DEMOGRAPHIC AND MOLECULAR GENETIC STUDIES OF NEURAL TUBE DEFECTS IN A COHORT OF MALAYSIAN PATIENTS

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ABSTRACT

Neural tube defects (NTDs) are among the most common birth defect which occur at a range of 0.5 – 10 or more in 1000 live births worldwide. According to our data from the Department of Patient Information University of Malaya Medical Centre (UMMC) between the years 2003 until 2016, there were 86 cases with spina bifida (1.33-6.4 per 1000 live births), 19 cases of anencephaly (0.38 per 1000 live births) and no report of craniorachischisis. Ethnicity of the patients was a factor of the incident whereby the highest numbers were Malays for both spina bifida and anencephaly. Most of the patients belonged to in mothers below 35 years of age. Additionally, males dominate the occurrence of spina bifida meanwhile almost equal number of male and female for anencephaly. For spina bifida cases, the highest number of diagnoses reported was lumbar myelomeningocele. Furthermore, 32.84% of patients were found to be mobile and 36.07% of patients received formal education. To date, the most studied human spina bifida risk variant is the *MTHFR* C677T (rs1801133). However, this variant was not well replicated in many populations across the world, indicating that the variant is not likely to be a major contributor of NTDs globally. Therefore, the candidate genes for screening NTDs worldwide has remained elusive. We screened for the potential genetic cause of spina bifida in 40 reported spina bifida risk genes in our patient cohort using Whole Exome Sequencing (WES) datasets. We managed to identify 10 non-synonymous variants in *MTHFR, ALDH1L1, MTRR, SARDH, XPD, CUBN* and *BRCA1* genes with potential pathogenic effects. The identified variants might be the risk factor for spina bifida in patients individually, but do not represent as common variants within the cohort. We also screened the potential spina bifida candidate genes using Whole-Exome Sequencing (WES) in three representative patients (triad study); (1) syndromic spina bifida aperta, (2) non-syndromic spina bifida aperta and (3) non-syndromic spina bifida occulta. Although our patient cohort is small, the power of the study is enhanced as it is

a triad study involving the proband and his/her parents. Case (1) involves a patient born with spina bifida aperta (myelomeningocele-type) with variant Turner syndrome. No likely candidate variant was identified for Patient 1. Case (2) is a non-syndromic spina bifida aperta. WES revealed six candidate variants but only *SEC63* (rs17854547) variant was chosen as the potential candidate variant by taking into account the literature surrounding the SEC family of genes and NTDs. The variant is considered uncommon but was predicted to be non-deleterious when subjected to the bioinformatics analysis. Case (3) is a patient born with spina bifida occulta (lipomyelomeningocele-type). WES identified a novel and deleterious variant in the *ZIC2* gene as the candidate variant for Patient 3. In summary, all variants found on their own might not be the spina bifida genetic risk factor. The act of these variants in combination with other events might be the possible causative factor(s). In the future, screening of these variants in large scale genome-wide association study is needed to confirm the variants as common genetic risk factors for spina bifida.

ABSTRAK

Kecacatan neural tube (Neural Tube Defects, NTDs) adalah kecacatan kelahiran yang tinggi berlaku di antara 0.5-10 atau lebih dalam setiap 1000 kelahiran hidup di seluruh dunia. Merujuk data yang kami ambil dari Jabatan Maklumat Pesakit Pusat Perubatan Universiti Malaya (UMMC) dari tahun 2003 hingga 2016, terdapat sejumlah 86 kes spina bifida (1.33-6.4 bagi setiap 1000 kelahiran), 19 kes anencephaly (0.38 dalam 1000 kelahiran) dan tiada laporan bagi kes craniorachischisis. Kaum melayu mencatatkan jumlah tertinggi bagi kes spina bifida dan anencephaly. Umur ibu yang terlibat pula kebanyakannya dibawah umur 35 tahun bagi kedua-dua jenis NTD. Tambahan pula, kaum lelaki mendominasi kes spina bifida. Jumlah pesakit lelaki dan perempuan adalah hampir sama bagi kes anencephaly. Bagi kes spina bifida, diagnosis tertinggi yang direkodkan adalah pada ruas tulang pinggang (lumbar) jenis myelomeningocele. Selain itu, 32.84% pesakit adalah mampu untuk bergerak dan 36.07% pesakit sedang menerima pendidikan formal. Sehingga hari ini, kelainan spina bifida yang paling banyak dikaji adalah kelainan MTHFR C677T (rs1801133). Walau bagaimanapun, kelainan ini tidak direplikasi secukupnya dalam kebanyakan populasi bermaksud kelainan ini bukanlah penyebab terbesar NTDs di seluruh dunia. Justeru, pemeriksaan calon gen NTDs masih sukar untuk dilakukan. Oleh itu, kami telah memeriksa sebanyak 40 gen yang telah direkodkan sebagai gene berisiko spina bifida dalam kohort pesakit kami. Kami menjumpai 10 kelainan-kelainan non-synonymous yang mungkin mempunyai kesan patogenik dalam gen *MTHFR, ALDH1L1, MTRR, SARDH, XPD, CUBN* dan *BRCA1*. Kelainan-kelainan yang ditemui mungkin faktor berisiko bagi pesakit spina bifida secara individu tetapi tidak bukanlah kelainan biasa yang ditemui dalam kohort pesakit kami. Kami juga memeriksa calon kelainan spina bifida yang berpotensi menggunakan analisa Jujukan Exom Menyeluruh (Whole Exome Sequencing (WES)) dalam tiga wakil pesakit;

