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Identification of a Novel Deletion Mutation in *AGPAT2* Gene Associated with Congenital Generalized Lipodystrophy Type I

By

Ibrahim Salama

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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

"Identification of a Novel Deletion Mutation in *AGPAT2* Gene Associated with Congenital Generalized Lipodystrophy Type I"

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Abstract

Background: Congenital generalized lipodystrophy (CGL) also known as Berardinelli-Seip Congenital Lipodystrophy (BSCL) is rare autosomal recessive disorder caused by mutation in AGPAT2, BSCL2, CAV1, and PTRF genes characterized by loss of adipose tissues from birth or early infancy, acanthosis nigricans, diabetes mellitus, muscular hypertrophy, hepatomegaly and hypertriglyceridemia. There are four subclinical phenotypes of CGL (CGL1-4) and mutations in four genes AGPAT2, BSCL2, CAV1 and PTRF have been assigned to each type respectively.

Methods: DNA was extracted from ten blood samples collected from the members of the AY family. Microarray technique was used to detect the suspected region that include the candidate gene then all the coding exons including splice junctions of AGPAT2 gene was PCR amplified and sequenced directly using an automated DNA sequencer ABI3730. In a second family clinically diagnosed as CGL all the coding exons including splice junctions of AGPAT2, BSCL2, CAV1 and PTRF genes were PCR amplified and sequenced. The mutation detected in the study was screened by Sanger Sequencing in 100 normal controls.

Results: Sequence analysis of AY family revealed a novel deletion mutation (delG 662) in exon 6 of AGPAT2 gene causing a frame shift and premature termination codon. The second family (CE) does not show any mutation that may cause CGL. The 100 controls were negative for the AGPAT2 variant.

Conclusion: Mutation identified here in AGPAT2 gene causing congenital generalized lipodystrophy is the first report in Palestinian population. The patients exhibited characteristic features of generalized lipodystrophy, acanthosis nigricans, hypertriglyceridemia, diabetes mellitus and hypertrophic cardiomyopathy.
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Keywords: Congenital generalized lipodystrophy, Deletion mutation and AGPAT2.

ملخص

خلفية: مرض الحثل الشحمي الكلي الوراثي والمعروف ايضا باسم Berardinelli-Seip وهو مرض وراثي نادر ذات صفة متنحية، ينتج من طفرة او خلل وراثي في احدى الجينات الاربعة AGPAT2, BSCL2, CAV1, and PTRF ويؤدي الى نقص الانسجة الدهنية من الولادة او من مرحلة الطفولة المبكرة، الشواك الأسود، داء السكري، تضخم العضلات، تضخم الكبد وزيادة شحوم الدم. وهناك اربعة انواع من هذا المرض تصنف حسب نوع الجين الذي يحمل الطفرة وهي CGL1-4 وكل نوع مسؤول عنه جين بالترتيب التسلسلي.

الطرق المستخدمة: تم استخراج الحمض النووي من عشر عينات دم والتي تم جمعها من أفراد الأسرة الاولى. تم استخدام تقنية Microarray للكشف عن المنطقة التي يشتبه بها أن تحتوي هذا الجين. وتم البحث عن الطفرات في جميع الاكسونات وتم ايضا استخدام تقنية sanger sequencing . اما العائلة الثانية فقد تم تشخيصها بمرض نقص الخلايا الدهنية Lipodystrophy وتم البحث عن طفرة في الاربع جينات المسؤولة عن هذا المرض AGPAT2, BSCLS,CAV1 and PTRF. وتم اختبار صحة الطفرة باستخدام 100 عينة ضابطة.

النتائج: تم الكشف عن طفرة جينية جديدة في جين AGPAT2 وهي طفرة حذف القاعدة النيتروجينية G في موقع 662 في الاكسون السادس. ومما ادى الى قصر البروتين المصنع نتيجة لظهور كودون ايقاف لكن هذه الطفرات كانت غائبة في ال 100 عينة الضابطة. والعائلة الثانية لم تظهر في عيناتها أي طفرة تسبب هذا المرض في الاربع جينات السابقة.

الاستنتاج: الطفرة الجديدة التي تم التعرف عليها في جين AGPAT2 هي المسببة لهذا المرض وهي الحالة الاولى التي يتم كشفها في فلسطين. هذه الطفرة ادت لضهور اعراض على المرضى منها الحثل الشحمي المعمم، شواك مسود، زيادة شحوم الدم، داء السكري واعتلال عضلة القلب الضخامي.

الكلمات الدالة: الحثل الشحمي المعمم الوراثي، طفرة حذف، موقع اللصق و AGPAT2

DECLARATION

I hereby certify that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others and to the extent that such work has been cited and acknowledged within the text of my work.

Name and signature: __Ibrahim Salama_____

Date: ____August. 2016_____

Dedication

I would to dedicate this thesis to my parents, who were always there for me.

And whom without their care I couldn't have achieved this.

And to everyone who has ever supported me.

Acknowledgment

I wish to express my sincere thanks to my supervisor Prof. Moien Kanaan for providing me with all the necessary facilities for the research.

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List of Abbreviations

CGL	Congenital Generalized Lipodystrophy
FPL	Familial Partial Lipodystrophy
APL	Acquired Partial Lipodystrophy
AGL	Acquired Generalized Lipodystrophy
BSCL	Berardinelli-Seip Congenital Lipodystrophy
PPARG	Peroxisome Proliferator Activated Receptor Gamma
ER	Endoplasmic Reticulum
DGAT	Diacylglycerol Acyltransferase
TAG	Triacylglycerol
WAT	White Adipose Tissues
DAG	Diacylglycerol
PAP	Phosphatidic Acid Phosphatase
FAS	Fatty acid Synthase
ACC	Acetyl Coenzyme A Carboxylase
LD	Lipid Droplet

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CHAPTER 1

Introduction

1.1 Background

Lipodystrophies are rare metabolic disorders described by loss of body fat from different sites of the human body. This loss of fat can occurs in one small area of the body and extends to be total absence of fat from the body. The severity of metabolic complication that result from lipodystrophy depend on the amount of fat loss. Some metabolic complications such as hypertriglyceridemia, low level of HDL, diabetes mellitus, insulin resistance and liver steatosis. (1, 2, 36)

Two major types of Lipodystrophy are inherited and acquired. Inherited lipodystrophy have two major subtypes, are familial partial lipodystrophy (FPL) and Congenital generalized lipodystrophy (CGL) and both of subtypes have its own subtypes.(4) Acquired lipodystrophy is more common than inherited lipodystrophy which caused by external factors like medication in HIV patient who receive antiviral therapy, also other types of acquired lipodystrophy that do not have direct genetic basis like acquired partial lipodystrophy, localized lipodystrophy and acquired generalized lipodystrophy. (2, 3, 4)

Lipodystrophy suspected in patients who are lean whit some symptoms like pre insulin resistance increased level of triglycerides ,decreased level of HDL, fatty liver, acanthosis nigricans, polycystic ovarian syndrome in females and hepatosplenomegaly. Many types of lipodystrophy starts with the clinical diagnosis and other types with genetic background need a molecular testing for the known genes such as AGPAT2, BSCL2, CAV1, PTRF for the fourth types of CGL and LMNA, ZMPSTE24, and PPARG for FPL subtypes.(2,32)

About 95% of CGL cases are result from mutation from AGPAT2 and BSCL2 genes. The rest of causes are due to mutation in CAV1, PTRF genes that form about 5% of CGL causes. Diagnosis of Congenital Generalized Lipodystrophy starts at birth or early stage of life. The prevalence of CGL is 1 in 10 million worldwide. (1, 2, 4)

In adipose tissues of higher eukaryotes Triacylglycerol (TAG) function is store of energy. Also, the main reserve form of energy in the eukaryotes is the white adipose tissues (WAT), that's storage and synthesis of triacylglycerol when energy excess occurs and hydrolyze it in period of energy deprivation to give fatty acid that's used by other organs. Excess amount of WAT increase the obesity that may lead to a major health problem such as insulin resistance, hypertension, diabetes mellitus and atherosclerosis. On the other hand, these metabolic disorders can also found in lipodystrophy as a result of TAG storage in the liver and muscles instead of adipocytes. (5, 33, 36)

On the other hand, the pathogenicity of lipodystrophy can be due to defect in one of the following steps or pathway, i) regulation of transcription in the adipocyte differentiation, ii) synthesis of triacylglycerol, iii) lipid droplet formation. So a mutation in one of these pathways may lead to Congenital Generalized Lipodystrophy. (5, 33, 34)

1.2 Literature review

1.2.1 Synthesis of triglycerides

The biosynthesis of triglycerides start with the catalyzation of glycerol 3-phosphate to form lysophosphatidic acid through enzyme called glycerol-3-phosphate acyltransferase (GPAT), this reaction occurs in the mitochondria an ER. Then Lysophosphatidic acid converted into phosphatidic acid in the ER by enzyme called AGPAT (1-acylglycerol-3-phosphate acyltransferase). Fig 1.A (4, 7)

1-acylglycerol-3-phosphate acyltransferase an enzyme present in the endoplasmic reticulum with different isoforms. AGPAT2 is highly expressed in the adipose tissue. So according to previous studies, a mutation in AGPAT2 gene was linked to CGL type I. The next step is the formation of 1,2-diacylglycerol from phosphatidic acid by an enzyme called Phosphatidic Acid Phosphatase. (4, 5, 37)

The final step is the formation of triacylglycerol (TAG) or triglycerides from 1,2-diacylglycerol (1,2-DAG) by enzyme called diacylglycerol acyltransferase or (DGAT). The importance of this pathway considered in the production of triacylglycerol and phospholipids. Five deferent phospholipids was produced from two different intermediate (Phosphatidic acid and 1,2-diacylglycerol). Fig 1.A (4, 5, 39)

Defecincy of AGPAT2 gene in adipose tissue lead to lipodystrophy by decrease TAG and phospholipid synthesis that's lead abnormal function of adipocytes and affect lipid droplet formation due to lack of membran phospholipids.(33,39).fig 1B.

The second mechanism that can directly affect lipodystrophy is the lipid droplet formation. As shown in figure 1 B, in normal cells, synthesis of TAG and phospholipid occurs in the LD and they used in the lipid droplet fusion and formation to form large LD as shown in panel A. this mechanism occurs in the ER.(4,5,39).

Panel B shows the deficiency in the AGPAT2 gene in case if CGL1 which lead to deficiency in the TAG and glycerophospholipids. So LDs fusion still occurs but slower than normal cells in present of AGPAT2 gene. As shown in panel B of figure 1B, the LDs

are white color which is due to deficiency in the TAG, on the other hand, some cells still have its normal color (yellow) which is due to AGPAT isoforms.(4,5,39).

On the other hand, there is a another possibilities in case of CGL1 are lipid droplet may not consist at all because of deficiency in the glycerophospholipids on the surface of LD or there is total deficiency in the mature adipocytes as a result of deficiency in the phospholipids that's needed for cell membrane formation.(4,5,39).

In case of CGL2, this occurs due to deficiency in seipin. Synthesis of TAG and phospholipids still occurs but small lipid droplet fusion to form one large LD may not happen due to defect in seipin gene. Figure 1B. (4, 5, 39).

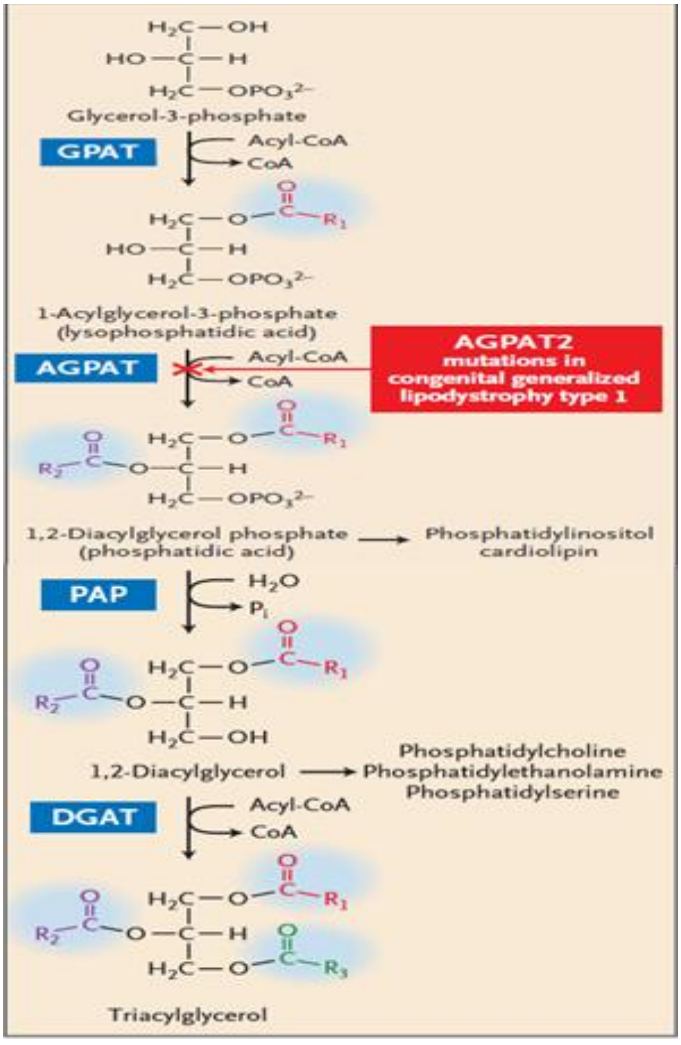


Figure 1-A: Synthesis of TAG and gylcerophospholipids pathway. (4)

