DEVELOPMENT OF ^{EZ}DNA DIAGNOSTIC KIT FOR THE DETECTION OF HOMOZYGOUS DELETION OF *SMN1* GENE IN SPINAL MUSCULAR ATROPHY (SMA) PATIENTS

by

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**Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science**

FEB 2012

DEDICATIONS

Alhamdulillah to Allah SWT for your bless.
To my beloved and wonderful parents, En. Marzuki Hj Daud and Pn. Sharipah Ab Kadir, who had never stop in supporting and motivating me throughout my study.

To my brothers, Nasri and Fikri, and also my lovely sister, Mardhiah who have given me the energy and courage for my study.

To my dear hubby, Mohd Syafiq who has offered me unconditional love and gave me strength to finish my study.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

%	:	percentage
°C	:	degree celcius
µl	:	microliter
A_{260}/A_{280}	:	ratio absorbance of genomic DNA
AE buffer	:	elution buffer
AS-PCR	:	allele specific polymerase chain reaction
BL buffer	:	lysis buffer
bp	:	base pair
BSA	:	bovine serum albumin
BW buffer	:	washing buffer I
CBs	:	cajal bodies
ddH_2O	:	deionized distilled water
dHPLC	:	denaturing high performance liquid chromatography
DNA	:	deoxyribonucleic acid
dNTP	:	dinucleotide triphosphatase
EMG	:	electromyography
Gems	:	Gemini of cajal bodies
kb	:	kilo base
kDa	:	kilo dalton
$MgCl_2$	:	magnesium chloride
min	:	minute
ml	:	mililiter
mM	:	milimolar
NCS	:	nerve conduction study
ng/µl	:	nano gram per microliter
PCR	:	polymerase chain reaction
PCR-RE	:	polymerase chain reaction restriction enzyme
pmol	:	pico mole
pre-mRNA	:	precursor messenger ribonucleic acid

RNA	:	ribonucleic acid
rpm	:	round per minute
SMA	:	spinal muscular atrophy
SMN	:	survival of motor neuron
SMN1	:	survival of motor neuron 1
SMN2	:	survival of motor neuron 2
snRNPs	:	small nuclear ribonucleoprotein
SYBR® Green I	:	SYBR® Green I nucleic acid staining
Taq	:	*thermophillus aquaticus*
TW buffer	:	washing buffer II
U	:	unit
UV	:	ultraviolet
V	:	volt

PEMBANGUNAN KIT ᴱᶻ DNA DIAGNOSTIK UNTUK PENGESANAN KEHILANGAN HOMOZIGUS GEN *SMN1* PADA PESAKIT ATROPI OTOT SPINA (SMA)

ABSTRAK

Spinal Muscular Atrophy (SMA) adalah penyakit yang mempunyai frekuensi yang kedua tertinggi yang menyebabkan kematian dikalangan kanak-kanak. Kadar kejadian penyakit ini adalah didalam anggaran 1 setiap 10000 kelahiran. SMA digambarkan melalui kelemahan otot secara beransur-ansur akibat daripada kemerosotan dan kehilangan neuron motor didalam bahagian hadapan tulang belakang. *Survival of Motor Neuron* (*SMN*) merupakan gen yang bertanggungjawab terhadap penyakit SMA. Gen *SMN1* dan *SMN2* berkongsi lebih dari 99.8% jujukan yang sama dan ia hanya boleh dibezakan dengan perubahan bes pada exon 7 dan 8. Gen *SMN1* tidak dapat dikesan pada kebanyakan kes SMA disebabkan oleh kehilangan atau penukaran bes tersebut. Kebiasaanya, kehilangan gen *SMN1* dapat dikesan menggunakan kaedah Reaksi Rantai Polimer-Enzim Pembatasan (PCR-RE). Kaedah ini memerlukan masa yang lama, kos yang tinggi dan memerlukan pencernaan enzim pembatasan yang memerlukan banyak jumlah DNA yang diamplifikasi dimana ia akan muncul selepas pencernaan dan berkemungkinan menunjukkan keputusan yang salah. Untuk mengatasi masalah tersebut, kaedah altenatif menggunakan primer alel-spesifik khusus untuk diagnosis molekul SMA lebih menjimatkan masa dan kos. Platform 'freeze-dry' telah digunakan pada multiplex Reaksi Rantai Polimer-Alel Spesifik (AS-PCR) untuk pembangunan kit yang stabil suhu. Sejumlah satu ratus empat puluh tiga (143) sampel daripada pesakit yang disyaki menghidapi SMA telah digunakan di dalam kajian ini. Kaedah diagnosis molekul menggunakan PCR-RE telah dilakukan untuk mengesan kehilangan dan