(1) spina bifida aperta bersindrom (syndromic spina bifida aperta), (2) spina bifida aperta tidak bersindrom (non-syndromic spina bifida aperta), (3) spina bifida occulta tidak bersindrom (non-syndromic spina bifida occulta). Walaupun kohort kami kecil, kekuatan kajian ini adalah pada kajian triad yang melibatkan pesakit dan kedua-dua ibu bapa mereka. Kes (1) melibatkan seorang pesakit spina bifida aperta (jenis myelomeningocele) yang juga memiliki kelainan sindrom Turner (variant Turner syndrome). Walau bagaimanapun, tiada kemungkinan calon kelainan yang ditemui bagi pesakit kes 1. Kes (2) adalah spina bifida aperta (jenis myelomeningocele) yang tidak bersindrom. Analisa WES mendapati 11 kelainan dalam pelbagai gen akan tetapi hanya gen *SEC63* (rs17854547) yang dianalisa merujuk kepada sejarah keluarga gen ini dengan NTDs. Kelainan ini jarang ditemui dan dijangka tidak membawa kesan patogenik berdasarkan analisa bioinformatik. Kes (3) pula melibatkan pesakit yang lahir dengan spina bifida occulta (jenis lipomyelomeningocele). Kajian analisa WES menemui kelainan pada gen *ZIC2* yang sangat jarang ditemui dan dijangka akan membawa kesan deleterious melalui analisa bioinformatik. Secara kesimpulannya, penemuan ini mencadangkan bahawa kesemua kelainan ini semata-mata bukanlah punca utama spina bifida. Gabungan kelainan-kelainan ini dengan faktor kelainan yang lain mungkin adalah faktornya. Saringan kelainan-kelainan ini dalam genome-wide association study (GWAS) yang berskala besar amat diperlukan bagi menentukan kelainan-kelainan ini sebagai risiko spina bifida.

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols:

µL	Micro litre
µm	Micro metre
µm	Micro mole
ml	Mili litre
nm	Nano meter
kg	Kilo gram
%	Percentage
°C	Degree Celsius

A:

A	Adenine
ABSL2	Animal Biosafety Level 2
ADPLD	Autosomal dominant polycystic liver disease
AKNA	**AT-Hook Transcription Factor gene**
Ala	Alanine
ALDH1L1	Aldehyde Dehydrogenase 1 Family Member L1 gene
ALDH1A2	Aldehyde Dehydrogenase 1 Family Member A2 gene
ANP	Anterior neuropore
Arg	Arginine

B:

BHMT	Betaine-homocysteine S-methyltransferase gene
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BRCA1	Breast cancer type 1 gene
BRCA2	Breast cancer type 2 gene
BWA	Burrows-Wheeler Aligner

C:

C	Cytosine
CBS	Cystathionine beta-synthase
CDC	Center for Disease Control
CELSR1	Cadherin EGF seven-pass G-type receptor 1 gene
CFL1	Cofilin 1 gene
CHKA	Choline Kinase Alpha gene
Chr	Chromosome
CIC	Clean intermittent catheterization
CNS	Central nervous system
CRL	Crown rump length
CSF	Cerebro-spinal fluid
CSMD1	CUB and Sushi Multiple Domains 1 gene
CTEV	Congenital Talipes Equinovarus
CUBN	Cubilin gene

D:

dbSNP	Single Nucleotide Polymorphism database
DHFR	Dihydrofolate reductase gene
DLHP	Dorsolateral hinge points
DMEM	Dulbecco's modified Eagle's medium

