Mutational analysis of human genes involved in Spinal Muscular Atrophy

A thesis Submitted for partial fulfillment of Master degree of Science in biochemistry

By

Ghada Mahmoud Metwally Al-Ettribi
Research assistant
Medical Genetics Department
National Research center

Under the supervision of

Prof. Dr. Amr Mahmoud Karim
Professor of Biochemistry
Biochemistry Department
Faculty of Science
Ain Shams University

Dr. Gamila Mohamed Labib Shanab
Assist. Prof. of Biochemistry
Biochemistry Department
Faculty of Science
Ain Shams University

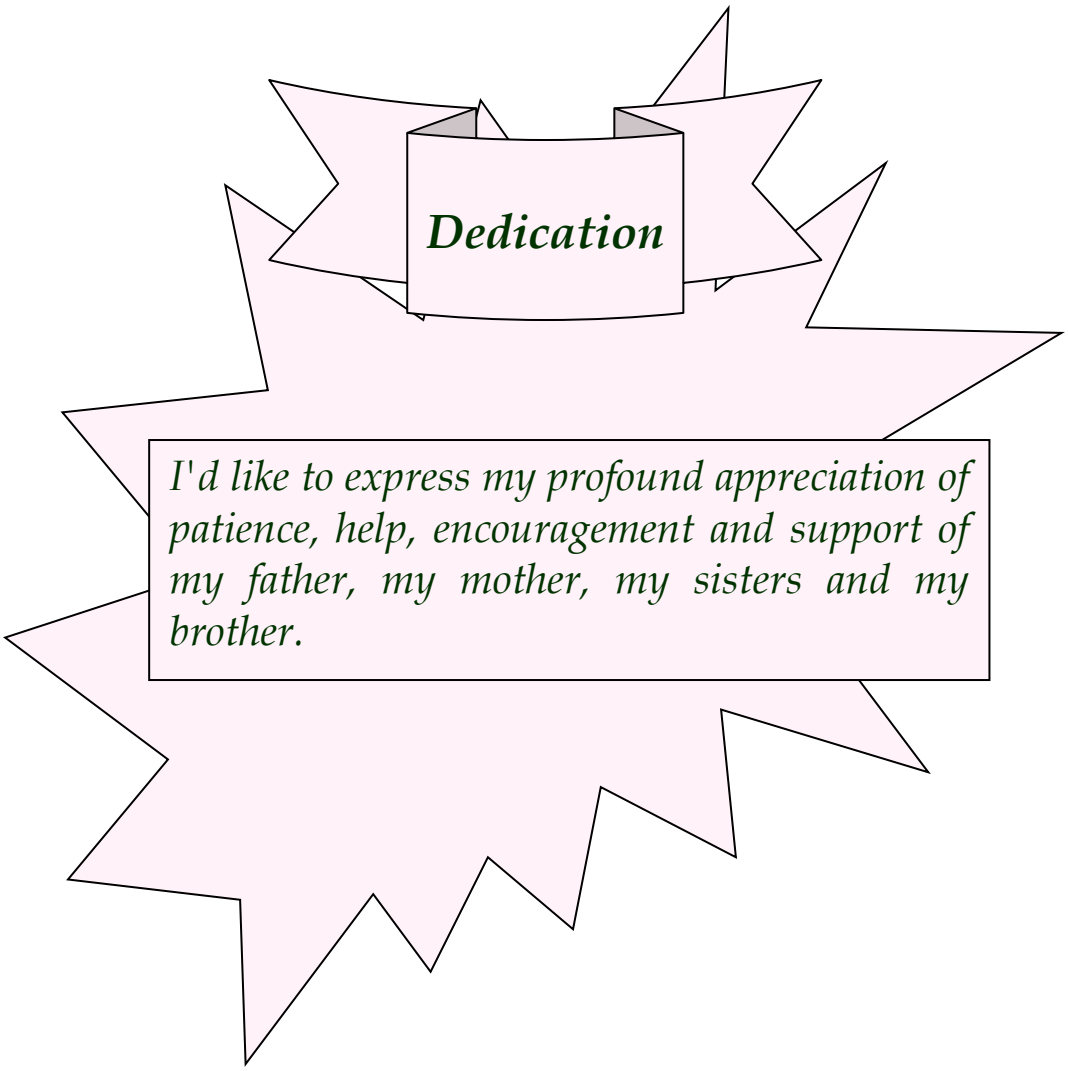
Dr. Mona Lotfi Essawi
Assist. Prof. of Molecular Genetics
Medical Genetics Department
National Research Center

Department of Biochemistry
Faculty of Science
Ain Shams University
2005

بسم الله الرحمن الرحيم

"فأما الزبد فيذهب جفاء و أما ما ينفع الناس فيمكث في الأرض"

(سورة الرعد أيه 17)


Dedication
I'd like to express my profound appreciation of patience, help, encouragement and support of my father, my mother, my sisters and my brother.