kehadiran gen *SMN1* didalam semua sampel tersebut. Lima puluh enam sampel telah dikesan mempunyai kehilangan gen *SMN1*, sementara 87 sampel lagi telah dikesan tidak mengalami kehilangan gen tersebut. DNA telah diekstrak menggunakan kit komersil yang sedia ada. Untuk menilai keberkesanan kaedah AS-PCR, kesemua 143 sampel telah dianalisa semula menggunakan kaedah AS-PCR. Untuk mengesan amplifikasi PCR dan mengelakkan keputusan yang salah, gene 'housekeeping' (*β-globin* gene) telah digunakan sebagai kontrol dalaman. Keputusan daripada kedua-dua kaedah kemudiannya telah di bandingkan dan dinilaikan dari segi masa kajian dan kos setiap sampel. Kemudian, analisis kesensitiviti, ketepatan dan kestabilan untuk kit stabil suhu AS-PCR telah diperhatikan dan dinilai. Seterusnya, kit stabil suhu AS-PCR telah dianalisis keatas sampel SMA Jepun. Kajian ini telah menunjukkan 100% keputusan yang sama diantara kaedah PCR-RE dan AS-PCR. Sementara, kit stabil suhu AS-PCR juga menunjukkan 100% ketepatan dan kesensitifan diantara kaedah PCR-RE dan AS-PCR biasa. Berdasarkan ujian kestabilan kit stabil suhu AS-PCR menunjukkan sekurang-kurangnya 2.5 tahun kestabilan pada suhu dibawah 4°C. Keputusan kajian menunjukkan kit stabil suhu AS-PCR adalah berkesan standing teknik PCR-RE untuk pengesanan kehilangan gen *SMN1*. Seterusnya, penilaian diagnostik keatas populasi Jepun menunjukkan ketepatan kit ini adalah 98% sementara kesensitifannya tetap pada 100%. Kesimpulannya, pengecualian pencernaan enzim telah menjadikan kaedah AS-PCR lebih murah dan pantas berbanding kaedah lama. Ia dapat mengurangkan lima kali ganda masa dan menjimatkan 69% kos setiap sampel. Kit stabil suhu AS-PCR juga menunjukkan keberkesanan dan juga kelebihannya berbanding kaedah PCR-RE didalam diagnosis molekul SMA yang mana menunjukkan ia boleh digunapakai di dalam diagnosis rutin SMA.

DEVELOPMENT OF ^{EZ}DNA DIAGNOSTIC KIT FOR THE DETECTION OF HOMOZYGOUS DELETION OF *SMN1* GENE IN SPINAL MUSCULAR ATROPHY (SMA) PATIENTS

ABSTRACT

Spinal Muscular Atrophy (SMA) is the second most frequent fatal autosomal recessive disorder of childhood. The incidence of this disease is approximately 1 in 10000 live births. SMA is characterized by progressive muscle weakness resulting from degeneration and loss of motor neurons in the anterior horn of spinal cord. The responsible genes for SMA are *Survival of Motor Neuron* (*SMN*). *SMN1* and *SMN2* genes share over 99.8% sequence homology and they can be distinguished by base changes in both exons 7 and 8. *SMN1* gene is not detectable in majority of SMA cases due to either deletion or conversion. Conventionally, the homozygous deletion of *SMN1* gene is detected using Polymerase Chain Reaction-Restriction Enzyme (PCR-RE) method. This method is time consuming, expensive and requires restriction enzyme digestion and a considerably high amount of amplified DNA to be visible after digestion. This may lead to false-negative results. To avoid these problems, we have developed an alternative method using an allele-specific primer for the molecular diagnosis of SMA which is more time-saving and cost-effective. The freeze dry platform was applied to the multiplex AS-PCR for the development of a thermostabilize diagnostic kit. A total of one hundred and forty three samples of clinically suspected SMA were included in this study. Conventional molecular diagnosis using PCR-RE was done to detect the presence or absent of *SMN1* deletion in these samples. Fifty-six samples were found to have deletion of the *SMN1*, while the remaining 87 samples were

found to have no deletion of the gene. To evaluate the reliability of the AS-PCR method, all 143 samples were then re-analyzed using AS-PCR. To monitor the PCR amplification and to avoid the false negative results, a housekeeping gene (*β-globin* gene) was used as an internal control. The results from both methods were subsequently compared and evaluated for the experimental duration and per sample cost. The sensitivity, specificity and stability of the thermostabilized AS-PCR were monitored and subsequently analyzed. This thermostabilized AS-PCR was then tested on Japanese SMA samples. Our study showed 100% similarity between those using PCR-RE and AS-PCR. The thermostabilized AS-PCR also showed 100% specificity and sensitivity with PCR-RE and AS-PCR methods. Based on the accelerated stability, the thermostabilized AS-PCR has the minimum of 2.5 years shelf life in temperature below 4°C. Our results suggest that the thermostabilized AS-PCR is a reliable technique when compared to the PCR-RE method in the screening of *SMN1* deletion. Furthermore, the diagnostic evaluation on Japanese population suggested that the specificity of the kit is 98% while the sensitivity remains 100%. In conclusion, the exclusion of digestion step makes AS-PCR method cheaper and faster compared to the conventional method. It can reduce almost five times of the time and 69% of the cost per sample. The thermostabilize AS-PCR also reliable and superior over PCR-RE method in the molecular diagnosis of SMA, suggesting that it could be applied in the routine diagnosis of SMA.

CHAPTER 1

INTRODUCTION

1.1   The Overview of Spinal Muscular Atrophy

Spinal Muscular Atrophy (SMA) is the autosomal recessive genetic disease which can lead to infant mortality (Robert *et. al.,* 1970; Pearn 1973, 1978; Czeizel and Hamula, 1989). It is the second most common lethal genetic disease leading to childhood death after cystic fibrosis (Steward *et. al.,* 1998). The incidence of this disease is 1 in 10,000 live births while 1 in 40 normal people carry one copy of this lethal gene (Adreassi *et. al.,* 2001). Markowitz *et al.* (2004) was reported, the early founder of SMA was described by Guido Werdnig and Johann Hoffmann. The main cause for SMA is the degeneration of alpha-motor neurons that leads to the progressive muscle weakness. This alpha-motor neuron was located at the anterior horn of the spinal cord. The degeneration of this neuron could lead to the failure of transmission of the impulse from brain to the muscle neuron. When the impulse did not normally function, the normal function of the muscle will reduce and muscle will slowly shrink and atrophy. Furthermore, most of the SMA patients commonly show the general symptoms such as

muscle atrophy, weakness of cry, poor muscle tone, scoliosis, hypotonia and feeding difficulties (Zerrez *et al.,* 1995). The proximal muscles of SMA patients are mainly weaker than the distal muscle due to the location which was closest to the spinal cord.

