The Development of an Amplification Specific PCR Protocol for the Detection of the Presence of Two Gaucher Mutations in the Ashkenazi Jewish Population

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Abstract

Gaucher disease is an autosomal, recessive, lysosomal storage disease most prevalent in the Ashkenazi Jewish population. Gaucher disease is caused by a deficiency in glucocerebrosidase enzymatic activity, with subsequent accumulation of glucosylceramide in various organs. In the Jewish Ashkenazi population there are four known mutations that cause the disease: 1226G (N370S), 84GG, IVS2+1 and 1448C (L444P). By using PCR mutagenesis, here we developed an amplification specific PCR protocol for the detection of the presence of two Gaucher mutations in the Ashkenazi Jewish population. Developing an amplification specific PCR protocol for the mutations of Gaucher disease is very important both for detecting carriers and homozygotes for the disease and for determine mutations frequencies.

Introduction

Gaucher disease is the most common lysosomal disease (Fig.1) and is most prevalent in the Ashkenazi Jewish (AJ) population. It is an autosomal recessive disorder and it caused by a deficiency in glucocerebrosidase enzymatic activity due to various mutations in the gene encoding lysosomal glucocerebrosidase (GBA). Glucocerebrosidase is found in the lysosomes of nearly all cells types, and is responsible for the degradation by hydrolysis of Glucosylceramide and Glucosylsphingosine – metabolic intermediate derived from the cellular turnover of membrane gangliosides and globosides (Zukerman et al, 2007).

Since Glucosylceramide and Glucosylsphingosine are crucial factors of biological membranes and are both important intermediates in the biosynthetic and degradative pathways of complex glycosphingolipids, their accumulation are likely to have severe pathological consequences (Jmoudiak & Futerman, 2005). These accumulations occur primarily in cells of mononuclear phagocyte origin. These macrophage cells containing these accumulations are also known as 'Gaucher cells' (Fig.2). These aberrant cells release pro-inflammatory factors causing inflammatory response which is a classical sign of Gaucher disease.
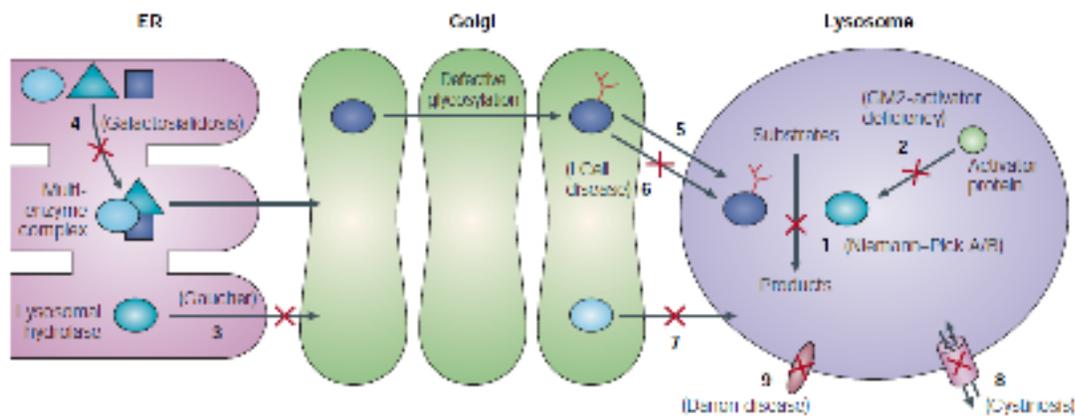

Fig.1 The biochemical and cellular basis of Lysosmal Storage disorders (LSD). Lane 3 – Gaucher disease is caused by the defective transport of Lysosomal Hydrolase out of the ER. (Futerman et al 2004).
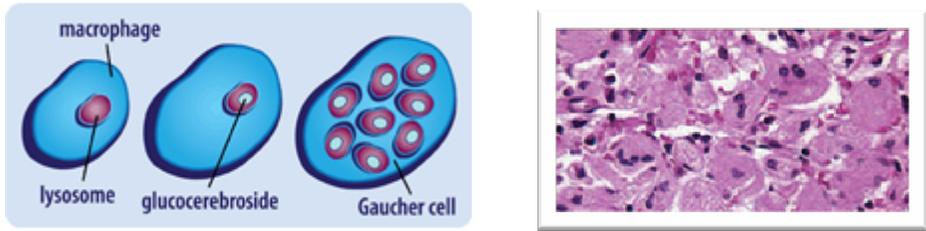
Fig.2 Gaucher Cells. From the web.