I declare that this thesis has been composed by me and the work therein has not been submitted for a degree at this or other university.

Ghada Mahmoud Metwally Al-Ettribi

Contents

Abstract…… I
Acknowledgement…….. II
List of abbreviations…… IV
List of figures…… VI
List of tables…….. VIII
List of charts…….. X
Introduction and aim of work…… 1
1. Review of literature……. 3
1.1 Clinical and molecular basis of SMA…….……... 3
1.1.1 Definition and classification of spinal muscular atrophies…… 3
1.1.2 History of the disease……. 6
1.1.3 Epidemiology of proximal SMA……... 9
1.1.4 Clinical features and phenotypic variability of proximal SMA……. 10
1.1.5 Pathogenesis of the proximal SMA……...……... 12
1.1.6 Molecular basis of SMA……...…….. 13
1.1.6.1 SMA Locus……..……... 15
1.1.6.2 Structure and organization of SMN gene……...… 16
1.1.6.3 SMN transcripts and alternative splicing process… 18
1.1.6.4 SMN-protein……..……... 20
1.2 The molecular basis of the disease……..…….. 27
1.2.1 Mutations and polymorphisms in SMN genes……..…. 27
1.2.2 Disease mechanisms……..……. 31
1.2.3 Genotype-phenotype correlation for SMA……..… 33
1.2.3.1 The relation between SMA severity and disease mechanisms……..……. 34
1.2.3.2 The relation between SMA severity and SMN2 copy number (The dosage effect of the SMN protein)…… 35
1.2.3.3 The relation between the disease severity and the SMA modifying genes of SMA……..… 39

1.2.3.3.1 The Neuronal Apoptosis Inhibitory Protein (NAIP) gene…… 39
1.2.3.3.2 Basal Transcription Factor subunit p44 (BTFp44) gene…… 40
1.2.3.3.3 H4F5 gene…… 41
1.2.3.3.4 Models of SMA alleles…… 42
1.2.4 Molecular diagnosis…… 44
1.2.4.1 Confirmation of the clinical diagnosis…… 44
1.2.4.2 Carrier testing…… 46
1.2.4.3 Prenatal testing…… 47
1.2.5 Therapeutic approaches…… 47
2. Subjects and Methods…… 53
2.1 Subjects…… 53
2.2 Samples…… 53
2.3 Reagents…… 53
2.3.1 Reagents used in genomic DNA extraction from blood…… 53
2.3.2 Reagents for genomic DNA extraction from amniocytes…… 54
2.3.3 Reagents for PCR…… 54
2.3.4 Reagents used in agarose gel electrophoresis…… 54
2.3.5 Reagents used in polyacrylamide gel preparation for SSCP analysis…… 55
2.3.6 Preparation of 50 ml total volume of 12% non-denaturing polyacrylamide gel…… 55
2.3.7 Reagents used in silver staining…… 56
2.4 Clinical Evaluation…… 56
2.5 Molecular Studies…… 57
2.5.1 Extraction of genomic DNA…… 57
2.5.1.1 From blood samples …… 57
2.5.1.2 From amniocytes in amniotic fluid…… 57
2.5.2 Measurement of DNA concentration and purity…… 58

2.5.3 Detection of the deletion of exon 5 of the NAIP gene……... 59
2.5.4 Detection of the absence of exons 7 and 8 of the SMN1 gene……... 60
2.5.4.1 Polymerase chain reaction (PCR)……... 60
2.5.4.2 Single Stranded Conformational Polymorphism (SSCP)……... 61
3 Results…… 66
3.1 Clinical Data……... 66
3.2 Molecular Studies Results…… 74
3.2.1 Results of blood DNA samples……... 74
3.2.2 Results of DNA analysis of the 2- prenatal cases…..…….. 84
4. Discussion…..…….. 89
Summary……...… 106
References……...…….. 109
Arabic Summary
Arabic Abstract

Abstract

Mutational Analysis of Human Genes Involved in Spinal Muscular Atrophy

This study aimed to determine the frequency of the homozygous absence of exons 7 and 8 of the telomeric survival of motor neuron (SMN1) gene and the deletion frequency of exon 5 of the neuronal apoptosis inhibitory protein (NAIP) gene in patients with the three different types of apinal muscular atrophy (SMA). It aimed also to assess the effectivness of the PCR-SSCP method in prenatal diagnosis of mothers at risk of SMA.

The study included 20 Egyptian SMA patients classified into 5 patients type I, 9 patients type II, and 6 patients type III. They were classified at clinical examination according to age at onset and severity of the disease. Two fetuses of 2 mothers at risk were also included in the study.