1.1.1 Classification of SMA

Spinal Muscular Atrophy is classified based on age of onset and the clinical severity, as described in the International SMA Consortium Meeting (26th to 28th June 1992) in Bonn, Germany (Munsat *et al.,* 1992) (Table 1.1). This guideline was very useful for the determination of the prognosis and management of SMA. However, the clinical symptoms of SMA commonly overlap with other neuromuscular disorders (Chien *et al.,* 2005). Hence, many affected patients died due to the misdiagnosis of the clinical symptoms and lack of awareness among the clinician. In some condition, the molecular genetic analysis is useful for the confirmation of the clinical diagnosis (Chien *et al.,* 1995).

1.1.1(a) Type I SMA

Type I SMA is the severe type of SMA and it is also known as Werdnig-Hoffmann disease or acute SMA. Patient with type I SMA is diagnosed by the early age of onset which started between 0 to 6 months of age. Type I SMA patient usually have a very limited life expectancy (less than 2 years). Most of the patients are not able to sit without support. Generally, patients diagnosed with this form have very little control of their heads and they also cough and cry weakly. They lose the ability to swallow and feed

before they reach 1 year of age. Over time, their trunk and limbs will be weakened and it normally spreads to the intercostals muscles, making an abnormal respiratory cycle. The risk of early mortality is usually associated with bulbar dysfunction and respiratory complications.

1.1.1(b) Type II SMA

An intermediate form of SMA is the type II SMA. Patient with type II SMA is generally diagnosed around 6 to 18 months of age, but it may start earlier. Some of these patients which have been classified to have SMA type II are able to sit up without support while others can remain sitting if they are positioned, but they still cannot sit without any support. In the other case, some of the patients could stand with support and this showed that they have a better muscle development than others. However, this patient is still unable to walk. In type II SMA patient, some of them will experience reducing weight due to the bulbar weakness which combined with the swallowing difficulties. Furthermore, these patients may have problems with coughing and with cleaning secretions from the trachea, and as well as tongue fasciculation and later they can suffer from scoliosis and contractures. Their life expectancy is around 10 to 40 years.

1.1.1(c) Type III SMA

Kugelberg-Welander syndrome is also known as type III SMA or juvenile SMA. The actual age varies but according to the consortium, the age of onset for type III SMA is after 18 months. Wirth *et al.* (2000) divided the type III SMA into two types, which is

type III(a) SMA and type III (b) SMA. Type III(a) SMA is when the disease started before 3 years of age, while if the onset is later, it is called type III(b) SMA. The major difference between both types is the preservation of the ability to walk. Patients with type III(a) are able to walk until they are 20, while type III(b) patients are able to walk for their whole life. The general problems in SMA type III patient are difficulties in swallowing and coughing when feeding. Scoliosis will develop later in their life.

1.1.1(d) Type IV SMA

The adulthood form of SMA is also known as type IV SMA. There is no consensus on the age of onset of type IV SMA. Several cases study have reported that this type of SMA started after 10 years of age (Russman *et al.,* 1995) while another report by Wang *et al.* (2007) stated that their weakness normally emerges during the second or third decade of life, or at about 30 years of age. Normally, the motor function involvement is mild and there are no problems with deglutition or respiration. Type IV SMA patients are able to walk normally and have normal life expectancy.

Table 1.1 Classification of Spinal Muscular Atrophy based on the International SMA Consortium Meeting (26th to 28th June 1992) in Bonn, Germany (Munsat *et al.,* 1992).

Phenotype	Age of Onset	Life Span	Motor Milestone
SMA I	Before six months	Reduced	Sit with support only
SMA II	6-18 months	Adolescence	Independent sitting
SMA III	After 12 months	Adult	Independent ambulation
SMA IV	Adulthood	Normal	Normal

Figure 1.1   A Malay boy with Type II SMA (Adapted from Watihayati *et al.,* 2007). This patient passed away in February 2010.

Figure 1.2   A Malay girl with Type III SMA (Adapted from Watihayati *et al.,* 2007). This patient is still undergoing treatment at Hospital USM.

1.2   SMA as an Autosomal Recessive Disease

Spinal Muscular Atrophy is one of the autosomal recessive diseases inheritance pattern, leading to the early death in children. In autosomal recessive disease, two copies of the gene must be mutated for a person to be affected. In most of the SMA cases, patient usually has unaffected parents but they carry a single copy of the mutated gene while the other gene is normal. They are called a carrier. When a carrier marries another carrier, they will have a 25% chance with each pregnancy of having an affected child.