The phenotypes of the disease are classified into three types based on the clinical signs and the symptoms. Type 1 of the Gaucher disease is the most frequent type occurring in approximately 90% of the patients. The Gaucher cells in type 1 patients are present in all organs, but accumulate particularly in the spleen, liver, lungs, and bone marrow. Type 1 Gaucher patients experience abdominal pain and exhibit splenomegaly, hepatomegaly, anaemia, thrombocytopenia. Skeletal disorders common to Type 1 patients include osteopenia, lytic lesions, pathological fractures, chronic bone pain, acute episodes of excruciating bone crisis, bone infarcts, osteonecrosis and various skeletal deformities. There is also lung involvement that includes interstitial lung disease and pulmonary hypertension (Zimran, 1997).

Types 2 & 3 are rare and violent forms of Gauchers disease. They include neurological involvement resulting in death in the first years of life in type 2, and in the forth decades in type 3 (Cox, 2010 and Jmoudiak & Futerman, 2005).

The glucocerebrosidase gene (*GBA*) is located on chromosome 1q21, and there are more than 200 mutations that are known to be associated with Gaucher Disease.
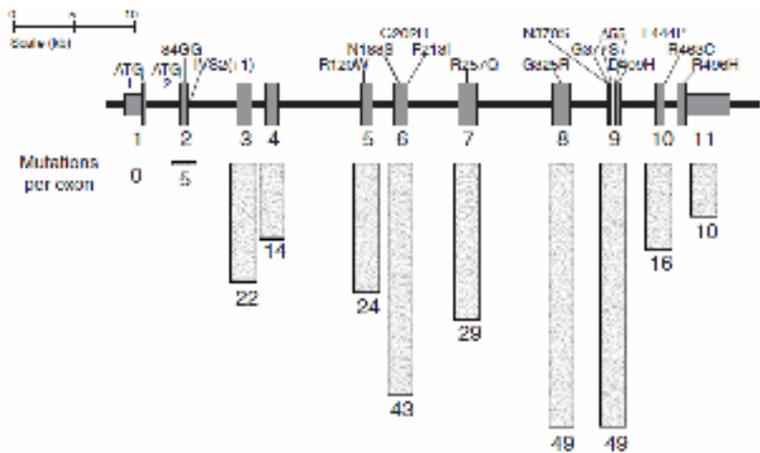

Fig.3 The exonic structure of GBA with 15 common mutations and the number of reported substitution, deletion, insertion, and splice site mutations per exon. (Hruska et al, 2008).

In the JA population the four most common mutations are: 1226G (N370S), 84GG, IVS2+1 and 1448C (L444P). The most common mutation, N370S, accounts for 70% of mutant alleles in AJ and 25% in non-Jewish patients (Beutler & Grabowski, 2001). This mutation results in type-1 Gaucher disease. The 84GG mutation, accounts for 10% mutant alleles in AJ and has not been found among non-Jewish patients. The IVS2+1 mutation constituted 2.26% of the disease alleles among Jewish patients and 1.43% among the non-Jewish patients. The prevalence of the 1448C mutation among non-Jewish Gaucher patients was 31.43%, while its prevalence among Jewish patients was only 4.24% (Horowitz,1993).

Carrier testing for diseases common in AJ exists for over a decade. Glucocerebrosidase enzymatic activity can be measured in leukocytes using fluorescent substrates, and demonstrating deficient enzymatic activity. (Zukerman et al, 2007).
In this experiment an amplification specific PCR protocol for the detection of the presence of two Gaucher mutations in the AJ population was developed.

Material and Methods

Genomic DNA

Genomic DNA was provided by Alex Bulanov of the Laboratory for Familial Dysautonomia Research, Department of Biological Sciences, Fordham University.