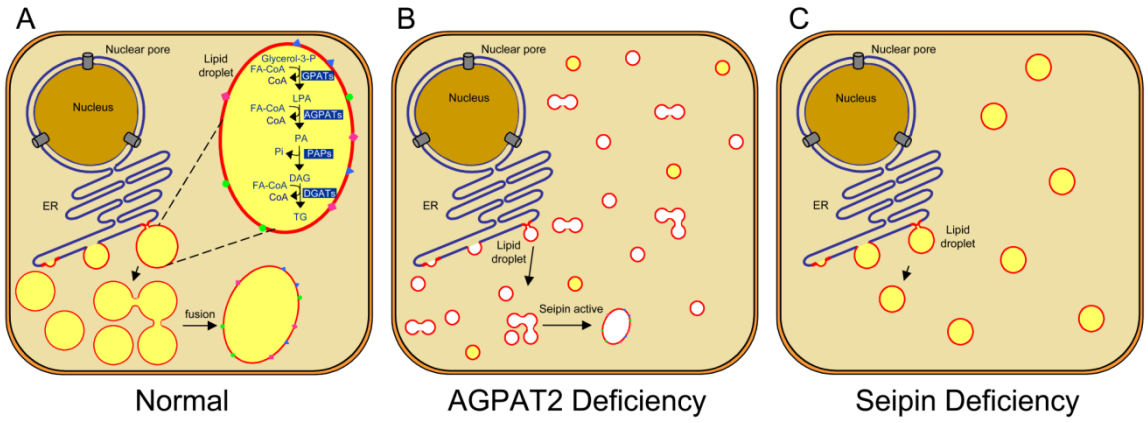


Figure 1 – B: Lipid droplet formation mechanism compared to CGL types. (39)

1.2.2 Role of lipogenic genes in the differentiation of adipocytes

This mechanism starts with the formation of pre-adipocytes from stem cells. Pre-adipocytes developed and form adipocytes as a result of changes like hormonal signal and transcription factor such as PPAR gamma and C/EBP (alpha, beta and delta) as shown in figure 2 (6,33,34).

Mature adipocytes were formed from adipocytes in corporation with lipogenic genes such as AGPATs and others that used in the synthesis of TAG and glycerophospholipids. So a defect in lipogenic genes like AGPAT can lead to deficiency in the mature adipocytes that lead to lipodystrophy by accumulation of the lipids in the blood stream. On the other hand, adipocytes deficiency can occur due to defect in this step or by death of mature adipocytes (apoptosis) (5, 6, 33, and 34).

As a result of adipocytes deficiency, hormones like leptin and adiponectin will be decreased, which has an effects on the metabolic organs like muscle pancreas and liver. These signals are important for fat metabolism and oxidation and when they decreased due to fat lose, many abnormalities may occurs in the affected patients. (7, 8, 9, 35)

In lipodystrophy syndrome there is a multiple metabolism abnormalities. Insulin resistance occurs may due to accumulation of fats in the pancreas, muscles and liver, that's lead to increase the level of triglycerides in the blood, fatty liver and DM. (4, 5, 6, 37)

Moreover, insulin resistance may occur due to absent of mature adipocytes that store fat and secrete adipocytokines such as leptin and adiponectin for normal metabolism. In the adipocytes leptin and adiponectin increase fat storage and decrease the distraction of fat in

other tissue like muscles and liver. So a mutation in gene responsible in differentiation of adipocytes can directly affects these hormones that lead to many metabolic disorders as in cause of Congenital Generalized Lipodystrophy type 1. (10, 11, 34, 35).

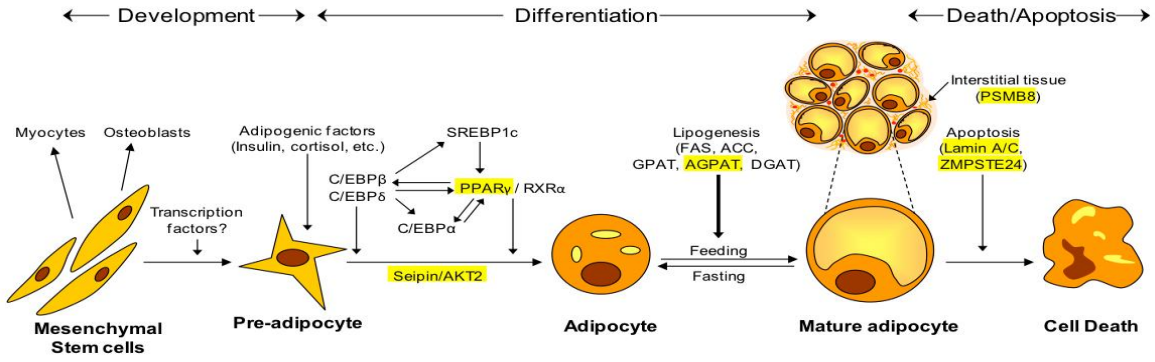


Figure 2: Differentiation and maturation of adipocytes and the role of lipogenic genes. (/http://nordphysicianguides.org/lipodystrophies/biochemistry)

1.2.3 Lipodystrophy types

Lipodystrophies are rare group of metabolic disorders described by loss of body fat from one site of the human body or generalized to hall body. Based on clinical and genetic features, lipodystrophies was classified into acquired and inherited and each has its own subtype. Table 1 (2,4).

1.2.3.1 Acquired Lipodystrophy:

This type of lipodystrophy is more common than the inherited lipodystrophy which may occurs due to some kind of medications or injections, viral infection and could be due to unknown mechanisms. Foer different subtypes of acquired lipodystrophy are localized lipodystrophy, Acquired Generalized Lipodystrophy (AGL), lipodystrophy in HIV infected patients and Acquired Partial Lipodystrophy (APL) (2,4).

1.2.3.2 Inherited lipodystrophy

This type of lipodystrophy is rare which include three subtypes: CGL, FPL and MAD. Table 4. (2, 4).

- **Congenital Generalized Lipodystrophy "CGL" (the Berardinelli–Seip Syndrome)**

Congenital Generalized Lipodystrophy (CGL) It was originally discovered by Berardinelli in (1954) and Seip in (1959). (4,29,30,38). CGL was clinically characterized by loss of fat (adipose tissues) near birth, muscular appearance, hypertriglyceridemia, DM, insulin resistance, hepatomegaly and acanthosis nigricans. (2,4,7)

Insulin resistance occurs in patients with lipodystrophy due to redistribution of fat and accumulation in other organs and tissues like liver, pancreas and muscles. (8,22)

Compound heterozygous or homozygous mutations can cause CGL, which is inherited as an autosomal recessive disorder and have four clinical subtypes (CGL1-4). Each type

linked to one gene of those AGPAT2, BSCL2, CAV1 and PTRF respectively to each type. (2,9,10,39)

1.2.3.3 Clinical and Molecular Basis of congenital generalized lipodystrophy types

1.2.3.3.1 CGL1 locus: AGPAT2

Clinically CGL1 is described by a complete loss of adipose tissue at birth. Moreover, infected patient characterized by hypertriglyceridemia, muscular hypertrophy, acanthosis nigricans, diabetes, depressed HDL cholesterol, splenomegaly, enlarged liver, hands, feet, and jaw. (2,11).

CGL1 is caused by mutation in AGPAT2 gene, which is composed from six exons and located on the chromosome 9 at this location (9q34) as described in figure 3. AGPAT2 gene encode an enzyme called 1-Acyleglycerol-3-Phosphate O-Acyltransferase 2, that's used in the formation of phospholipids and TAG in the TAG synthesis pathway. Defect in AGPAT2 enzyme due to mutations in the gene was linked to CGL1 and lead to appearance of different phenotypes by lack of glycerophospholipids and TAG synthesis in adipocytes, that's lead to damage in the signaling system between cells by alteration in the structure of the membrane or due to abnormal formation of lipid droplet. All of these abnormalities prevent the body from storing fats normally in the adipose tissue. That's will lead to appears of CGL1 symptoms and sings. (2, 7, 11, 38)

Different mutations have been identified in AGPAT2 gene in patients with CGL1 of different origins. This rare metabolic condition was characterized by complete loss of fat and other metabolic symptoms. AGPAT2 gene mutations that cause congenital generalized lipodystrophy type 1 greatly reduce or eliminate the activity of the AGPAT2 enzyme.(1,7,11)

Many mutations were observed previously in AGPAT2 gene. These mutations were observed from affected patients from different pedigrees from previous studies. Some of the affected patient have compound heterozygotes for novel mutations and other with

splice site mutation, IVS3 - 1G>A. and other had the homozygous IVS4-2A>G mutation. And other mutations were identified as shown in table 1. (1, 2, 7, 10, 11, 40, 41)

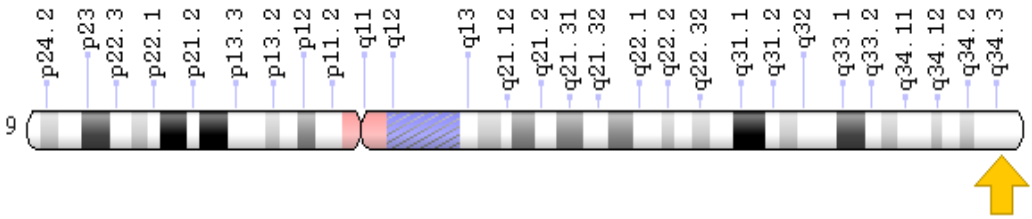


Figure 3: Genomic location of AGPAT2 gene on chromosome 9 (https://ghr.nlm.nih.gov/gene/AGPAT2#location).

Table 1: List of known mutations of BSCL2 gene.

Exon	**Mutation**	**Amino acid change**	**Phenotypes**	**Reference**
Intron 1	IVS1 _2A→ G	F60fsX102	CGL1	2,40
Intron 1	IVS1 _1G→ A	NA	CGL1	2
-	418delTTC	140delPhe	CGL1	11
Exon 2	194G →A	Trp65X	CGL1	2,40
Exon 2	202C → T	Arg68X	CGL1	2,40
-	406G→A	Gly136Arg	CGL1	11,41
Intron 3	IVS3_1G → C	delLeu165-Gin196	CGL1	2,40
-	504delGA	Val167fsX183	CGL1	11,41
Exons 3–4	del 317→588	Gly106fsX188	CGL1	40
Intron 5	IVS5-2A→C	221delGlyThr	CGL1	11
Exon 4	514G → A	Glu172Lys	CGL1	2,40
Intron 4	IVS4-2A→G	Gln196fsX228	CGL1	2,11
Intron 4	IVS4 _2 A-G	F109fsX452	CGL1	2,40
-	716C→T	Ala239Val	CGL1	11
Exon 5	645A → T	Lys216X	CGL1	40
-	683T>C	L228P	CGL1	41
Intron 5	IVS5 _2 T → G	F109fsX452	CGL1	40
Exon 6	676 C→ T	Gln226X	CGL1	40
-	377insT	L126fsX146	CGL1	41
Exon 6	712 C→ G	Ala238Gly	CGL1	40
Exon 6	Del G 662	Protein truncation	CGL1	This study

1.2.3.3.2 AGPAT2 protein domains:

AGPAT2 protein is highly expressed in the adipose tissues, liver and pancreas. This protein was predicted to have 3 domains as shown in figure 4. Tow transmembrane helix regions and PIsC domain which is phosphate acyltransferase. Composed from 116 amino acid as shown in table 2 and have a specific role in the biosynthesis of phospholipids and activity of glycerol phosphate. (38)

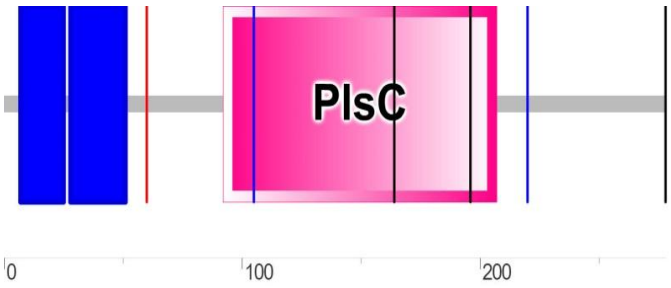


Figure 4: Graphical view of AGPAT2 protein predicted domains.
http://smart.embl.de/smart/show_motifs.pl?ID=O15120

The table below show two different transmembrane helix domains of AGPAT2 protein count about 18 and 23 amino acid respectively.