As an example in Figure 1.3, 'A' is the normal gene and 'a' is the mutated gene. When both the parents have an 'Aa' gene which mean they are a carrier, they will have 25% chance to get a normal homozygous 'AA' child, 50% chance a carrier 'Aa' heterozygous child and 25% an affected child (aa) which carry both mutated genes from the parents (Figure 1.3).
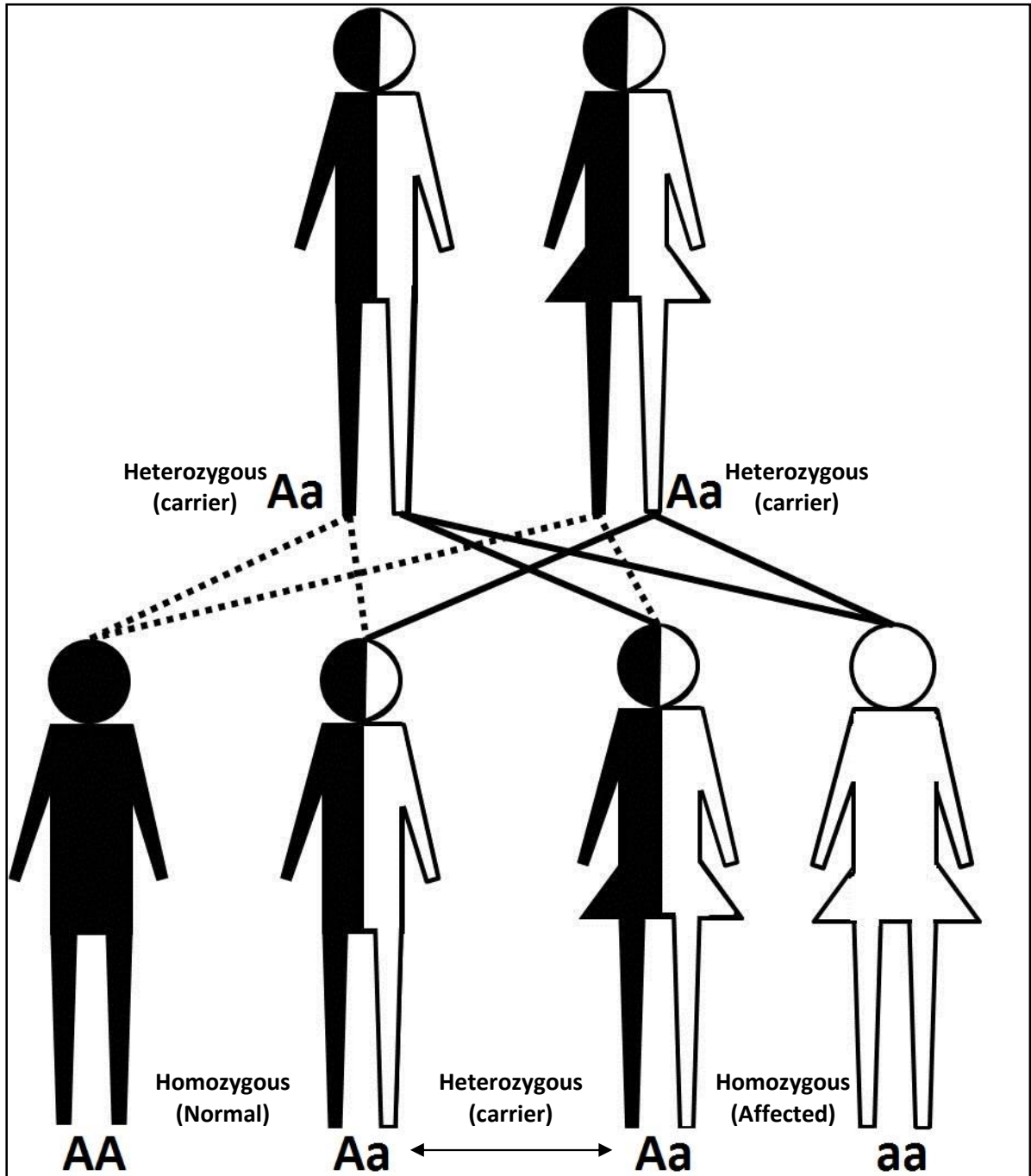
Figure 1.3 This figure shows an autosomal recessive inheritance pattern in Spinal Muscular Atrophy.

1.3   Diagnosis of SMA

In order to diagnose patients with SMA, the general symptoms of SMA need to be clinically present. Since SMA is caused by the progressively degeneration of the alpha-motor neuron, the affected neuron is only the motor neuron, not the sensory neuron. SMA patients therefore commonly present with weakness and atrophy of the voluntary muscle of the legs, arms and trunk. Most of the SMA cases could be identified with the molecular genetic testing of *SMN1* gene while in some cases, the clinical diagnosis are essential for the confirmation of SMA due to the heterozygous deletion or other mutations in the *SMN1* gene (Hahnen *et al.,* 1995; Rodrigues *et al.,* 1995). In addition, due to the low prevalence of SMA, the diagnosis of SMA becomes quite challenging. There are numbers of neurogenetic diseases that could present with similar symptoms of SMA. Hence, diagnosis of clinical presentation of SMA should be investigated with care (Araújo *et al.,* 2005) since these clinical signs can also be observed in other neuropathologies (Baioni *et al.,* 2010). Generally, SMA patients manifest clinical characteristic signs such as hypotonia, tongue fasciculation and scoliosis.

1.3.1   Clinical Features

1.3.1(a) Hypotonia

One of the clinical features of SMA is hypotonia. Hypotonia is a symptom where the muscle state is very low, which is caused by the defect of the motor nerve control by the brain. Most of the SMA cases which was detected in the infant state, the patient will have 'floppy infant syndrome' which is described an abnormal limpness when an infant is put in prone position. They easily slip through one's hand as showed in figure 1.4. The patient usually could not control the movement of their head and have difficulties in feeding. Usually, this patient is classified in the type I SMA and died before 6 months of age.