DNA	Deoxyribonucleic acid
DVL1	Dishevelled-1 gene
DVL2	Dishevelled-2 gene
DYNC1H1	Dynein Cytoplasmic 1 Heavy Chain 1 gene

E:

E	Embryonic day
ERCC2	Excision Repair Cross-Complementing Rodent Repair Deficiency2
ESP	Exome Sequencing Project
ESV	Exome Variant Server
et al.	*et alia;* and others
EUROCAT	European Surveillance of Congenital Anomalies
ExAC	Exome Aggregation Consortium

G:

G	Guanine
Gln	Glutamine
Glu	Glutamic acid
GLUT1	Glucose transporter 1 gene
Gly	Glycine
GRCh37	Genome Reference Consortium Human Build 37
GWAS	Genome-wide association study

H:

His	Histidine
hg19	Human Genome version 19
HK1	Hexokinase 1 gene
HNP	Hindbrain neuropore
HOXA10	Homeobox A10 gene
HSPA6	Heat Shock Protein Family A (Hsp70) Member 6 gene

I:

ICD10	International Statistical Classification of Diseases and Related Health Problems 10th Revision
InDel	Insertions or deletions
Ile	Isoleucine

L:

L3	Lumbar 3
LEP	Leptin gene
LEPR	Leptin receptor gene
LMNB1	Lamin-B1 gene
LSCS	Lower segment Caesarean section
Lys	Lysine

M:

MAF	Minor allele frequency
MEC	Medical Ethics Committee
Met	Methionine
MHP	Median hinge point
MRC	Medical Research Council
MRI	Magnetic Resonance Imaging

MTCH2	Mitochondrial Carrier 2 gene
MTHFD1	Methylenetetrahydrofolate dehydrogenease 1 gene
MTHFR	Methylenetetrahydrofolate reductase gene
MTRR	Methionine synthase reductase gene

N:

MRC	Medical Research Council
MRI	Magnetic Resonance Imaging
MTCH2	Mitochondrial Carrier 2 gene
MTHFD1	Methylenetetrahydrofolate dehydrogenease 1 gene
MTHFR	Methylenetetrahydrofolate reductase gene
MTRR	Methionine synthase reductase gene

P:

PAX3	Paired box gene 3
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PBS	phosphate buffered serum
PCYT1A	Phosphate cytidylytransferase 1, choline, alpha gene
PDGFRA	Platelet derived growth factor receptor alpha gene
PNP	Posterior neuropore
POAF	Point of adhesion and fusion
Pro	Proline
PT-L2P	PrepIT DNA extraction kit

R:

RNA	Ribonucleic acid
RORA	RAR Related Orphan Receptor A gene
rs	Reported SNP

S:

S5	Sacral 5
SARDH	Sarcosine dehydrogenase gene
SB1A	Proband with spina bifida number 1
SB2A	Proband with spina bifida number 2
SB3A	Proband with spina bifida number 3
SB4A	Proband with spina bifida number 4
SB5A	Proband with spina bifida number 5
SB7A	Proband with spina bifida number 7
SB13A	Proband with spina bifida number 13
SB17A	Proband with spina bifida number 17
SB25A	Proband with spina bifida number 25
SB27A	Proband with spina bifida number 27
SB31A	Proband with spina bifida number 31
SBRR	The Spina Bifida Research Resource
SCRIB	Scribbled planar cell polarity gene
Se	Surface ectoderm
Sec23a	S. Cerevisiae 23 Homolog a gene
Sec24b	S. Cerevisiae 24 Homolog b gene
SEC63	S. Cerevisiae 63 gene
Ser	Serine
SGVP	Singapore Genome Variant Project

SIFT	Sorting Tolerant From Intolerant
SLCA19A1	Solute carrier family 19 member 1 gene
SMART	Simple Modular Architecture Research Tool
SNV	Single nucleotide variants
SOD1	Superoxide dismutase 1, soluble gene
SOD2	Superoxide dismutase 2, mitochondrial gene
SOX18	SRY-Box 18
SPSS	Statistical Program for the Social Sciences
STARD9	START Domain-Containing Protein 9 gene
SVD	Spontaneous vaginal delivery
SYMPK	Symplekin gene

T:

T	Thymine
T (Brachyury)	*T* gene that encode for Brachyury protein
TAE	Tris-Acetate- EDTA
TAS2R19	Taste 2 Receptor Member 19 gene
TOP	Termination of pregnancy
TRDMT1	TRNA Aspartic acid (D) methyltransferase 1 gene
TXN2	Thioredoxin 2 gene
TYMS	Thymidylate Synthetase
Tyr	Tyrosine