Table 2: Characteristics of the AGPAT2 protein domains:

Name	Start	End
Transmembrane region	7	24
Transmembrane region	28	50
PIsC	92	207

http://smart.embl.de/smart/show_motifs.pl?ID=O15120

By sequence similarity, four motifs of AGPAT2 protein was shown in figure 5. The first motif is HXXXXD motif which has a binding site for phosphate group of glycerol 3 phosphate in the TAG synthesis pathway also its important for activity of acyltransferase. Another motif is EGTR motif which have role in binding of LPA for AGPAT and G3P for GPAT. (42)

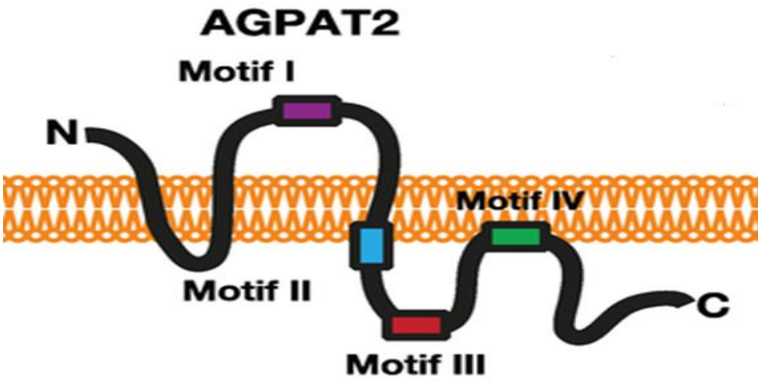


Figure 5: predicted motifs of AGPAT2 protein.(42)

http://www.mdpi.com/2079-7737/3/4/801/htm.

1.2.3.3.3 CGL2 locus: BSCL2

CGL2 is the second type of Congenital Generalized Lipodystrophy. Patients with CGL2 suffer from extreme loss of fat tissue since birth, diabetes mellitus, hypertriglyceridemia, liver steatosis, cardiomyopathy and mild mental retardation. (2,12,13)

CGL2 is autosomal recessive disease caused by mutation in BSCL2 gene, which is composed from eleven exons and located on the chromosome 11 at this location (11q13) as described in figure 6. BSCL2 gene encode an enzyme called Seipin, that's highly expressed in the adipose tissues, brane and testis and have a role in the formation of the large lipid droplet, fat storage and catabolism, adipocyte differentiation and lipid droplet maintenance. (2,12,14)

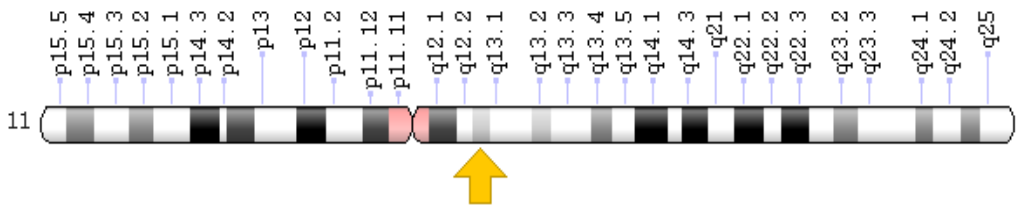


Figure 6: Genomic location of BSCL2 gene

(https://ghr.nlm.nih.gov/gene/BSCL2#location)

List of different types of mutation was identified for BSCL2 gene in patients with CGL2. Most of the BSCL2 gene mutations that cause congenital generalized lipodystrophy type 2 lead to production of a nonfunctional seipin protein or prevent cells from making any of this protein. Table 3. Loss of functional seipin disrupts normal adipocytes function and development and lipid droplets formation, which stop normal fat storage in adipose tissue. The resulting lack of body fat, that's lead to show signs and symptoms of congenital generalized lipodystrophy type 2. Shortage of adipose tissue leads to multiple health problems, including high levels of fats called triglycerides circulating in the bloodstream (hypertriglyceridemia) and diabetes mellitus. (1, 2).

Table 3: List of known mutations of BSCL2 gene.

Exon	mutation	Protein	phenotypes	References
	Missense			
2	c.232A >G	p.Thr78Ala	CGL2	(26)
2	c.272T>C	p.Leu91Pro	CGL2	(26)
3	c.412C>T	p.Arg138X	CGL2	(27)
4	c.560A>G	p.Tyr187Cys	CGL2	(28)
4	c.565G>T	p.Glu189X	CGL2	(27)
5	c.634G > C	p.Ala212Pro	CGL2	(27)
7	c.823C > T	p.Arg275X	CGL2	(25)
6	c.684C > G	p. Tyr228X	CGL2	(7)
10	c.1171C > T	p.Gln391X	CGL2	(26)
	Insertion			
1	c.154_155insTT	p.Tyr53SerfsX39	CGL2	(7)
3	c.301_302insAA	p.Met101LysfsX10	CGL2	(27)
3	c.325insA	p.Thr109AsnfsX5	CGL2	(27)
6	c.782dupG	p.Ile262HisfsX12	CGL2	(7)
	Deletion			
3	c.315_316delGT	p.Tyr106SerfsX7	CGL2	(27)
3	c.317_321delATCGT	p. Tyr106CysfsX6	CGL2	(27)
5	c.636delC	p.Tyr213ThrfsX20	CGL2	(27)
5	c.652_662del11	p.Ala218TrpfsX51	CGL2	(31)
	Splice site			
IVS2 -11A>G	Exon skipping	Protein truncation	CGL2	(23)
IVS4 +1G>A	Exon skipping	Protein truncation	CGL2	(27)
IVS5 -2A>G	Exon skipping	Truncation of protein	CGL2	(26)
IVS5- 2A>C	Exon skipping	Truncation of protein	CGL2	(24)
IVS6 +5G>A	Exon skipping	Truncation of protein	CGL2	(27)
IVS6 -3C>G	Exon skipping	Truncation of protein	CGL2	(27)
IVS6 -2A>G	Exon skipping	Truncation of protein	CGL2	(7)
	Complex rearrangements			
1	c.192_193delCCinsGGA	----	CGL2	(27)
1	c.193delCinsGGA	----	CGL2	(7)
4-6	Deletion of exons 4-6	----	CGL2	(27)
5-6	Indel mutation leads to exons 5–6 deletion	----	CGL2	(27)

1.2.3.3.4 CGL3 locus: CAV1

Congenital Generalized Lipodystrophy 3 which occurs due to mutation in CAV1 gene. CAV1 gene is located on chromosome 7 at this location (7q31). and provides instructions for making a protein called caveolin-1. This protein appears to have diverse functions in cells and tissues throughout the body. Caveolin-1 is highly expressed in caveolae, which is an invagination of the cell membranes of adipocytes and other type of cells. Adipocytes make up most of the body's fatty (adipose) tissue. In these cells, caveolae appears to be essential for the normal transport, processing, and storage of fats. Caveolae plays a role in the formation of lipid droplet through transport of fat from outside to inside of the cell. (15,16)

Caveolin-1 is also found in many other parts of cells, where it regulates various chemical signaling pathways. The functions of caveolin-1 likely differ depending on the type of cell and the part of the cell where the protein is found. It is unclear how a lack of this protein leads to the particular features of congenital generalized lipodystrophy type 3. However, the absence of caveolin-1 likely disrupts normal function and development of adipocytes, which would prevent fats from being stored normally in adipose tissue. (15,16)

1.2.3.3.5 CGL4 locus: PTRF

CGL4 a rare autosomal recessive disorder occurs due to mutation if gene called PTRF gene. This gene encodes a protein polymerase I and transcript release factor (or cavin-1). This protein is found in cells and tissues throughout the body. It is most abundant in several types of cells: osteoblasts, which are cells that build bones; muscle cells; and adipocytes, which are cells that store fats for energy. (17,18)

PTRF localized to caveolae component in the plasma membrane and have a critical role in the formation of caveolae. Moreover, this protein important in the lipid metabolism modification, and may has a role lipid droplet formation. (17, 18)

Different mutations in the PTRF gene have been found to cause CGL type 4. This rare condition is characterized by almost total absence of fat tissues and very muscular appearance. A shortage of adipose tissue leads to multiple health problems, including high levels of fats called triglycerides circulating in the bloodstream (hypertriglyceridemia) and diabetes mellitus. . (17, 18, 29)

All of the identified PTRF gene mutations prevent cells from producing any functional cavin-1. A lack of this protein probably impairs the formation of caveolae. Researchers suspect that a shortage of these important structures on the cell membrane disrupts many cell functions. However, it is unknown specifically how CGL4 with all of the symptoms occurs due to absence of cavin-1. (17,18)

1.2.3.3.6 Other mutations

A very rare types of inherited lipodystrophy was described like Familial Partial Lipodystrophy (FPL) which is an autosomal dominant disease with five subtypes are Kobberling variety due to unknown mutation, Dunnigan variety which occurs due to LMNA mutation and FPL3 due to PPARG gene mutation. Table 4 (2,4,36)

FPL Type 4 and 5: PLIN1 mutation s, AKT2 mutation are others extremely types of FLP. (2,36)

Table 4 . Clinical and molecular features of common lipodystrophies.

Type of lipodystrophy	Inheritance pattern	Gene involved	Clinical characteristics	Molecular basis
Acquired				
Lipodystrophy in HIV	None	None	Fat loss from the arms, legs, and face with excess fat at abdomen and neck regions	Occurs due to antiviral therapy like protease inhibitor that inhibits the adipocytes differentiation and alter the abiogenesis mechanism.
APL	None	None	Fat loss from the trunk, arms, legs, and face.	May occurs due to autoantibody C3 nephritic factor and reduces level of Complement 3, suggest autoimmune loss of fat tissues.
AGL	None	None	Fat loss from different area in the body (generalized)	Could be due to Panniculitis or due to autoimmune disease Or mechanism remain to be discovered.
Localized lipodystrophy	None	None	Fat loss from a small area in the body	Most cases occur due to injections like insulin and may due to drugs injection

				at specific area in the body.
Inherited				
CGL 1	Autosomal recessive	AGPAT 2	Loss of adipose tissues from body from birth, acanthosis nigricans, hypertriglyceride mia and insulin resistance.	CGL1 occurs due to mutation in AGPAT2 gene. Gene expressed in the adipose tissues. Has a key role in biosynthesis of TAG and phospholipids.
CGL 2	Autosomal recessive	BSCL2	The same symptoms as CGL1 with mental retardation, and cardiomyopathy	CGL2 occurs due to mutation in seipin gene. Gene highly expressed in the adipose tissues. Responsible for formation of large lipid droplet and can affect the central nervous system due to deficiency of this gene.
FPL or (Dunnigan variety)	Autosomal dominant	LMNA	Fat loss occurs from different area of the body include, legs, arms and trunk.	Rare form of inherited lipodystrophy. Occurs due to mutation in LMNA gene, which encode nuclear lamina C and A.
PPARg mutation	Autosomal dominant	PPARg	Fat loss occurs from different area of the body include, legs, arms and face.	This type of lipodystrophy occurs due to mutation in PPARg gene, which has a role in abiogenesis and adipocytes development and differentiation.

In this study I was worked in tow family (YA and CE), both of them from Bethlehem area and clinically diagnosed as Congenital Generalized Lipodystrophy. AY family was resolved and we found the causative mutation that segregate in all affected member of the family. CE was not resolved until now and we don't found the causative mutation in 4our genes for lipodystrophy.

Chapter 2

2.1 Problem statement and objectives

Generalized lipodystrophy (CGL; Berardinelli-Seip syndrome) is an autosomal recessive disorder is characterized by the almost complete absence of body fat deposition and is usually evident at birth or during early infancy. In addition, the syndrome often includes the following features: organomegaly, muscle hypertrophy, acanthosis nigricans, hyperlipidemia, and hyperinsulinemia or insulin-resistant diabetes. (1,2,4)

The disease has an extremely rare incidence. the estimated worldwide prevalence is about 1 in 10 million. (1,2,4). On the other hand, there has been a few documentations of some CGL cases in Palestine but not one that addresses causes or prevalence. This is the first study to highlight the genetic and molecular causes of these conditions in Palestine, which is important due to high consanguineous marriage rate.

The study aims to achieve the following objectives:

1. This study aims to resolve the causative mutation of Congenital Generalized Lipodystrophy in a Palestinian family from Bethlehem area with an autosomal recessive inheritance.
2. Validate mutation novelty and study its segregation in the affected family using Sanger sequencing.
3. Carry out 100 control sample genotyping to exclude any chance that mutation is normal SNPs in the Palestinian population.
4. For future perspective, resolve the causative mutation of the second family (CE) that clinically diagnosed as CGL .

Chapter 3

MATERIAL AND METHODS

3.1. Materials

3.1.1. Gels, buffers and solutions.

A- DNA extraction by salt base method

- Red Blood Cells lysis buffer (RBCs lysis buffer)

1. Add 8.28gr of NH4Cl (155mM)
plus 0.79gr NH4HCO3 (1mM) to 1L
flask.
2. Then mix with 0.2 ml EDTA
(0.5mM, pH 7.4).
3. Complete the volume to 1L with
double distilled water (dd H2O).
4. Store at 4 ^{o}C.

- **DNA lysis buffer (1x)**

1. Mix 50ml Tris-HCL (50mM,
7.5pH) with 33.4ml NaCl (100mM).
2. Then add 2ml EDTA (1mM).
3. Complete the volume to 1L with dd
H2O.
4. Store at room temperature.

- **50X TAE Buffer**

1. Mix 242gr Tris base (8.0pH) with 57.1ml Acetic Acid (1M).
2. Then add 100ml EDTA (0.5M, 8.0pH)
3. Complete to 1L with ddH2O
4. Store at room temperature

- **Proteinase K**

Proteinase K was dissolved in double distilled sterile water to a 5mg/ml final concentration.

Agarose Gel

1.5%, 3% agarose

1X TBE buffer

B. Electrophoresis

Ethidium Bromine

Ethidium bromide was dissolved in the double distilled sterile water to a final concentration of 1mg/ml.

