for the timed diagnosis and management of the disease. It also provides a foundation for future investigations, improving patient care and underscoring the importance of personalized approaches in addressing the challenges posed by this rare but clinically significant disease. The primary goal of genetic and clinical research is to establish a phenotype-genotype association. The use of gene therapy would be an effective strategy for

treating the disease once the genes linked to these rare diseases are found. Providing genetic counselling for the affected families as well as other families with similar characteristics in the community is made easier by studies that look for the causing genes and variations.

Chapter 05

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5. References

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