Detection of homozygous absence of exons 7 and 8 of SMN1 gene was carried out using the PCR-SSCP technique, whereas, deletion of NAIP exon 5 was detected through PCR-agarose gel electrophoresis. Homozygous absence of SMN1 exons 7 and 8, or exon 7 only, was found in 80% of patients (4/5 type I, 6/9 type II, and 6/6 type III SMA patients). NAIP exon 5 deletion was observed in 45% of patients (4/5 type I, 2/9 type II, and 3/6 type III SMA patients). One of the two fetuses included in the study was diagnosed as having SMA using the PCR-SSCP assay, while the other was diagnosed as genotypically normal. In conclusion, the frequency of homozygous absence of SMN1 exon 7 and 8, or exon 7 only, in concordance with deletion of NAIP exon 5 was higher in type I SMA than in types II and III. SSCP technique was effective in the prenatal diagnosis. Determination of the subtle mutations in the compound heterozygous patients and quantitation of the number of SMN2 copies are recommended for promoting our understanding of genotype-phenotype correlations in SMA patients.

Acknowledgement

My grateful acknowledgement for ***Dr. Amr Mahmoud Karim***, Professor of Biochemistry, Biochemistry Departement, Ain Shams University, for his kind supervision, precious guidance, helpful instructions, and powerful support.

I would like to express my great thanks to ***Dr. Mona Lotfi Essawi***, Assistant Professor of Molecular Genetics, Medical Molecular Genetics Department (MMGD), Human Genetics and Genome Research division (HGGRD), National Research Center (NRC), for her sincere guidance, great support, invaluable advice, and great help under her continous supervision to finish this work.

My profound and sincere thanks to ***Dr. Gamila Mohamad Shanab***, Assistant Professor of Biochemistry, Biochemistry Departement, Ain Shams University, for her sincere guidance, valuable discussion, great support, abounding patience, efforts, and time she spent in reviewing the thesis.

I wish to express my deep gratitude to ***Dr. Laila Kamal Al-Deen Effat***, Assistant Professor of Molecular Genetics, MMGD, HGGRD, National Research Center (NRC), for her great support, kind help, and strong encouragment.

A word of thanks to ***Dr. Ashraf Al-Haroni***, Professor of Clinical Genetics and Deputy Head of DHGGR, NRC, for his help in providing us with the blood samples and in interpreting clinical data of the patients.

A word of thanks to ***Dr. Khaled Gaber***, Professor and Head of Prenatal Diagnosis and Fetal Medicine Department, DHGGR, NRC, for his help in providing us with the amniotic fluid samples.

I would like to express my deep gratitude and sincere appreciation to ***Dr. Yehia Zakaria Gad***, Professor of Molecular Genetics and Head of the MMGD, HGGRD, National Research Center (NRC), for his valuable guidance and kindly encouragement and support.

I would like to thank all the staff members of Medical Molecular Genetics Department, for their kind encouragement.

Finally, I can not forget to thank the patients and their family members who participated in this work, praying Allah to help all parents taking care of their diseased children and to get them happy with other healthy children.

List of abbreviations

5q13	: The long arm of chromosome 5, region 1 band 3
ADP	: Adenosine diphosphate
AHCs	: Anterior Horn Cells
AS-PCR	: Allele Specific-Polymerase Chain Reaction
ATP	: Adenosine triphosphate
BA	: Sodium Butyrate
Bax	: Bcl-2 associated x protein
Bcl-2	: B-cell Leukemia / Lymphoma 2
bp	: base paire
BTFp44t	: The telomeric Basal Transcription Factor p44
BTFp44c	: The centromeric Basal Transcription Factor p44
CBs	: Cajal Bodies
cDNA	: complementary deoxyribonucleic acid
CK	: Creatine Kinase
CNS	: Central Nervous System
DEAD-box	: Aspartic acid-Glutamic acid-Alanine-Aspartic acid tetrapeptide
EMG	: Electromyography
ENMC	: Eropean Neuro Muscular Center
ESE	: Exonic Splicing Enhancer
ESS	: Exonic Splicing Silencer
ESSENCE	: Exon Specific Splicing Enhancement by Small Chimeric Effectors
FUSE binding protein	: the Far Upstream Element binding proteins
GDB	: The Human Genome Data Base
hnRNPs	: heterogenous nuclear ribonucleoproteins
Htra2β1	: Human Transformer 2 β1
IAP	: Inhibitor of Apoptosis
IRF-E	: Interferone Regulatory Factor binding motif
ISRE	: Interferone Stimulated Response Element
kb	: kilobase
kDa	: Kilo Dalton
LMNs	: Lower Motor Neurons