C. PCR products running gel

- **5X loading buffer**

  0.25% bromophenol blue with 0.25% Xylene cyanol FF in 30% Glycerol in water.

D. DNA Storage

- 0.02 % Sodium Azide.

E. Ethanol/EDTA Precipitation in the cleaning step

125mM EDTA, 100% Ethyl Alcohol (EtOH), and 70% EtOH are needed for this step.

3.1.2 Reagents, Instruments and Kits

Table 5: Reagents, Instruments and Kits

Reagent	**Supplier**	**Product specification**
Ready Mix PCR	ABgene	Cat# AB-0575- DC-LD
Agarose	Amresco	Cat# : 9012-36-6
Sodium Azide	Sigma-Aldrich	Cat# : S2002
Hi-Di-Form amide	Applied Bio system	P/N#:4311320
Gene ruler 100bp DNA ladder	Thermo Scientific	Cat # :SM0241

Instruments	**Supplier**	**Product specification**
Thermal cycler	Applied Bio system	Gene Amp PCR system 9700
Spectrophotometer –DNA Concentration measurement	Nano Drop technologies	Nano Drop. ND-1000
Automated Sequencing-Electrophoresis	Applied-Biosystems /HITACHI	ABI Prism 3130 DNA sequencing (S/N:20355-023)

kits	Supplier	Product specification
PCR purification kit	New England Bio labs	Antarctic phosphates Cat# M0289L
Sequencing kit	Applied bio system	Big Dye® Terminators V1.1 Cycle Sequencing Reaction Kit. Cat# 433750
cDNA synthesis kit	Applied bio system	High Capacity cDNA Reverse Transcription Kit, cat#(4368814)

3.2. Methods

3.2.1 Blood collection

In collaboration with the Caritas Baby Hospital samples were collected from patients affected with Congenital Generalized Lipodystrophy at the hospital.

Blood samples were collected from affected and non- affected individuals of the tow family from Bethlehem area showing a lipodystrophy phenotypes inherited in one generation of the family (MERC-AY Family) and (MERC-CE Family) in an autosomal recessive pattern as displayed in the pedigree in Figure 7 and 8 respectively.

The lipodystrophy phenotype was first determined by doctors in the Caritas Baby Hospital at Bethlehem city, and then 5 ml blood was mixed with EDTA (0.1 ml 0.5M EDTA for 20 ml blood) to prevent clotting. (Miller 1988). samples were collected from the members of the two families, who have participated in this study after obtaining informed consent, that was signed by each family member who agreed to participate in the study. For those individuals who are under 18 years old; the consent form was signed by one of their parents.

The first family is AY family was come to Caritas Baby Hospital with an inherited disease and has symptoms like acanthosis nigricans and muscular appearance. So the affected members were diagnosed with progressive lipodystrophy. Clinical examination was done by the doctors and abdominal ultrasound was done for AY3 member and the result was good with slightly enlarged liver. Moreover, blood test was done and the result as shown in the table 13.

On the other hand, AY family has a family history of heart problem, cancer liver, diabetes and FMF. Also the first pro band (AY1 and AY2) with three children (AY3, AY4 and AY5) have enlarged liver and high level of lipid in the blood. After that, ten blood samples were drawn from the AY family include affected and non-affected members and transformed to the HRL at Bethlehem University after signing the consult form by AY family.

The second family is CE family was come to Caritas Baby Hospital with an inherited disease with symptoms like acanthosis nigricans and muscular appearance. CE3 is the only affected member of the family. CE3 was diagnosed as lipodystrophy patient after clinical examination. Abdominal ultrasound was done for the patient and the result was increased medullar and cortical echogenicity and enlarged liver. Also CE3 was diagnosed with mild ventricular septal hypertrophy. Moreover, blood test was performed the results shown in table 13.

On the other hand, CE family has a history of epilepsy, liver enlargement, splenomegaly, miscarriages and many abortions. After signing the consult form by the family, five blood samples were drawn from the affected and non-affected member of the family and the samples were transported to HRL at Bethlehem University to detect the genetic causes of the lipodystrophy.

3.2.2. DNA isolation by salting- out technique

Ten blood samples were collected in a sterile vacutainer tubes contained EDTA to prevent blood clot and then Red Blood Cell lysis buffer was added 4 times the volume of the blood and mixed gently (section 3.1.1.a), then tubes were kept on ice for 10-20 minutes , being shaken by hand from time to time, till the blood becomes transparent. Then tubes were centrifuged at 2000g for 10 minutes at 4°C and the resulted supernatant was carefully removed and the pellet was re-suspended in 3 ml Red Blood Cell lysis buffer and re-centrifuged at same conditions. The pellet was then suspended in a mix of 1x lysis buffer, 100µl of 20%SDS and proteinase K. (section 3.1.1.a), followed by incubation at 37°C overnight. After that, 1ml of 6M NaCl was added to the lysate and vigorously mixed until the solution get a foamy appearance, then centrifugation was done at 3000g for 20 minutes at room temperature. The clear supernatant (upper phase) then was transferred carefully to a 15ml tube, avoiding the salt protein deposit. Cold 100% ethanol (EtOH) was added twice the volume to the upper phase and 15 mixed gently by tube inversion till fine threads appear which are the DNA that will be fished using glass Pasteur pipette and washed in 70% EtOH and let it for air dry for few minutes on the Pasteur pipette, DNA then was dissolved in 200-1000ml 0.02% Sodium Azide (depending on the amount of the DNA) and left at room temperature overnight.

3.2.3. RNA extraction from Peripheral Blood

RNA was isolated from 5 ml of fresh blood using RNeasy® Mini Kit (Qiagen-Cat# 74104).

RNA extraction start with sample preparation by suspension cells (cells grown in suspension) so cells was isolated by centrifugation and then lyse in Trizol using 1ml per $10X10^7$ centrifugation (pellet 10^7 cells by centrifugation). And then re suspend pellet in 100 µl supernatant. After that pellet was transferred to 1.5 ml tubes and 1 ml of Trizol was added, shake vigorously by hand for 1 minute and wait 5 min at room temperature. 0.2 ml of chloroform was added per tube, shake vigorously by hand for 15 second and wait 3 minutes. the resulting mixture was centrifuged at 10,000g for 15min at 4^0c. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase (DNA site), and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of Trizol reagent used for homogenization. The aqueous phase was transferred into new 1.5 ml tube and 500 µl of isopropanol was added to it. Incubate samples at 15 to 30ºC for 10 minutes and centrifuge at no more than 12,000 g for 10 minutes at room temperature. Supernatant was removed. RNA pellet was washed once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of Trizol reagent used for the initial homogenization. Mix the sample by vortex and centrifuge at full speed for 10 minutes at RT. Then supernatant was completely removed and the pellet was kept to dry for 5 minutes at RT. The pellet was re dissolved in 25 µl of water. Finally, 1 µl was taken to measure the UV absorbance at 260/280 nm.

3.2.4. Reverse transcriptase-PCR

The RNA extracted from blood (WBCs) was used as a template for reverse transcription, using the High Capacity cDNA Reverse Transcription Kit, (Cat# 4368814). 2X reverse transcription master mix were prepared to synthesis single strand cDNA from total RNA. Total RNA was added to the 2X master mix to make it 1X mix. Reverse transcription was performed in a thermal cycler. To prepare 2X RT master mix, kit components allowed to thaw on ice. Then, components were mixed together in a specific amount as shown in table 6.

Table 6: Standard reaction for preparation of 2X master mix.

Component	**Volume/reaction (µl)**
10✕ RT Buffer	2.0
25✕ dNTP Mix (100 mM)	0.8
10✕ RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
Nuclease-free H2O	4.2
Total per Reaction	**10**

Then 2X RT master mix was gently mixed and placed on ice and it's ready to use. To prepare cDNA RT reaction, 10 µL of 2✕ RT master mix was transported into each well of 96 well plates or into individual tube. Then 10 µL of RNA sample was mixed to each tube or plate. Tubes were centrifuged to spin down the content. Reactions were loaded onto thermal cycler and RT start run at reaction volume 20 µl at specific program. **Table 7**.

Table 7: Program of RT cDNA.

	Step 1	**Step 2**	**Step3**	**Step 4**
Temperature (°C)	25	37	85	4
time	10 min.	120 min.	5 min.	∞

After run on thermal cycler we can storage cDNA RT plates or tubes for long time at (-15 to -25 °C) or for short time at (2 to 6°C)

3.2.5 Excluding known mutations

Mutations in AGPAT2 gene are known to be the major cause for autosomal recessive CGL , so this genes was scanned by direct sequencing for the first family (AY).

For the second family (CE), AGPAT2, BSCL2, CAV1 and PTRF genes were scanned by direct sequencing. BSCL2 primers were described earlier (12). Primers are presented in table 10. CAV1 and PTRF genes were sequenced using primers that were designed at the Hereditary Research Lab/Bethlehem University.

Table 8: Primers used for AGPAT2 gene amplification

Gene	Template	Primer sequence	Size of template
AGPAT2			
Exon 1	DNA	F: GCGTTGTTGGGGACAGTG R: AAAGTTAGGGAAGCGGAAGC	435 bp
Exon 2	DNA	F: CTGTGTCTCCCGGTCTCCT R: CCCTGAGGGAGGTACTGAGG	408 bp
Exon 3	DNA	F: GGCCTCAGTACCTCCCTCAG R: TGATGTGGGGGTCTTGTTTT	455 bp
Exon 4	DNA	F: CCAGACCCCTACATCATCCA R: AGTGCAGGAAGGGGCAAG	401 bp
Exon 5	DNA	F: GCAGGACAGCCACAAGGAC R: GGAAATGGGAACGATGAGG	301
Exon 6	DNA	F: TAGGGAGTCCAGGGGAAGAG R: CCGGACAGAGTGGTATTTGG	412 bp
Exon 4	cDNA	F: AGGGTACTCGCAACGACAAT	3280 bp
UTR	cDNA	R: GAGGGCTCGGACAGTGTG	3280 bp

Table 9: Standard PCR reaction mix per 25µl of total volume

Reagent	Volume in µl
Ready mix	12.5
Forward primer	0.50
Reverse primer	0.50
Nuclease free H2O	11.2
100ng/ µl DNA Template	0.3

Table 10: Primers used for BSCL2 amplification

Gene	Template	Primer sequence	Size of template
BSCL2			
Exon 1	DNA	F: ACTCTGACACAGCACTTAGCACCT R: TAAAGTCCTTCAGAGGCAAGGCCA	440 bp
Exon 2	DNA	F: TGGCAGAGTGGCACAATCATTTGG R: TGTGAAAGTTGAGAGGCCCTGGAA	614 bp
Exon 3	DNA	F: GAAGGGTGCCTGTTCTGA R: GTCTCGAACTCCCAACCT	389 bp
Exon 4	DNA	F: ACAGATTGAAAGGCCCGTTAGGCT R: ACTCAACCTCCCAGGTTTAAGCGA	572 bp
Exon 5	DNA	F: TGGAATGGTTGGATGAGACTGAGG R: GTTAGCCCCCGTGAAGAGTT	373 bp
Exon 6	DNA	F: AAGCCGCCTTCATAGACT R: GGAGGGAAGATGAACAGG	727 bp
Exon 7+8	DNA	F: TTTCTGGGACATGAGAAGGCTGGT R: TCACCTTCCTCGCCTTTCCTTTGA	558 bp
Exon 9+10	DNA	F:TCAAAGGAAAGGCGAGGAAGGTGA R: TGGGAAAGTGCTGGAATGTGAGGA	632 bp
Exon 11	DNA	F: AAGATGCAGCTTTGCTGACGGA R:TCCCTCCTTGGACTTCCTAGGCTTAT	404 bp

Table 11: Primers used for CAV 1 amplification

Gene	Template	Primer sequence	Size of template
CAV 1			
Exon 1	DNA	F1: TCAGTTCCCTTAAAGCACAGC R1: GTGCTCCGAAGTGGAGAGAG	231 bp
Exon 2	DNA	F2: GGCTGACTTCTCATCGCTTG R2: ACGGCAATGCTAAAGAAGGA	345 bp
Exon 3	DNA	F3:GAACGAACTCATAAATGCTAATACAG R3: ACTTGAAATTGGCACCAGGA	521 bp

Table 12: Primers used for PTRF amplification

Gene	Template	Primer sequence	Size of template
PTRF			
Exon 1	DNA	F1 : CTGCTTCTCTCCGGGTCTC R1: GTCTCCCCACCCCAACTC	620 bp
Exon 2 a	DNA	F2a:CTCTCTCCAGGATCGTCACC R2b: GGTTTTCCTTGGTCTTGAGG	405 bp
Exon 2 b	DNA	F2 a: GGAGAAGACCAAGGTGCGTA R2 b: AATGCGAAAGAGGAAGTTCG	510 bp

3.2.6 Affymetrix Microarray

SNP array is a type of DNA array and based on DNA hybridization, fluorescence microscopy, and solid surface DNA capture. SNP array contains props or allele specific oligonucleotides, target fragmented nucleic acids that was labeled with florescent dye and a software that used to record and interprets the hybridization signal. This type of array used for studying variations within whole genomes and for determining disease susceptibility. Moreover, SNP array can be used to map disease loci based on linkage analysis and determine the responsible genes in a disease. (43)

There are about 10 million common SNPs, so it is very hard and expensive to cover all of them for every individual but through knowing the tag SNPs that can identify common haplotypes it's become easy and less expensive. Information about genetic variation can be covered by about 300.000 to 600.000 tag SNPs. (43)

Affymetrix chromosome analysis Suite (CHAs) software was used to define a possible candidate homologous area. Many candidate genes resulted from the microarray, AGPAT2 gene was selected for direct Sanger sequencing using primers that were designed at the Hereditary Research Lab/Bethlehem University. Microarray was performed on the some affected members of AY family (AY1, AY2, AY4 and AY5) by HRL team in BU.