Primers

Two mutations (positive controls) were created by PCR mutagenesis, using two sets of designed primers (Table no. 1)

Table 1:

Mutation		Sequence	Reverse Primer
IVS2 G to A	Normal	TGTCGTGGGCATCAG**G**TGAGTGAGTCAAGGCAG	AGTACAAGCAG AGTGAGG
	Mutated primer	TGTCGTGGGCATCAG**A**TGAGTGAGTCAAGGCAG	
1226 A to G	Normal	TGTCCTTACCCTAGA**A**CCTCCTGTACCATGTG	CTGTTGCTACCT AGTCAC
	Mutated primer	TGTCCTTACCCTAGA**G**CCTCCTGTACCATGTG	

PCR

The two mutations (positive controls) were produced by PCR using the two sets of primers.

PCR was performed according to the manufacturer's protocol for 50 cycles: [94°C (30 sec) 58°C (30 sec) 72°C (30 sec)], 72°C for 7 minutes, and 4°C.

Agarose Gel Electrophoresis

1% agarose gel containing ethidium bromide was prepared and 5μl of each product was loaded on the gel, and then electrophoresis was performed at 170V for 45 minutes to assure the presence of the right size bends. PCR products were then visualized in a UV trans-illuminator.

Gel Purification, PCR Purification, and Sequencing

Mutation 1226G was gel purified from the 1% agarose gel using QIAquick Gel Extraction Kit© according to the manufacturer's protocol.

Mutation IVS2+1 was purified using the PCR Purification Kit© (QIAGEN) according to manufacturer's protocol.

All samples were quantified and sent out for sequencing to Genewiz Laboratory.

Sequencing results were analyzed by NCBI BLAST program.

Ligation and transformation reaction

The purified PCR products were ligated to the pGEM®-T easy vector and transformed to JM109 *E.coli* cells.

Plasmid DNA purification

Plasmid purification was performed on five colonies for each mutation following the QIAprep Spin Miniprep Kit© according to the manufacturer's protocol.

Plasmid Sequencing

All samples were sent out for sequencing to Genewiz Laboratory.

Sequencing results were analyzed by NCBI BLAST program.

For the development of an amplification specific PCR protocol, six different sets of primers were designed for each mutation in order to detect the positive control (the plasmid) among normal genomic DNA (primers are presented in the results part).

Detecting the positive control among genomic DNA was performed by a PCR using the six sets of primers for each mutation.

PCR was performed according to the manufacturer's protocol for 50 cycles: [94°C (30 sec) 58°C (30 sec) 72°C (30 sec)], 72°C for 7 minutes, and 4°C.

Positive controls were added in appropriate molar ratios.

PCR was performed containing the following reagents (Table 2):

Table 2:

Tube	Go Taq master mix	DNA	Positive control	Mutated forward primer	Reverse primer	dH20
1	✔	✔	✔	✔	✔	✔
2	✔	✔		✔	✔	✔
3	✔			✔	✔	✔

Results

PCR using two sets of primers (Table 1) was performed to produce two Gaucher mutations –
positive controls. The PCR products were then analyzed on an agarose gel (Fig. 4). Three bands
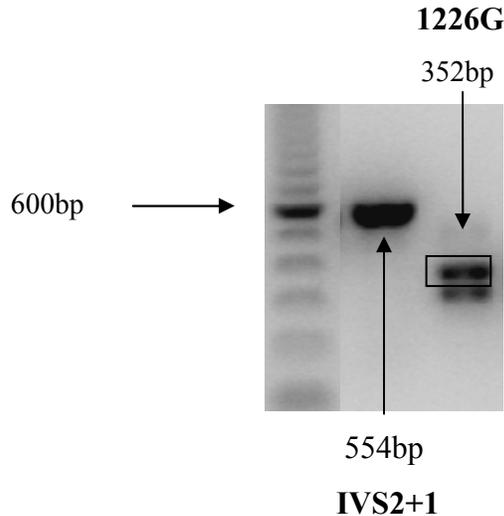were produced; two of them are at the right band size that matches the expected products.

Fig. 4: Three PCR products. Arrows indicate the number of base pairs. Negative control was
performed; no contamination was detected (not shown).