1.3.1(b) Scoliosis

Scoliosis is a condition which a person's spine is curved from side to side. The spine will form as an 'S' rather than a straight line (Figure 1.5). It is classified as either congenital (vertebral anomalies present at birth), idiopathic (unknown cause) or neuromuscular. The severe cases of scoliosis can lead to diminishing lung capacity, which put pressure on the heart and restricting physical activities. Patient with scoliosis is examined by physical examination involving the skin to indicate neurofibromatosis, the feet for cavovarus deformity, abnormal reflexes and muscle tone spacity. Generally there is no medical treatment for scoliosis because of its complexity. Patient who suffers from scoliosis usually will have a physical therapy, using a brace to support the body

and surgery to increase their self care activities and to prevent the scoliosis to become worsen.

Figure 1.4   The figure shows the SMA patient with hypotonia. (Adapted from http://www.nlm.nih.gov/medlineplus/ency/images/ency/fullsize/17229.jpg)
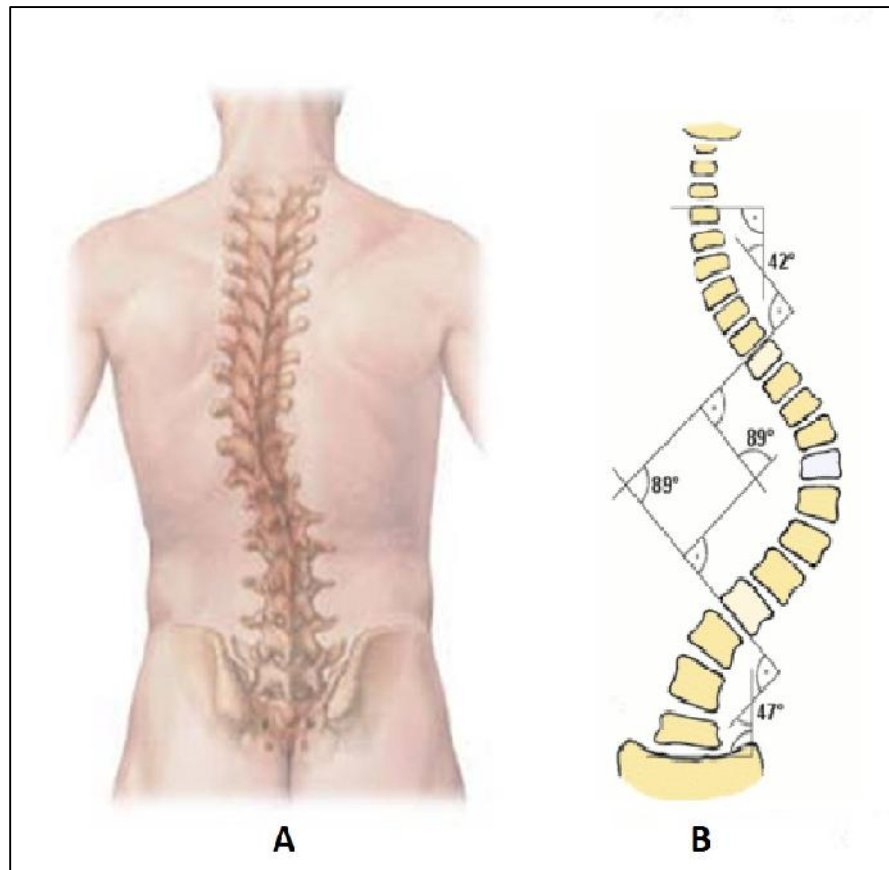
Figure 1.5   This figures show SMA patient spine who suffered from scoliosis. Figure **A** shows the actual patient spine (Adapted from http://www.nlm.nih.gov/medlineplus/ency/imagepages/1114.htm) and Figure **B** shows the cob angle for the measurement of levoscoliosis (Adapted from http://en.wikipedia.org/wiki/File:Scoliosis_cobb.gif)

1.3.1(c) Tongue Fasciculation

Tongue fasciculation is an abnormal pattern of non rhythmical, unorganized, contraction of individual muscle fibres across the surface of the tongue. The involuntary contraction of the muscle fibres are generally innervated by a motor unit. The fasciculation can often be visualised and take the form of a muscle twitch or dumpling under the skin, but usually do not generate sufficient force to move a limb. The tongue fasciculation may represent a condition such as in SMA.

1.3.2   Muscle Biopsy

A muscle biopsy is a procedure to remove a piece of muscle tissue from the respective target muscle. The procedure of muscle biopsy is involving a small surgical incision using a biopsy needle (figure 1.6). A muscle biopsy is generally used in the determination of a muscle and nerve problem such as nerve system, connective tissue, vascular system or musculoskeletal system (Araújo *et al.,* 2005; Wirth *et al.,* 2006). In SMA patient, a muscle biopsy can help the clinician to distinguish the myopathies (pathology is in the muscle tissue) and neuropathies (pathology is at the nerves innervating the muscle). In some cases, muscle biopsy is unnecessary but in other cases, it can help to clarify the muscle type and get a clear picture of which type of muscle dominates their body (Pons *et al.,* 1996).

Figure 1.6 The figure shows the procedure for muscle biopsy (Adapted from http://www.hes.cahs.colostate.edu.html)

1.3.3   Electromyography (EMG)

Electromyography (EMG) is a technique to evaluate and record the electrical activity produced by the skeletal muscle. EMG is performed by an electromyograph and the signal which is produced is called an electromyogram (figure 1.7). Electromyograph will detect the electrical potential that is generated by muscle cells when these cells are electrically or neurogically activated (Reed, 2002).