Different homozygosity regions were suspected to have the candidate gene that cause lipodystrophy. These regions were selected according to the high number of SNPs and to the physical length which should be more than 2 mega base pair. Table (14)

Affymrtrix SNP array was performed on AY1, AY2, AY4 and AY5. Procedure starts with the preparation of plate. This stage start with thoroughly mix the genomic DNA by vortex at high speed for three second. Followed by determine the concentration of each genomic DNA sample. Based on OD measurements, each sample was diluted to 50 ng/µL using reduced EDTA TE buffer. Then the diluted samples was thoroughly mixed using vortex at maximum speed for three second.
The next stage is the digestion by restriction enzymes. At this step the digestion of genomic DNA occurs using Nsp I or Sty I. then master mix was prepared and added to the

samples. Then the samples are placed on a thermal cycler and the 500K Digest program is run.
The next stage is the ligation using Nsp or Sty Adaptor to ligate the digested samples. Then ligation master mix was prepared and added to the samples. The samples was placed on the thermal cycler and the 500K Ligate program is run. After the program is finished the ligated samples was diluted with AccuGENE® water.

The fourth step is the PCR step. Ligated samples were transferred in equal amount to a three new 96 plate. Then PCR master mix was prepared and added to the samples. Each plate was placed onto the thermal cycler and the 500K PCR program is run.

PCR product was purified by adding the diluted EDTA to each PCR product. The PCR product was pooled back to clean up plate. Then clean up plate was placed onto a manifold and concentrate the PCR products. The PCR product was washed three times using AccuGENE® water. The PCR products was eluted using RB Buffer and was transferred t to a new 96-well plate.

During the stage of quantitation and normalization, three separate dilutions was prepared of each PCR product in optical plates. Then quantitation of the diluted PCR products were done and OD measurement was observed to each plate. After determine the concentration of each reaction, each reaction normalized to 2 µg/µl in RB buffer.

The next step is fragmentation of the normalized PCR product using fragmentation reagent, after dilution of the fragmented buffer with Accu GENE® water. Then the diluted fragmented reagent was added to each reaction on plate that was placed onto a thermal cycler and fragment program was run.

The next step is labeling of the fragmented samples with Gene Chip® DNA Labeling Reagent. Labelled master mix was prepared previously and was added to each samples. These samples was placed onto a thermal cycler and labeled program was run. The final step is target hybridization. This step start with loading of each sample onto Gene Chip® Human Mapping 250K NSP Array by using applied bio system thermal cycler or we can use Gene Amp® PCR System 9700 or by Using Heat Blocks. Data analysis was done by using affymetrix chromosome analysis Suite (CHAs) software.

3.2.7 Mutation Validation by direct Sanger sequencing

3.2.7.1 Polymerase Chain Reaction (PCR)

Forward and reverse primers for AGPAT2 gene was designed at the Hereditary Research Lab/Bethlehem University using primer 3 software.

Gene	Left primer	Right primer
AGPAT2	GGTCTTGGAGATGTGGAGGA	AAAATAACCCACCACGGCTA

Here is the tow PCR program that was performed on the affected members of both families by using the PCR machine Gene Amp-PCR system 9700 from Applied Bio system.

Programs, (T.D 55. 30 sec.)

AGPAT2 family	**BSCL2/ CE family**
94 °C 5 min[1]	94 °C 5 min[1]
94 °C 30sec[2] 63 °C 30sec[3] 72 °C 30sec[4] X2	94 °C 30sec[2] 63 °C 30sec[3] 72 °C 30sec[4] X2
94 °C 30sec 61 °C 30sec 72 °C 30sec X2	94 °C 30sec 61 °C 30sec 72 °C 30sec X2
94 °C 30sec 59 °C 30sec 72 °C 30sec X2	94 °C 30sec 59 °C 30sec 72 °C 30sec X2
94 °C 30sec 57 °C 30sec 72 °C 30sec X2	94 °C 30sec 57 °C 30sec 72 °C 30sec X2
94 °C 30sec 55 °C 30sec 72 °C 30sec X35	94 °C 30sec 55 °C 30sec 72 °C 30sec X35
72 °C 7 min[5]	72 °C 7 min[5]
4 °C ∞	4 °C ∞

1) Initial denaturation
2) An initial denaturation of 30 seconds at 94°C is sufficient for most amplicons from pure DNA templates.
3) The annealing step is typically 15–60 seconds. Annealing temperature is based on the T_m of the primer pair and is typically 45–68°C.
4) Extension, the recommended extension temperature is 68°C. Extension times are generally 1 minute per kb.
5) Final Extension.

3.2.7.2 Electrophoresis of PCR products using agarose gel.

The concentration of agarose gel was determined depending on the sizes of the PCR products. 1.5 % of agarose was prepared as concentration, then 2.25 g of agarose was mixed with 150 ml of 1X TEA buffer and then boiled until transparent, after a short cooling period ,Tow drops of ethidium bromide was added to the dissolved gel that poured to electrophoresis tray for a period of time until become solid. Then 3 µl of our PCR product were loaded onto the wells in the gel associated with 3µl of DNA ladder in the first well and run in 1X TAE running buffer at 120V for 20 to 30 minutes depending on the size of the fragment. At the end, DNA fragments were visualized and documented using BioRad ultraviolet imaging system.

3.2.7.3 PCR product cleaning

Cleaning of the PCR product is a critical step before sequencing by cleaning of the remaining primers, salts and free nucleotides by using tow enzymes Exonuclease I to degrade the remaining primers and Antaractic Phosphatase that removes the left over nucleotides. Table 13.

Table 13: Master Mix of enzymes per 5 µl of PCR

Reagent	Volume in µl
Exonuclease I	0.25
Antaractic Phosphatase	0.25
Nuclease free H2O	1.5
PCR Product	5.0

The total volume for whole reaction is 7 µl.
Enzyme purification of PCR Program.

37 $_{o}$C 30min

80 ^{o}C 20min

4 ^{o}C ∞

3.2.7.4 DNA Sequencing

DNA sequencing was done using 10ng of PCR product per 100 bp of the length of PCR fragment, for 1X reaction a 1.5 µl of 5X buffer, 0.75µl 64X buffer, 0.2µl Big Dye Terminater V1.1, 0.50µl Forward or reverse primer , 2.00µl DNA template were added to 11.05µl Nuclease Free H2O to reach 16 µl as a total volume. Table 14. Then the mix was run on ABI 3130 DNA sequencer (Applied Biosystem) at 64X program:

Sequencing PCR Reaction Program:

96 ^{o}C 1min

96 ^{o}C 10sec
50 ^{o}C 5sec
60 ^{o}C 4min
} X30

4 ^{o}C 10min

Table 14: Standard Sanger sequencing reaction mix per 16µl total volume

Reagent	Volume in µl 1X
5X buffer	1.5 µl
64X buffer	0.75µl
Big Dye Terminater V1.1	0.2µl
Forward primer	0.50µl
DNA template	2.00µl
Nuclease Free H2O	11.05µl

3.2.7.5 EDTA/Ethanol Precipitation of Cycle Sequenced Product

Cleaning up the PCR products from primers, excess dNTPs, and unincorporated dyes was done by using EDTA/Ethanol precipitation method. This step is the final step before running the PCR on PCR machine which start with adding 5 µl of 125mM EDTA and 100 µl of absolute ethanol to 16 µl of sequencing reaction and gently mixed. then the mixture was incubated at -20° for 30 minutes followed by centrifugation at 3800 RPM for 30 minutes at 4°C. The supernatant was discarded and 60 µl of 70% ethanol were added to each sample and the centrifuged directly at 3800 RPM for 20 min at 4C°. Then supernatant was discarded and the samples were inverted on tissue paper and centrifuged at 500 RPM for 1 minute. The samples were dried at 95oC for 5 minutes at hot plat to remove any remaining ethanol. 16 ul of Hi-Di Formamide (Applied Biosystems, Cat # 4311320) were then added, and the samples were dried again at 95°C for 2 minutes and they were placed on ice for 5 minutes before run on sequencing machine.

CHAPTER 4

Results

4.1 Clinical Diagnosis and Description

Family AY and CE were clinically diagnosed to have lipodystrophy by Dr. Nader Handal at Caritas Baby Hospital in Bethlehem. Family pedigree was constructed through the information that was obtained from interviews with families. The pedigrees shown in **Figure 7** and **figure 8** demonstrates the inheritance of lipodystrophy phenotype through one generation in AY family and CE family in an autosomal recessive pattern.

Clinical examination for AY3 shows symptoms of Congenital Generalized Lipodystrophy like acanthosis nigricans, muscular appearance due to lack of body fat, enlarged abdomen and splenomegaly. Figure 9.A Biochemistry tests was done on AY3 patient and the results on table 15.

The affected member of the CE family showed symptoms of CGL2 disease. The affected member CE3 has acanthosis nigricans (velvety thickening and hyperpigmentation of the skin) which was more prominent around neck and in body folds including axillae, antecubital fossae and popliteal fossae.

The affected individuals had prominent veins, rough dry skin, lack of body fat, and abdominal distension with protruding navel, muscular appearance, and splenomegaly. Figure 9.B. Also with high level of triglycerides (842 mg/dl) and high level of alanine amino transferase (124 U/L). Table 15. (2, 12, 13)

Table 15: Clinical data of AY3 and CE3.

Test	AY3 (III-9)	CE3 (IIII-7)	Reference rang
Cholesterol	160 mg/dl	122 mg/dl	< 200 mg/dl
Triglycerides	91 mg/dl	842 mg/dl	< 180 mg/dl
GOT	40 U/L	124 U/L	< 41 U/L
GPT	19 U/L	N/A	< 31 U/L
Alkaline phosphatase	258 IU/L	N/A	44-147 IU/L
CK	97 U/L	N/A	< 175 U/L
LDH	693	N/A	450 U/L
HGB	9.6 mg/l	N/A	< 12 mg/L
MCV	60 fl	N/A	75 fl
Electrocardiogram (ECG)	N/A	Normal	N/A

N/A: Not Available.

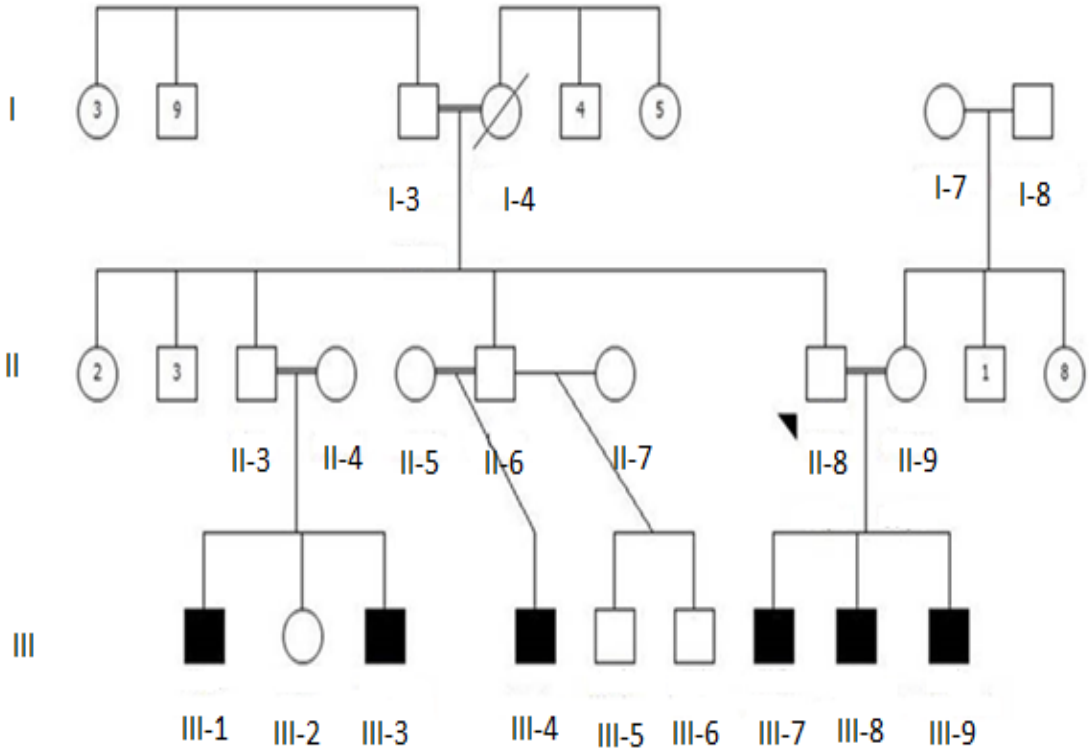


Figure 7: Pedigree of AY family:

Lipodystrophy phenotype appears in the third generation of AY family in an autosomal recessive pattern with segregation of the genetic disorder in 6 members of the family.