Lsm proteins	**:** smith antigen-like proteins
MDa	**:** Mega Dalton
NAIP	**:** Neuronal Apoptosis Inhibitory Protein
NLS	**:** Nuclear Localization Signal
OMIM	**:** Online Mendelian Inheritance in Man
p^{53}	**:** Phosphoprotein 53
PCR-SSCP	**:** Polymerase Chain Reaction – Single Stranded Conformational Polymorphism
PFN II	**:** Neuron specific proflilin II
PNA	**:** Peptide-Nucleic Acid
PB	**:** 4-Phenyl Butyrate
Pre-mRNA	**:** preliminary messenger ribonucleic acid
RBD	**:** RNA Binding Domain
RFLP	**:** Restriction Fragment Length Polymorphism
RNA	**:** Ribonucleic Acid
RS-domain	**:** Argenine-Serine domain
SF2/ASF	**:** Splicing factor Argenine-Serine rich 2/ Alternative Splicing Factor
SIP1	**:** SMN Interacting Protein 1
sm proteins	**:** smith antigen core proteins
SMA	**:** Spinal Muscular Atrophy
SMN1 or SMNt	**:**The telomeric Survival Motor Neuron
SMN2 or SMNc	:The centromeric Survival Motor Neuron
smN	**:** Neuron Specific smith antigen
snoRNP	**:** small nucleolar ribonucleoprotein
snRNA	**:** small nuclear ribonucleic acid
snRNP	**:** small nuclear ribonucleoprotein
SR-proteins	**:** Serine-Argenine rich proteins
TFIIH	**:** Transcription Fator IIH
TOES	**:** Targeted Oligonucleotide Enhancers of Splicing
UsnRNPs	**:** Uridine small nuclear Ribonucleoproteins
WD-repeat protein	**:** Tryptophan-Aspartic acid repeat protein
YG- rich box	**:** Tyrosine-Glycine rich box
ZPR1	**:** Zinc Finger Protein 1
ΨNAIP	**:** Psudo Neuronal Apoptosis Inhibitory Protein

List of Figures

Fig (1): A. Schematic representation of the SMA region on 5q13.
B. Nucleotide differences between SMA1 and SMN2… 15

Fig (2): Schematic representation of SMA chromosome showing the location of the Ag-CA (C272) and C212 markers corresponding to SMN genes…… 17

Fig (3): Genetic basis of SMA…….. 19

Fig (4): SMN2 exon 7 skipping due to the disrupted ESE…….. 20

Fig (5): SMN is localized in distinct nuclear organelles of cultured motor neurons…….. 21

Fig (6): Domains of the SMN protein……. 24

Fig (7): Schematic representation of the most frequently observed SMA chromosomes in acute SMA (type I) and mild SMA (type II/III)…… 34

Fig (8): Genomic organization of the SMA locus. ……... 36

Fig (9): Genomic organization of H4F5 relative to existing genes in SMA critical region……. 42

Fig (10): Correction of defective SMN2 splicing…… 52

Fig (11): SSCP-analysis indicating the difference between a wild type and a mutant PCR product based on their unique conformation…… 62

Fig (12): Agarose gel electrophoresis of amplified PCR products of exons 5 & 13 of the NAIP gene from eight SMA patients……... 75

Fig (13): Agarose gel electrophoresis of amplified PCR products of exons 7 & 8 of the SMN genes from four SMA patients……... 76

Fig (14): SSCP analysis of SMN genes exon 7 on 12% polyacrylamide gel……... 77

Fig (15): SSCP analysis of SMN genes exon 8 on 12% polyacrylamide gel…… 78

Fig (16): Agarose gel electrophoresis of the amplified products of exons 5 & 13 of the NAIP gene from two fetuses and their brothers……. 85

Fig (17): Agarose gel electrophoresis of the amplified products of exon 7 & exon 8 of the SMN genes for the 2 fetuses……... 86

Fig (18): SSCP analysis of exons 7 & 8 of the SMN genes for the 2 fetuses……. 87

Fig (19): A diagrammatic representation of the mutation patterns of the twenty SMA Egyptian patients included in this study……... 99

List of Tables

Table (1):	Classification of SMAs according to the clinical criteria and the mode of inheritance……...	5
Table (2):	Classification of spinal muscular atrophies according to chromosomal location……	6
Table (3):	Overview on the history of the disease……	6
Table (4):	Criteria for classification of childhood SMA……	10
Table (5):	Milestones in SMA research……	14
Table (6):	RG-rich-domain-containing substrates of the SMN complex…….	23
Table (7):	Frequency of homozygous absence of the SMN1 gene found in types I, II and III SMA patients……..	28
Table (8):	List of small intragenic SMN1 mutations……	29
Table (9):	Polymorphisms identified in both SMN1 and SMN2 genes……	32
Table (10):	Distribution of SMN2 copies in patients with SMA….	37
Table (11):	Oligonucleotide primers……..	65
Table (12):	Clinical data of the patients…….	67
Table (13):	Classification of SMA patients according to type of the disease, showing the frequency of patients classified in each type……...	72
Table (14):	The relation between age at onset and type of the disease…….	72