The muscle abnormalities, activation level, recruitment order and biomechanics of human movement can be analyzed by EMG. Normal muscle tissue is electrically inactive while in the resting condition while in the neurophatic disease, the action potential amplitude and duration is twice the normal because of the increased number of fibres per motor unit because of the innervations of denervated fibres (Araújo *et al.,* 2005; Wirth *et al.,* 2006). In contrast, the myophatic disease shows the action potential amplitude and duration is decreased than the normal level.

1.3.4   Nerve Conduction Study (NCS)

Nerve Conduction Study (NCS) is the procedure to evaluate the function and the ability of electrical conduction of the normal and sensory nerve of the human body (figure 1.8). In NCS test, the Nerve Conduction Velocity (NCV) measurement was made to set the actual mean of the test but in certain cases, this measurement may be misleading because it is the only one measurement in the test suite.

The NCS consist of four components which are motor NCS, sensory NCS, F-wave study and H-reflex study. All of these components were done to stimulate and record the wave and reflex of the motor nerves and muscle potential. The interpretation of NCS is complex, but generally the abnormality in motor nerves is indicated by the slowing of the NCV wave (Araújo *et al.,* 2005; Wirth *et al.,* 2006). This test is not invasive, but can be painful due to the electrical shock associated with the low electrical current.
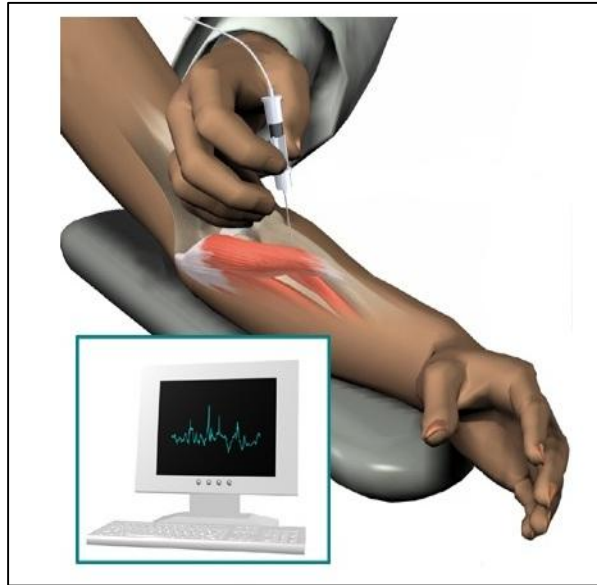
Figure 1.7   The figure shows the example of the procedures for Electromyography (EMG) (Adapted from http://cdn.nursingcrib.com/wp-content/uploads/emg.jpg)
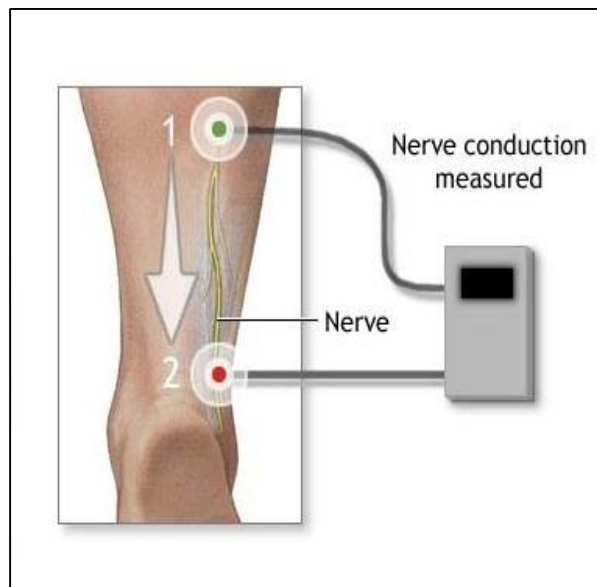
Figure 1.8   The figure shows the example of the procedures for Nerve Conduction Study (NCS) (Adapted from http://www.drbezner.com/ncs.jpg)

1.4 **Molecular Analysis of SMA**

1.4.1 **Survival of motor neuron gene (*SMN*)**

Almost sixteen years back, the inverted duplication of a 500kb element in a long arm of a normal chromosome 5 (5q13) have been discovered by Lefebvre *et al.* (1995) which contains the gene for type I SMA patient (figure 1.9). They found a 20kb gene which encode 294 amino acid protein within the telomeric portion that known as survival of motor neuron gene (*SMN* gene). This gene is also known as telomeric *SMN* gene (*SMNT*) (Lefebvre *et al.,* 1995). *SMN* gene is a component of a large highly stable protein complex that can be found in both cytoplasmic and nuclear compartments (Liu *et. al.,* 1997; Lefebvre *et. al.,* 1997; Meister *et. al.,* 2000). The human *SMN* gene was reported to have 8 exons (Lefebvre *et al.,* 1995). However, Burglen *et al.* (1996) have characterized the *SMN* gene and showed that it has 9 exons because exon 2 is devided into two exons; exon 2a and 2b. The *SMN* gene stop codon was found in the exon 7 while the exon 8 is not translated.