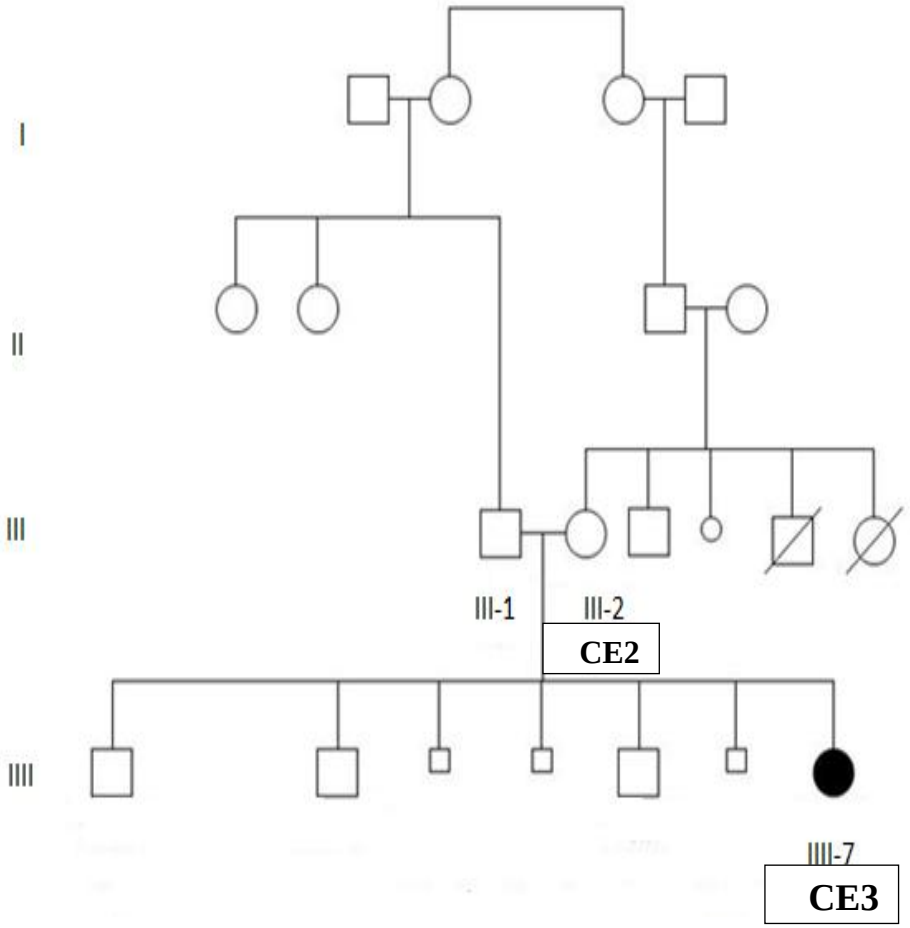


Figure 8: Pedigree of CE family:

Lipodystrophy phenotype appears in one patient of CE family this patient was clinically diagnosed to have Congenital Generalized Lipodystrophy

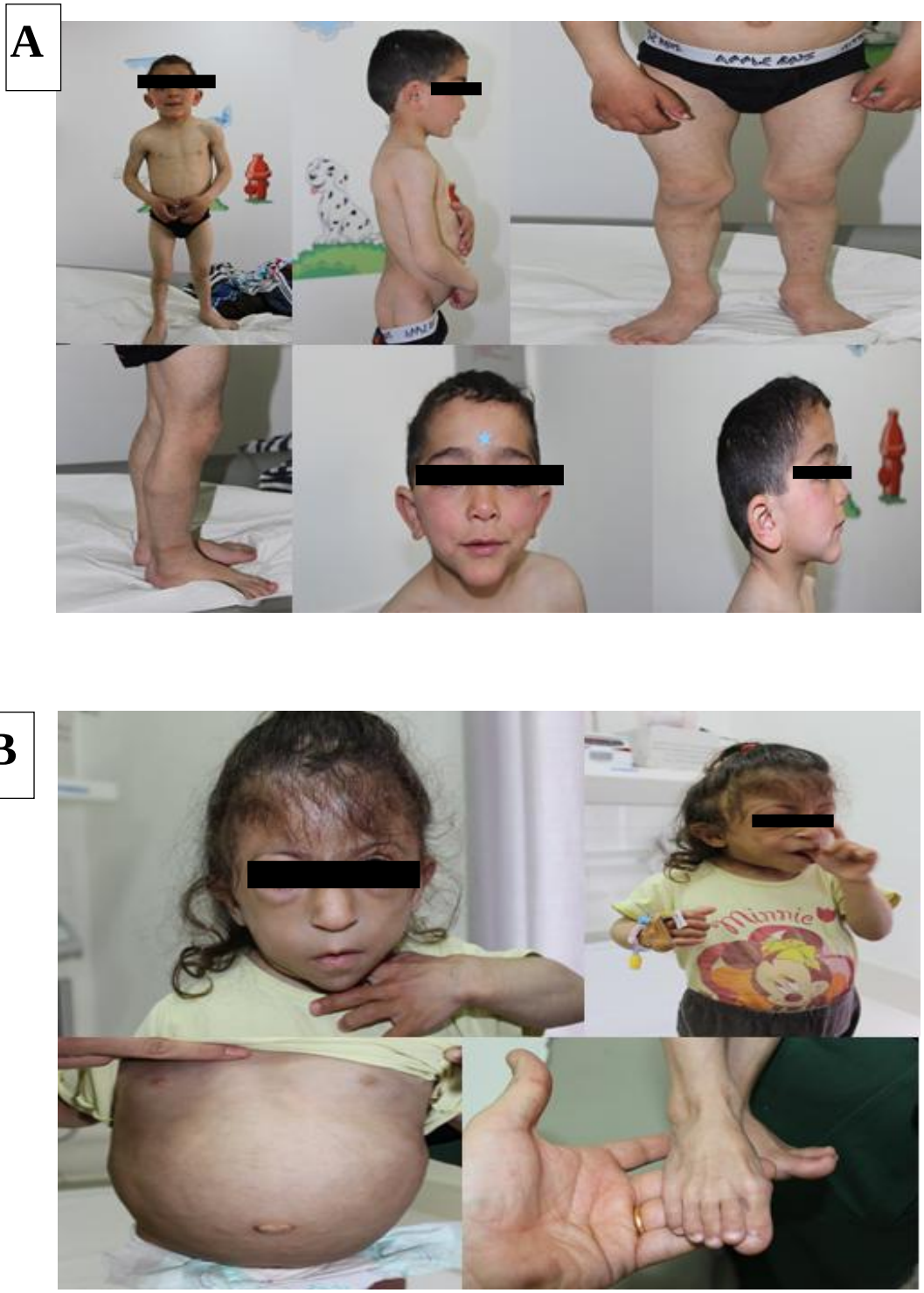


Figure 9: Picture of the affected members of AY and CE families.
Figure 9-A: is AY 3 from family AY show symptoms of CGL
Figure 9-B: CE3 patient from CE family clinically diagnosed as CGL

4.2 SNP microarray result

After diagnosis of the affected members of AY family to have lipodystrophy. SNP microarray was performed on AY1, AY2, AY4 and AY5 to map disease loci based on linkage analysis and to determine the responsible gene of the disease in the family.

The result show different homozygosity regions of different chromosome. Every homozygous region on different chromosome has a high number of SNPs and increased in length, and these regions have a number of suspected genes as shown in table 14.

There is four genes linked to autosomal recessive Congenital Generalized Lipodystrophy, and based on clinical diagnosis, the causative mutation of the clinically diagnosed member of AY family may be in one of the four genes that cause CGL.

Every homozygous region was screened gene by gene to find our candidate gene that linked to the disease and we are lucky to find AGPAT2 gene in a homozygous region of chromosome 9. Table 16. This gene was linked to CGL1 and the patients with a mutation in this gene have symptoms match with the symptoms of the affected members of AY family. So we select AGPAT2 gene for direct Sanger sequencing.

Table 16: SNP microarray result**:**

Chromosome number	Start position	End position	Number of SNP	Length	Number of genes
chr 12.3	32,358,237	58,332,351	1710	25,974,114	476
chr 16.6	31,845,697	48,306,394	98	16,460,697	171
chr 15.13	94,827,885	10,2374,592	700	7,546,707	72
chr 6.5	149,027,366	155,387,239	634	6,359,873	78
chr 9.7	137,248,166	141,027,939	105	3,779,773	151
chr 15.7	42,412,606	45,542,312	112	3,129,706	93

chr 6.6	164,021,403	167,130,749	356	3,109,346	16
chr 5.7	146,600,210	149,446,119	304	2,845,909	40
ch r1.3	32,143,138	34,271,055	60	2,127,917	55
chr 15.1	23,814,372	25,922,331	170	2,107,959	36

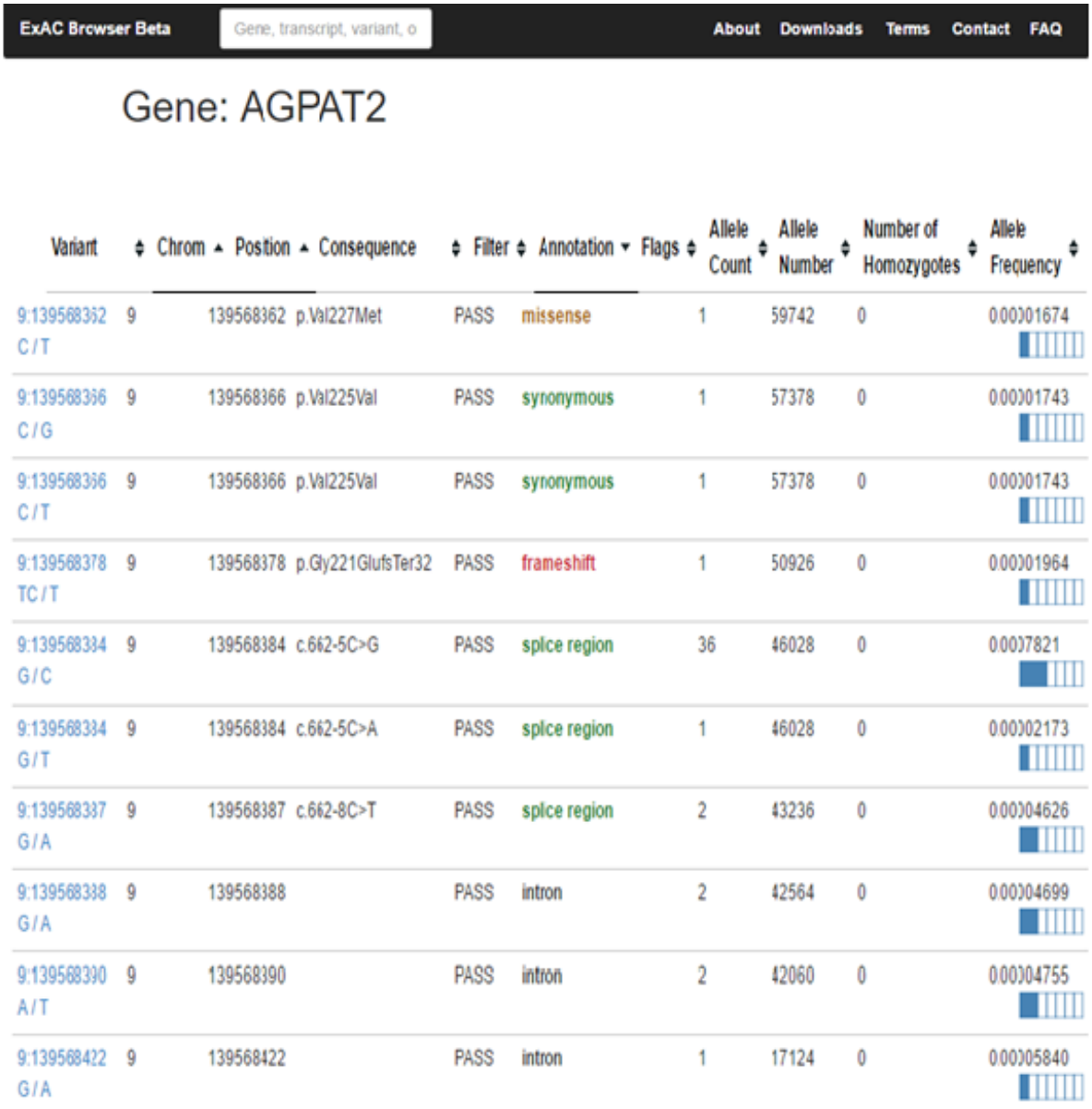
This table of SNP microarray results showing a number of homozygous regions of different chromosomes. We dose not found the candidate gene that match with the phenotypes of the affected members of AY family. By looking to homozygous region number 5 at chromosome 9 it's contain 151 genes. After screening of these genes we found AGPAT2 gene, a mutation in this gene was linked to CGL1.

Because we found a gene that match with the phenotypes of the affected members of AY family we design primers for the six exons of the candidate gene (AGPAT2 gene). Sanger sequencing was performed on all the affected members and on parents of the affected and other member who not show any lipodystrophy phenotypes. And to check if AGPAT2 gene is our candidate gene that have the causative mutation that responsible for the appearance of lipodystrophy phenotypes.