Sequencing results of PCR and Gel Purification products:

BLAST Partial Alignment of PCR Products:

1. Mutation IVS2 +1:

```
Query  497     TTGACTCACTCAT 524
               ||||||||||||
Sbjct  45640   TTGACTCACTCAC 45667
```

2. Mutation 1226G:

```
Query  253     -TC-TA-GGG-TA-AGGACAAGGTTCCAGTCGGTCCATCCGACCACATGGTACAGGAGGC
307
                || |  ||| |   ||| |||||||||||||||||||| ||||||||||||||||||||
Sbjct  40807   CTCCTTCGGGGTTCAGGGCAAGGTTCCAGTCGGTCCAGCCGACCACATGGTACAGGAGGT
40866
```

Sequencing results of Plasmid DNA purification:

1. Mutation IVS2 +1:

```
Query  55      TGTCGTGGGCATCAGA114
               ||||||||||||||
Sbjct  45667   TGTCGTGGGCATCAGG 45608
```

7

2. Mutation 1226:

```
Query  51      TTTGTCCTTACCCTAGAG  110
               ||||||||||||||||||
Sbjct  40883   TTTGTCCTTACCCTAGAA
40824
```

The amplification of the two positive controls

For the amplification of the two positive controls, PCR was performed using six different specific primers for each positive control. The PCR was performed using genomic DNA with the positive control and without it for each set of primers. PCR products were then run on a 1% agarose gel.

Mutation IVS2+

Primers 2B and 2D produced a band in the presence of the mutation but not in the absence of the mutation. Primers 2C and 2E produced a band in the presence of the mutation and a different size band in the absence of the mutation. Primers MF and 2A produced a band in the presence and in the absence of the mutation. Primers 2C and 2D did not produce any band in the presence or the absence of the mutation (Fig. 6).

A.

Primers: MF   2A   2B   2C   2D   2E


Genomic DNA	Positive Control
+	+
+	−

B.

MF – GCAGTGTCGTGGGCATCAGA**A**

2A – GCAGTGTCGTG**A**GCATCAG**A**

2B – GCAGTGTCGTAGGCATCAGA

2C – GCAGTGTC**A**TGGGCATCAG**A**

2D – GCAGTGT**A**GTGGGCATCAG**A**

2E – GCAGT**A**TCGTGGGCATCAG**A**

Fig. 5: PCR products using six different primers in the absence of and in the presence of a positive control (the plasmid). Band size 554bp. Negative control was performed; no contamination was detected (not shown) (A). Six mutated primers for IVS2+1 mutation (B).

Mutation1226G

Primer 2C' produced a band in the presence of the positive control but not in the absence of it. Primers MF', 2A', 2B' and 2D' produced a band in the presence and in the absence of the positive control. Primer 2E' produced a band only in the absence of the positive control.

A.

Genomic DNA	Positive Control
+	+
+	–
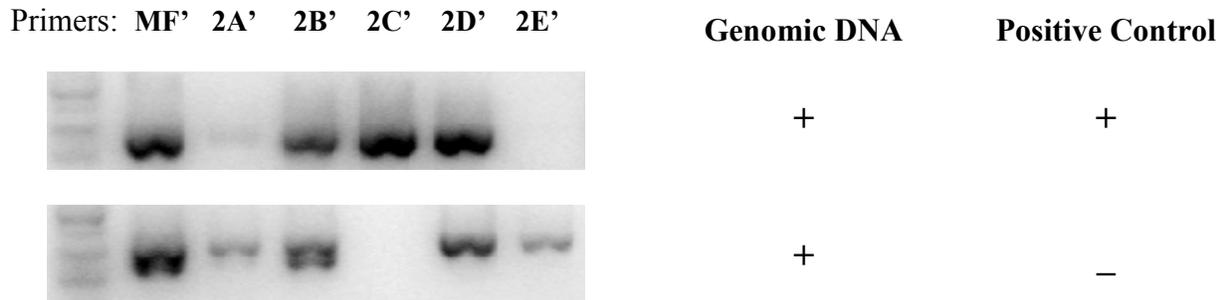
B.

MF' – CCTTTGTCCTTACCCTAGA**G**

2A' – CCTTTGTCCTTA**T**CCTAGA**G**

2B' – CCTTTGTC**A**TTACCCTAGA**G**

2C' – CCTTTGTCCTTAC**A**CTAGA**G**

2D' – CCTTTGTCCTTAC**T**CTAGA**G**

2E' – CCTTTGT**A**CTTACCCTAGA**G**

Fig. 6: PCR products using six different primers in the absence of and in the presence of a positive control (the plasmid). Band size 352bp. Negative control was performed; no contamination was detected (not shown) (A). Six mutated primers for 1226G mutation (B).