The *SMN* protein is found throughout the body but in higher level in the spinal cord (Coovert *et al.,* 1997). The function of *SMN* protein in the spinal cord is in the maintenance of the nerve cells that is called motor neurons. Healthy motor neurons are critical because they will control the movement of the muscle. In cells, the *SMN* protein is found in a sub nuclear form known as Gemini of Cajal bodies (Gems) because of its similarities in number and size with Cajal bodies (CBs) (Liu and Dreyfuss, 1996). The Gems also have the same response and metabolic condition as CBs. However, the

presence of small nuclear ribonucleoprotein (snRNPs) in CBs has made it different from Gems. The snRNPs is a complex of snRNP which is consisted of U1, U2, U4/U6 and U5 and it plays an important role in assembling the cellular machinery in the processing molecules which is called pre-messenger RNA (pre-mRNA) (Liu and Dreyfuss, 1996). The full length of *SMN* protein is needed for the interaction of this protein with other protein to form a large complex of multiprotein.

The *SMN* protein, also has an additional function in nerve cells that is important for dendrites and axons which are important for the transmission of impulses from nerve to nerve and from nerves to muscles (Fan and Simard, 2002).

In the fetus period, the *SMN* gene is normally inactive and allows normal apoptosis in the developing fetus (Soler-Botija *et al.,* 2002). This gene becomes active in the healthy mature fetus to stabilize the neuronal population. In its absence, programmed cell death persists. The mechanism and timing of abnormal motor neuron death remain unknown (Brzustowicz *et al.,* 1990; Pern *et al.,* 1978).
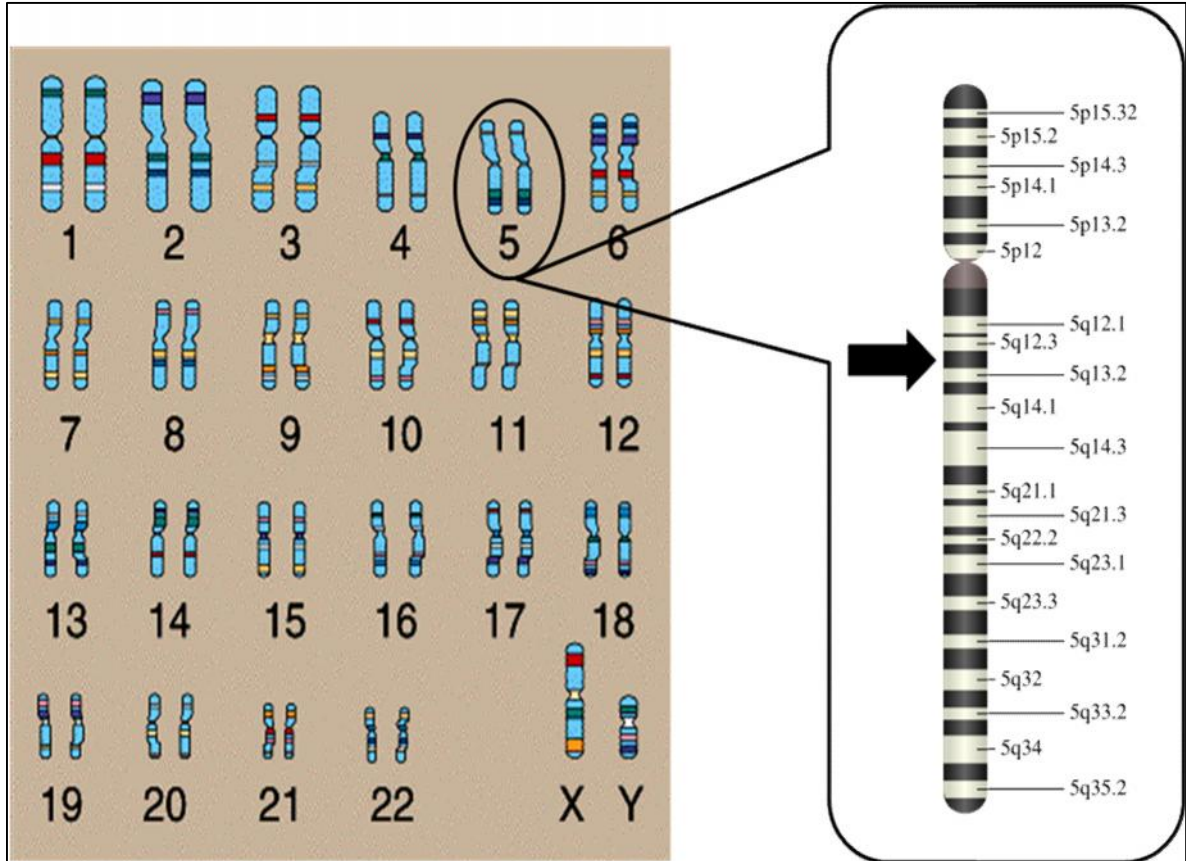
Figure 1.9 This figure shows the location of the human chromosome 5 and the location of the *SMN* gene in the chromosome. (Adapted from http://www.ensembl.co.uk/)

1.4.2   The *SMN1* and *SMN2* gene

A study by Coovert *et al.* (1997), found that the *SMN* protein is expressed from both *SMN1* and *SMN2* genes. However, the exon 7 *SMN1* gene is not detectable in 95% of SMA cases due either deletion or conversion of the sequences (Lefebvre *et. al.,* 1995). The other 5% of non deleted SMA patients is believed to have intragenic mutations; missense mutation, frameshift mutation, nonsense mutation, Alu-mediated deletion and donor splice site mutation (Wirth *et. al.,* 1999). The study by Coovert *et al.* (1997) also demonstrated that the *SMN2* gene was found in more than 95% of the controls at the duplicated centromeric region of the chromosome 5 (Coovert *et al.,* 1997), hence there is no report about the patients who absent both of these genes.