4.3 Sanger Sequencing Results:

After sequencing of the six exons of all affected and non-affected members of AY family. Analysis of the sequencing and screening for the causative mutation was done using Chromoas 2 and Finch TV software's. The result of the screening is a deletion mutation in the first base pair of exon 6. This deletion is occurs in guanine base pair delG 622 at this coordinate (chr9:139568379) and occurs near a splice sit at exon 6. And by do sequencing for the rest of the family members we found that delG 622 occurs in all affected members of the family.

Using ExAC database we know that deletion mutation that segregate in all the affected members of AY family is novel mutation and was not described before in the literature. (chr9:139568379) is the coordinate of the deletion mutation. By looking at the figure 10 we can't find this coordinate in the known mutation.



Variant	Chrom	Position	Consequence	Filter	Annotation	Flags	Allele Count	Allele Number	Number of Homozygotes	Allele Frequency
9:139568362 C / T	9	139568362	p.Val227Met	PASS	missense		1	59742	0	0.00001674
9:139568366 C / G	9	139568366	p.Val225Val	PASS	synonymous		1	57378	0	0.00001743
9:139568366 C / T	9	139568366	p.Val225Val	PASS	synonymous		1	57378	0	0.00001743
9:139568378 TC / T	9	139568378	p.Gly221GlufsTer32	PASS	frameshift		1	50926	0	0.00001964
9:139568384 G / C	9	139568384	c.662-5C>G	PASS	splice region		36	46028	0	0.0007821
9:139568384 G / T	9	139568384	c.662-5C>A	PASS	splice region		1	46028	0	0.00002173
9:139568387 G / A	9	139568387	c.662-8C>T	PASS	splice region		2	43236	0	0.00004626
9:139568388 G / A	9	139568388		PASS	intron		2	42564	0	0.00004699
9:139568390 A / T	9	139568390		PASS	intron		2	42060	0	0.00004755
9:139568422 G / A	9	139568422		PASS	intron		1	17124	0	0.00005840

Figure 10: known mutations of AGPAT2 gene by ExAC database.

Sanger sequencing was performed for affected and unaffected members of AY family to see the segregation of the mutation and to validate the mutation in AY family, also Sanger sequencing was performed to 100 healthy controls to ascertain absence of the mutation. Sanger sequencing has revealed that AGPAT2 delG 662 mutation segregate perfectly in an autosomal recessive manner in lipodystrophy AY family, Figure 11. Moreover, all affected individuals of AY family were homozygous for the mutation and the unaffected parents were heterozygous and the unaffected siblings were heterozygous or wildtype for the mutation and the 100 control as wild type. Figure 11 shows Sanger sequencing results of homozygous and heterozygous sequences.

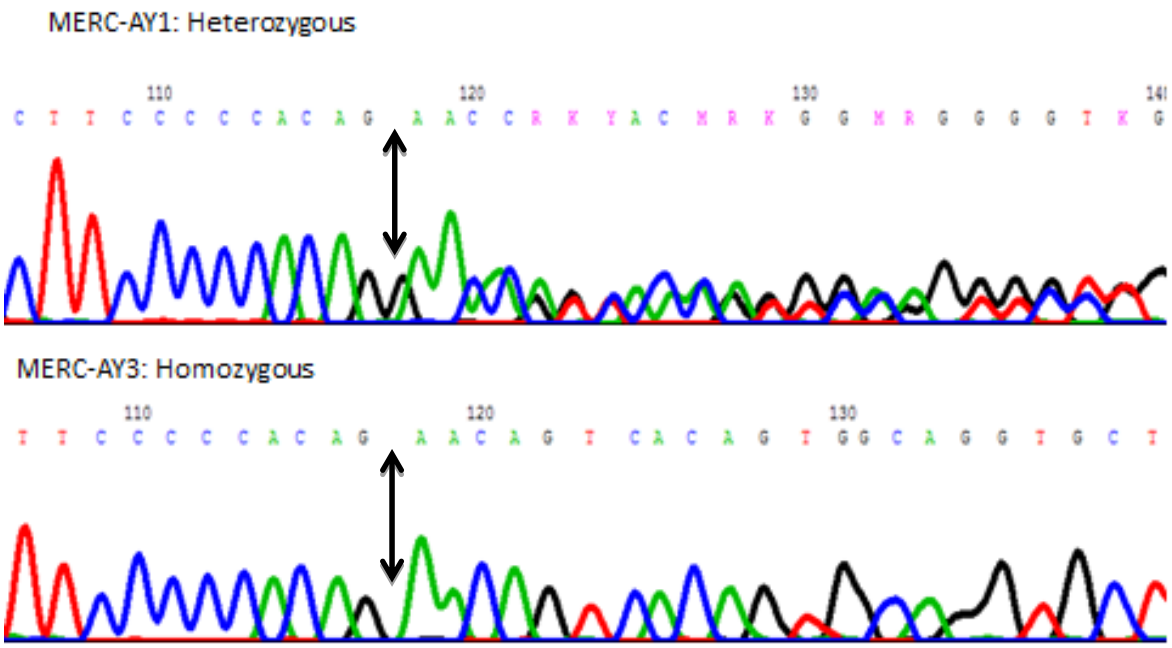


Figure 11: Sanger sequences for heterozygous individual AY1 and Homozygous affected AY3: The upper arrow indicates the site of the mutated nucleotide in the heterozygous member. The lower arrow indicates the site of the deletion in the affected member. In the heterozygous affected member there is single nucleotide deletion in one allele and other allele still have a guanine for this reason the sequence show a lot of homozygosity after the deletion of guanine base pair.

The figure below show the segregation of the novel deletion mutation delG 662 at this coordinate (chr9:139568379) in all affected members of AY family. Figure 12

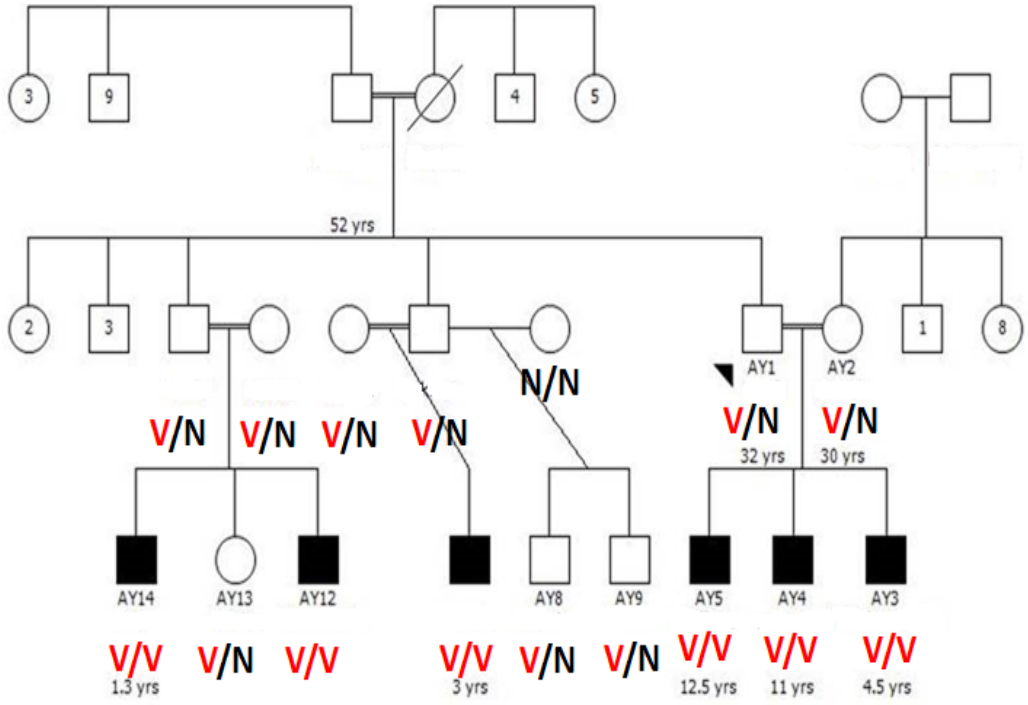


Figure 12: Segregation of the genotype with the phenotype. Where NN is the unaffected wild type, NV is heterozygous affected and the VV is the homozygous affected.

After knowing that delG 662 is a deletion mutation near a splice site at exon 6, Sanger sequencing was performed to check if this mutation has splice site effect like exon skipping, partial exon deletion and intron exon inclusion and exclusion.

Sanger sequencing results for cDNA of one homozygous affected (AY14) of the AY family compared with healthy control. Figure 13. The novel deletion mutation is around the splice site of all affected members of AY family, this mutation lead to shortcut the protein by 27 amino acid less than the normal functional one. And no difference was observed on gel electrophoresis between homozygous and heterozygous. So the mutation was not affect the splice site and dose not lead to any splice site mutation.

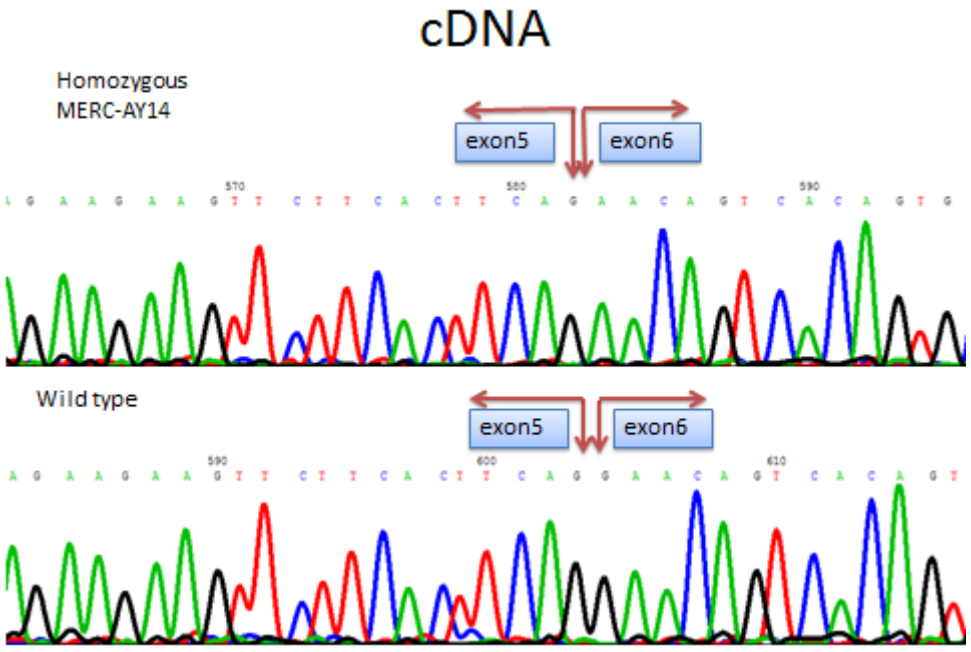


Figure 13: Sanger sequencing results for cDNA of one homozygous affected AY14 of the AY family compared with healthy control.

Mutation tester browser was used to compare the nucleotides and protein sequence of the AGPAT2 gene between the mutated and wild type sequences as illustrated in the tables below. (Table 17 and 18)

Table 17 : Mutated and original DNA sequence

WT/MU	DNA Sequence
Original gDNA sequence snippet	GTCTGTTGCTTCCCCCACAG**G**AACAGTCACAGTGCAGGTGC
altered gDNA sequence snippet	GTCTGTTGCTTCCCCCACAGAACAGTCACAGTGCAGGTGC
original cDNA sequence snippet	GAAGAAGTTCTTCACTTCAG**G**AACAGTCACAGTGCAGGTGC
altered cDNA sequence snippet	GAAGAAGTTCTTCACTTCAGAACAGTCACAGTGCAGGTGC

As shown above in the table the deleted guanine base pair appears bold in red color, which leads to change all amino acid sequence after the deletion.

The deletion occurs in the second guanine base pair in the GGA codon that give glycine protein, so glycine replaced with glutamic acid.

Table 18: Mutated and original amino acid sequence.

WT/MU	Amino Acid Sequence
wildtype AA sequence	MELWPCLAAA LLLLLLLVQL SRAAEFYAKV ALYCALCFTV SAVASLVCLL RHGGRTVENM SIIGWFVRSF KYFYGLRFEV RDPRRLQEAR PCVIVSNHQS ILDMMGLMEV LPERCVQIAK RELLFLGPVG LIMYLGGVFF INRQRSSTAM TVMADLGERM VRENLKVWIY PEGTRNDNGD LLPFKKGAFY LAVQAQVPIV PVVYSSFSSF YNTKKKFFTS GTVTVQVLEA IPTSGLTAAD VPALVDTCHR AMRTTFLHIS KTPQENGATA GSGVQPAQ*
mutated AA sequence	MELWPCLAAA LLLLLLLVQL SRAAEFYAKV ALYCALCFTV SAVASLVCLL RHGGRTVENM SIIGWFVRSF KYFYGLRFEV RDPRRLQEAR PCVIVSNHQS ILDMMGLMEV LPERCVQIAK RELLFLGPVG LIMYLGGVFF INRQRSSTAM TVMADLGERM VRENLKVWIY PEGTRNDNGD LLPFKKGAFY LAVQAQVPIV PVVYSSFSSF YNTKKKFFTS EQSQCRCWKP SPPAASLRRT SLRSWTPATG P*

This sequence of the translated protein after the frameshift deletion mutation of the AGPAT2 gene of the first lipodystrophy family (AY), so this deletion as show above its leads to change all the sequence after the deletion and lead to pre stop codon and give slightly truncated protein shorter than the WT in -27 amino acid.