U:

UCSC	University of California, Santa Cruz (Genome Browser)
UK	United Kingdom
UMMC	University of Malaya Medical Centre
USA	United States of America
USP49	Ubiquitin Specific Peptidase 49 gene
UV	Ultra violet

V:

VANGL1	Vang-like 1 gene
VANGL2	Vang-like 2 gene
Val	Valine
VP	Ventriculo-peritoneal

W:

WES	Whole exome sequencing

xg	*Times gravity*
XIAP	X-Linked Inhibitor of Apoptosis
XPD	Xeroderma pigmentosum group D

Z:

ZIC2	Zic family member 2 gene
ZIC3	Zic family member 3 gene

CHAPTER 1: INTRODUCTION

The neural tube, the precursor of the brain and the spinal cord is generated by a process of structural changes called neurulation that is conventionally divided into two phases; primary and secondary neurulation (Copp *et al.*, 2003; Greene & Copp, 2014). In mouse embryos, the events of primary neurulation include (1) neuroepithelium thickening and convergence, (2) elevation of the neural folds bilaterally, (3) bending at the dorsolateral hinge points (DLHP), (4) adhesion and fusion of the neural folds, and (5) remodeling of the neural tube (Copp *et al.*, 2003; Greene & Copp, 2014). This process creates the brain and most of the spinal cord (Copp *et al.*, 2003). Secondary neurulation includes canalization whereby the formation of condensed rod of mesenchymal cells takes place and later forms a canal structure of the neural tube in the caudal eminence (Copp *et al.*, 2003).

Incomplete closure at any part of the neural tube leads to neural tube defects (NTDs). NTDs are a group of common and severe congenital defects of the central nervous system that comprise of craniorachischisis, anencephaly and spina bifida (Copp & Greene 2010). The clinical phenotypes of NTDs depend on the points of embryonic neural tube closure (Greene & Copp, 2009). The most severe phenotype is craniorachischisis where the defect includes most of the brain and the entire spinal cord followed by anencephaly of which is the resulting phenotype of a degenerative and exposed brain (Copp *et al.*, 2003; Greene & Copp, 2014). Both craniorachischisis and anencephaly are incompatible with postnatal life. Spina bifida is the most common and less severe form of NTD with a higher chance of survival (Greene & Copp, 2009; Copp & Greene, 2013).

The epidemiology of NTDs vary across worldwide geographical location. The highest prevalence is in the Luliang Prefecture, Shanxi province in China with prevalence rate of 19.94 per 1000 births (Chen *et al.*, 2009). In USA, the NTDs prevalence rate was reported to be 0.53 per 1000 births (Canfield *et al.*, 2014) while a higher prevalence was reported in the United Kingdom (Dolk *et al.*, 2010). In general, NTDs occur in 0.5 to more than 10 cases per 1000 births worldwide (Copp *et al.*, 2015).

In Malaysia, a study of a small population in the Kinta district reported an incidence of NTDs at 0.73 per 1000 births but does not specify the NTD phenotypes (Thong *et al.*, 2005). A prospective cohort study of neonates with NTDs using the data from the Malaysian National Neonatal Registry revealed the overall NTDs prevalence at 0.42 per 1000 live births where anencephaly dominate the occurrence (0.19 per 1000 births) followed by spina bifida which occur at 0.11 per 1000 births (Boo *et al.*, 2013). However, the study has included the prevalence rate of encephalocele although it is not a NTD phenotype hence suggesting a potentially lower preponderance. Encephalocele is the result of a post-neurulation defect of the cranial mesoderm and thus does not arise from the same presumptive tissue as the brain and the spinal cord (Greene & Copp, 2009, 2014).

This thesis aims to determine the distribution and genetics of NTDs in Malaysia by focusing on a subset of the Malaysian NTD cohort; in this case, the NTD patients of University of Malaya Medical Centre (UMMC). This study determines the latest prevalence rate and distribution of NTDs including anencephaly and spina bifida cases in UMMC, a single major referral hospital in the capital of Malaysia, Kuala Lumpur from 2003 to 2016.