Discussion

In this experiment, two amplification PCR protocols were developed for two Gaucher mutations in the Jewish Ashkenazi population. Mutations IVS2+1 and 1226G, were specifically detected by two different sets of primers that were designed to detect them.

Out of six primers that were designed to detect each mutation, only one primer (2C') detected mutation 1226G and four primers detected mutation IVS2+1. Out of those four primers, two (2B and 2D) specifically detect the mutation only in the presence of the positive control and the other two (2C and 2E) detected a different size bands. Those results require future experiment and sequencing of the two different size bands to identify the PCR products that they produced.

Different PCR conditions for the primers that did not detect the mutations should also be considered in future experiments in order to develop more primers that can be used in detection of Gaucher disease mutations.

In addition, more experiments should be performed using new designed primers in order to develop more amplification PCR protocols for other Gaucher mutations in the JA population and in non Jewish population.

Detection of Gaucher disease mutations is a valuable tool for determining gene frequency for the disease and for carrier screening in order to prevent severe, untreatable Gaucher disease by identifying couples at risk before the birth of an affected child.

References

Arlyn Sibille, Christine M. Eng, S.-J. Kim, Gregory Pastores, and Gregory A.Grabowski Pt (1993). *Phenotype/Genotype Correlations in Gaucher Disease Type I: Clinical and Therapeutic Implications Am. J. Hum Genet. 52:1094-1101.*

Anthony H. Futerman and Gerrit van Meer (2004*). The cell biology of lysosomal storage disorder. Nature Reviews Molecular Cell Biology* 5, 554-565.

E Beutler, T Gelbart, W Kuhl, A Zimran and C West (1992). *Mutations in Jewish patients with Gaucher disease.* Blood, Vol 79, No 7**:** 1662-1666.

Ernest Beutler, Nicole J. Nguyen, t Michael W. Henneberger, t Jo Marie Smolec,t Richard A. McPhersonT Carol West, and Terri Gelbart (1993*). Gaucher Disease: Gene Frequencies in the Ashkenazi Jewish Population.* Am. J. Hum.Genet. 52:85-88.

Ernest Beutler, Anna Demina, and Terri Gelbart (1994). *Glucocerebrosidase Mutations in Gaucher Disease*. Molecular Medicine, Volume 1, Number 1:82-92.

Kathleen S. Hruska, Mary E. LaMarca, 1 C. Ronald Scott,2 and Ellen Sidransky (2008). *Gaucher Disease: Mutation and Polymorphism Spectrum in the Glucocerebrosidase Gene (GBA)* Human Mutation 29(5), 567-583.

Mingyi Chen, MD, PhD; Jun Wang, MD (2008). *Gaucher Disease Review of the Literature* Arch Pathol Lab Med—Vol 132, 851-853.

Marina Jmoudiak and Anthony H. Futerman (2005). *Gaucher disease: pathological mechanisms and modern Management.* British Journal of Haematology, 129, 178–188.

Mia Horowitz, Galil Tzuri, Nurit Eyal, Alan Berebi,t Edwin H. Kolodny, Roscoe O. Brady, Norman W. Barton, Aya Abrahamov, and Ari Zimranil (1993*). Prevalence of Nine Mutations among Jewish and Non-Jewish Gaucher Disease Patients* Am. J. Hum. Genet. 53:921-930.

Shachar Zuckerman, MSc Amnon Lahad, MD, MPH Amir Shmueli, PhD Ari Zimran, MD Leah Peleg, PhD Avi Orr-Urtreger, MD, PhD Ephrat Levy-Lahad, MD (2007). *Carrier Screening for Gaucher Disease **-** Lessons for Low-Penetrance, Treatable Diseases*. JAMA, Vol 298: 1281-1290.

Susan J. Gross, MD1, Beth A. Pletcher, MD2, and Kristin G. Monaghan, PhD3, for the Professional Practice and Guidelines Committee (2008). *Carrier screening in individuals of Ashkenazi Jewish descent Genet Med*: 10(1):54 –56.

Timothy M Cox (2010). *Gaucher disease: clinical profile and therapeutic developments Biologics: Targets & Therapy:4 299–313.*