On the other hand, Sanger sequencing was performed for all genes that's linked to CGL in the second family (CE). To search for potential sequence variants, all coding exons and splice junction sites of AGPAT2, BSCL2, CAV1 and PTRF genes were sequenced initially in CE3 the affected individual and one unaffected individual (CE2) in the family.

4.4 Bioinformatics analysis of results

Our results show that deletion mutation of the first base pair in the exon six of AGPAT2 gene in all the affected members of AY family is what causes the lipodystrophy phenotypes in the affected patients. This mutation leads to premature stop codon and gives a truncated protein shorter than the WT in -27 amino acid. The deletion occurs in the second guanine base pair in the GGA codon (chr9:139568379) that give glycine amino acid, so glycine replaced with glutamic acid and change the amino acids sequence after the deletion.

By using UCSC genome browser, we found that glycine is completely conserved among the species from human to zebra fish as seen in Figure 12 Consequently, any changes in this amino acid will may lead to damage in the structure and function of the AGPAT2 protein. Figure 14.

On the other hand, to predict the possible impact of delG 662 on the length and function of AGPAT2 we used mutation tester browser and we found the mutated amino acid sequence is less than the normal about 27 amino acid when it's compared with wildtype as seen in the Table 17, 18.

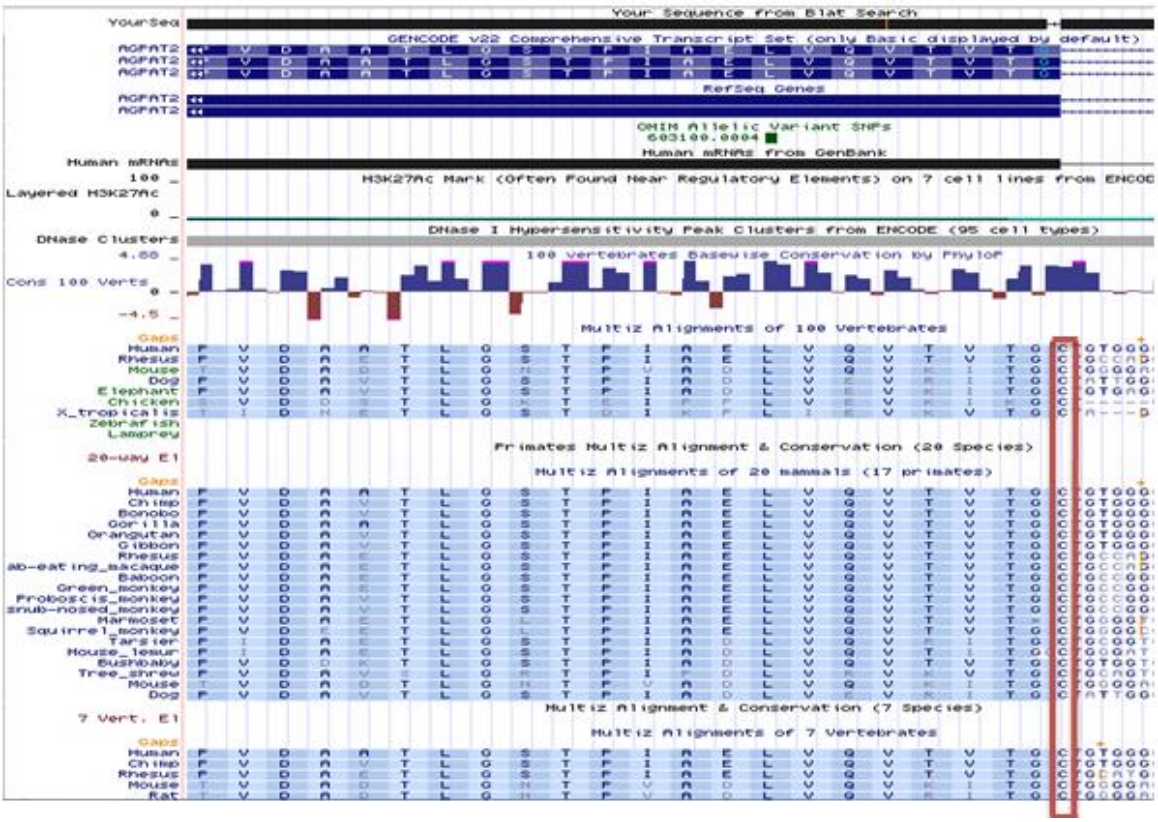


Figure 14: Multiple alignments of AGPAT2 protein for variable organisms from human to zebra fish showing the conservation of the mutant site (delG 662).

At protein level, the deletion dose not lead to delete any domain or motifs, its lead to deletion of the C-terminal due to truncation of the protein as a result of pre termination codon. Figure 15. The deletion in C-terminal end of the protein may affect protein structure and function. C-terminal has a role in protein sorting which contain signal for protein retention, this signal keep the protein in the ER. Some modification can occurs at the C-terminal like adding of lipid anchor which help protein insertion into a membrane without helps of transmembrane domains. So deletion of the C-terminal of AGPAT2 protein may affect its normal function in TAG synthesis and lipid droplet formation, but to know the real effect of the deletion on the protein level we must do protein functional assay.

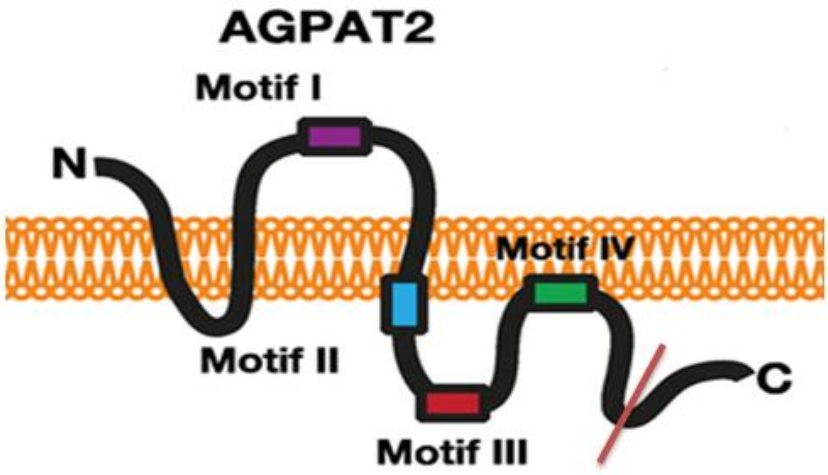


Figure 15: Predicted AGPAT2 protein motifs showing the expected location of delG 622 on C-terminus. (42)

CHAPTER 5

Discussion:

In a previous study by (Ramanathan N. et al. 2012) in Germany. They identified a novel pathogenic mutation in AGAPT2 in patient with insulin resistance, and server fat loss. This mutation is a substitution mutation p.Cys48Arg. Wild type and mutant AGPAT2 were expressed in control and AGPAT2 deficient pre adipocyte cell lines. Also they measure protein and mRNA level and they found protein levels of p.Cys48Arg AGPAT2 was significantly reduced compared with wild-type AGPAT2.

Another study by (Corte´s V. A. et al. 2014) in Chile. They found two sisters with CGL1 due to double AGPAT2 homozygous mutations. The first mutation is Missense mutation (c.299G.A) changes a conserved serine in the acyltransferase motif of AGPAT2 (p.Ser100Asn). and other one is a splice site mutation lead to skipping of exon 4 and deletion of substrate binding domain of AGPAT2 gene. Also they measure the amount of plasma leptin level in both sister and they found it lower than the normal level.

In this study, we have identified a novel deletion mutation in AGPAT2 gene using microarray and Sanger sequencing methods. by using Sanger sequencing the mutation was confirmed and found to be perfectly segregated with the Congenital Generalized Lipodystrophy type 1 phenotypes. This novel frameshift deletion mutation (delG 662) in exon 6 of AGPAT2 gene occurs in the second guanine base pair in the GGA codon that give glycine amino acid, so glycine replaced with glutamic acid and consequently changes the protein sequence after the deletion causing frameshift.

A novel deletion mutation was detected in a Palestinian family (Fig.7) diagnosed with Congenital Generalized Lipodystrophy type 1 that inherited it in an autosomal recessive way. Next, using Sanger sequencing the (del G 662) mutation was proved to segregate with the CGL1 phenotype. This deletion mutation is damaging because it causes protein truncation and give pre mature stop codon. This deletion mutation was segregate in the six affected members of the AY family.

This deletion dose not lead to deletion of domains or motifs, it's may lead to deletion of the cytoplasmic C-terminal end of the protein. This deletion may affect AGPAT2 protein function by affect the stability of the structure. So the protein may not able to work correctly in TAG synthesis pathway and in lipid droplet formation pathway.

AGPAT2 gene play critical role in adipocytes differentiation pathway, which is activate adipocytes to be mature adipocytes for synthesis of triglycerides and phospholipids. So as a result of a mutated AGPAT2 gene, deficiency in adipocytes occurs that lead to health problems like hypertriglyceridemia and insulin resistance as what occurs in patients with CGL1. (21,39)

Hepatic steatosis is one symptom of the CGL, but liver enzymes in the AY3 was not elevated. We must remember that the aminotransferases are not reliable predictors of steatohepatitis and several patients with normal aminotransferases may have steatohepatitis when undergoing liver biopsy. On the other hand, CGL has already started since birth, in contrast to insulin resistance and hypertriglyceridemia that worsens with age. (21,39)

Moreover, AGPAT2 gene has a role in synthesis of triglyceride pathway, which is catalyzes the conversion of lysophosphatidic acid to phosphatidic acid, a key step in the synthesis of triglycerides and glycerophospholipids from glycerol-3-phosphate. Reduced AGPAT2 activity in adipose tissue, lead to lipodystrophy either due to lack of triglyceride synthesis or due to abnormal adipocyte function from lack of phospholipids also, loss of AGPAT2 may initially restrict the lipid droplet formation due to decrease phospholipid synthesis. (21, 39)

The defect of TAG synthesis therefore provides a very plausible explanation for lipodystrophy in patients with genetic defects in the AGPAT2 gene. However, cellular studies have also demonstrated that disruption of AGPAT2 or BSCL2 inhibits not only lipid synthesis but also the normal induction of adipogenic gene expression during fat cell development. This may reflect the co-regulation of gene expression and lipid droplet formation in developing adipocytes or indicate that these proteins possess functions in addition to their proposed roles in lipogenesis. (21, 39)

The second family (CE) that does not show any mutation segregates with the phenotype of the affected member. So all coding exons and splice junction sites of AGPAT2, BSCL2, CAV1 and PTRF genes were sequenced initially in the affected member (CE3) and one

unaffected individual of the family. this may indicate that the mutation was occurs in the intron region or in the promoters and enhancers. Also it could be a novel gene responsible of the phenotypes that's appears in the affected member of the family.

One of the most important limitations of the study is the correct diagnosis of CGL, because it's not easy to doctors to clinically diagnose those patients. Moreover, lack of genetic analysis of one some parents of the patients who clinically diagnosed CGL. So the genetic analysis of parents and other uninfected family members is important for best clinical/genetic diagnosis of the disease. We hope that earlier clinical/genetic diagnosis and more efficient treatment options can reduce complications, prevent early deaths, and thus increase the expectation and quality of life of these patients.

Lack of functional protein assay is a major limitation in the study. By studying the protein level of AGPAT2 gene we can know the effect of the deletion mutation on the deleted C-terminal so we can validate this mutation is the causative mutation of the lipodystrophy phenotypes in AY family. To overcome this limitation we can do protein functional assay on the affected members of AY family.

Moreover, lack of some clinical data and parameters like adipocytokines such as leptin and adiponectin. These parameters are so important for best clinical diagnosis because they are important in metabolism and oxidation and they are reduced in case of fat loss. To overcome such a limitation, a new fresh blood sample can be taken from the patients to measure the amount of each parameter in the blood stream.

As a conclusion, Mutations in the AGPAT2, BSCL2, CAV1, and PTRF genes cause congenital generalized lipodystrophy types 1 to 4, respectively. The proteins produced from these genes play important roles in the development and function of adipocytes, which are the fat-storing cells in adipose tissue. Mutations in any of these genes reduce or eliminate the function of their respective proteins, which impairs the development, structure, or function of adipocytes and makes the body unable to store and use fats properly as occurs in AY family. These abnormalities of adipose tissue disrupt hormones and affect many of the body's organs, resulting in the varied signs and symptoms of congenital generalized lipodystrophy include hypertriglyceridemia, acanthosis nigricans and insulin resistance.

A novel deletion mutation (delG 662) in exon 6 of AGPAT2 gene was detected in the affected patients in AY family and segregates in 6 members of the family. This deletion

mutation cause a frame shift and premature termination codon, that's leads may lead to un functional protein. (delG 662) mutation cause Congenital Generalized Lipodystrophy type 1 associated with acanthosis nigricans and hypertriglyceridemia.

Some of the genes associated with congenital generalized lipodystrophy also play roles in other cells and tissues. In some people with congenital generalized lipodystrophy, no mutations have been found in any of the genes listed above. Researchers are looking for additional genetic changes associated with this disorder.

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