Both of these genes share over 99.8% of the similar nucleotide and are different in only five nucleotides. *SMN1* and *SMN2* can be distinguished by only 5 nucleotide differences located in one in intron 6 (G>A), one in exon 7 (C>T), two in intron 7 (A>G; A>G) and one in exon 8 (G>A)(Figure 1.10).

SMN1 and *SMN2* expressed the same peptide sequence for the *SMN* gene. However, because of one nucleotide difference in exon 7 (C>T) between *SMN1* and *SMN2* gene, the alteration of one nucleotide in exon 7 *SMN2* gene will cause the splicing of exon 7 during the transcriptional process, and later produce a truncated *SMN2* protein (Lorson *et al.,* 1999). This truncated *SMN2* gene is not stable *in vivo* and *in vitro* and it also could not self-oligomerized (Hofmann *et al.,* 2000).

Rochette *et al.* (2001) reported that *SMN2* is unique in man since the chimpanzee has only multiple copy of *SMN1* gene. The *SMN1* gene is not present in most of SMA patients; however they retain at least one copy of *SMN2*. (Figure 1.10)

However, in 10% of the cases, the *SMN2* may also produce full lengh *SMN* protein (Lefebvre *et al.,* 1995). The severity of the disease depends on the efficiency of splicing of this *SMN2* in order to produce the full lengh *SMN* protein. The low level of *SMN* could still allow the development of the embryo but the survival of motor neuron sufficiency was still abnormal.
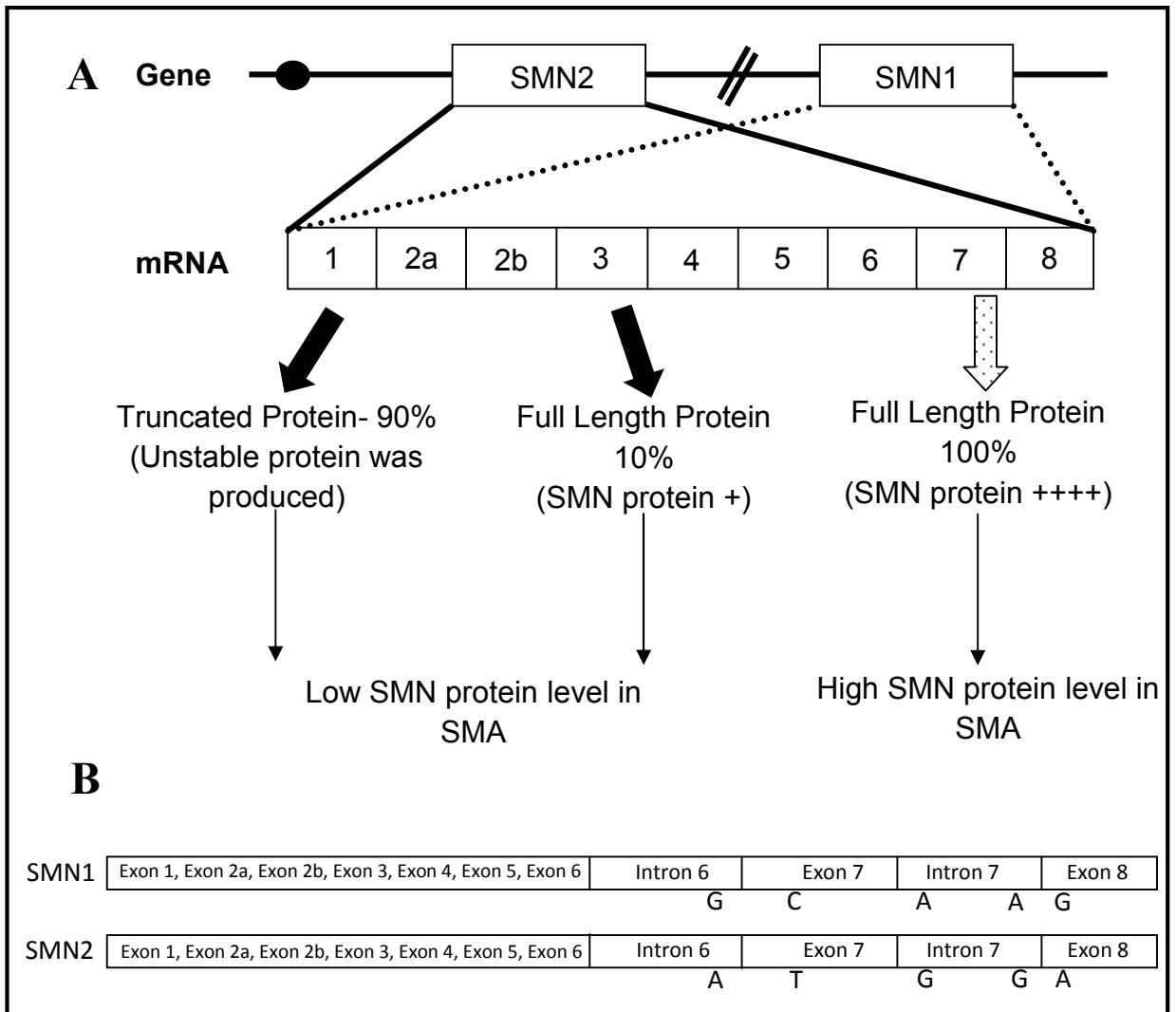
Figure 1.10   This diagram shows **(A)** the inverted duplication of *SMN1* and *SMN2* gene and **(B)** the nucleotide differences between both genes.