Over the past 20 years, most studies involved the identification of candidate genes in the cohort of NTDs patients. This study primarily focused on one of the gene (methylenetetrahydrofolate reductase (*MTHFR))* that participate in folate one carbon metabolism as lack of folic acid been considered as the main NTD causing factor (Boyles *et al.*, 2005; Copp & Greene, 2010). The most studied genetic variant of *MTHFR* is the C677T (rs1801133) variant (Amorim *et al.*, 2007; Yaliwal & Desai, 2012; Yang *et al.*, 2015). The C677T variant has been reported to cause increased NTD risk in the Irish and Dutch populations but not well replicated in many other populations across the world, such as Mexicans and Italians (van der Put *et al.*, 1995; Shields *et al.*, 1999; Gonzalez-Herrera *et al.*, 2002; Grandone *et al.*, 2006). In addition, the planar cell polarity genes (PCP genes) including *CELSR1, VANGL1, VANGL2, DVL2,* and *SCRIB* were also identified as genetic risk candidates for NTDs (Doudney & Stanier, 2005; Kibar *et al.*, 2009; Lei *et al.*, 2013; De Marco *et al.*, 2014; Lei *et al.*, 2014). These genes involved in the PCP pathway control the polarization of the epithelial cells and play an important role during neurulation (Mlodzik, 2002; De Marco *et al.*, 2014). Despite the many and varied genetic studies, a candidate or candidates for screening neural tube defects worldwide has remained elusive.

In addition, this study involved the screening of spina bifida genes in three spina bifida patients representing (1) syndromic spina bifida aperta, (2) non-syndromic spina bifida aperta and (3) nonsyndromic spina bifida occulta. The selection of distinct clinical phenotype particularly between syndromic and non-syndromic spina bifida is extremely important because syndromic spina bifida has a more well-defined genetic causative components compared to non-syndromic spina bifida. The causative factors of non-syndromic spina bifida are heterogeneous and appears to be caused by both genetic and environmental factors including nutritional status, drug intake, maternal health status, and

presence of environmental toxicants (Greene & Copp, 2014). Although our patient cohort is small, the power of the study is enhanced as it is a triad study involving the proband and his/her parents.

We opted for whole-exome sequencing (WES) technology to find candidate variants in the patient-parents trios (triads). The use of WES is relevant and of value when triads are utilised for the understanding of NTDs (Krupp *et al.*, 2014). The use of WES in recent studies contributed to the identification of common and rare variants as well as *de novo* variants associated to a wider range of diseases (Veltman & Brunner, 2012). Exome sequencing identified DNA variants in genes associated with several disorders including primary open-angle glaucoma (Zhou *et al.*, 2017), cutaneous neurofibromas (Faden *et al.*, 2017) and breast cancer (Kim *et al.*, 2017).

1.1 Objectives

1.1.1 To study the prevalence and distribution of NTDs in the University of Malaya Medical Centre (UMMC) from 2003 until 2016.

1.1.2 To screen for mutations in known spina bifida risk genes.

1.1.3 To identify candidate variants in genes by whole exome sequencing in the patients and correlate any findings with the clinical phenotype.

1.1.4 To further understand the potential role of *Sec63* in NTD using antisense oligonucleotide technology.

CHAPTER 2: LITERATURE REVIEW

2.1 Neural development and neural tube closure

The development of the primordium of the central nervous system which is the neural tube occurs through an event called neurulation. The neural tube is the precursor of brain and spinal cord (Copp *et al.*, 1990). The neurulation process is divided into two phases; the primary neurulation and secondary neurulation (Copp *et al.*, 2003). Primary neurulation involves a series of structural changes (Figure 2.1) which is defined originally in amphibian and avian embryos. First is the convergent and extension process whereby the polarized cells which form the neural plate move medially and intercalate in the midline (Copp *et al.*, 2003) (Figure 2.1A). This intercalation process has been detected in the frog and has yet been experimentally shown to occur in the mouse (Shih & Keller, 1992; Wallingford & Harland, 2001). Convergent extension leads the neural plate to narrow mediolaterally and lengthen rostrocaudally which subsequently assist neural fold elevation through axial elongation (Kibar *et al.*, 2001). However, studies in amphibian embryos suggest elevation of neural folds are not actually assisted by the axial elongation of the neural plate (Wallingford *et al.*, 2002). The normal process of neural folds elevation still occurs despite of the failure of convergent extension hence suggesting the convergent extension and neural fold elevation are two distinct processes (Wallingford *et al.*, 2002; Zohn *et al.*, 2003).

The second event of primary neurulation is the formation of bilateral neural folds through bending at the hinge points. In the mouse, at high levels of the spinal neuraxis, Mode 1 neurulation occurs during spinal neural tube formation in 6 to 10 somite stage embryos whereby the lateral aspects of the neural folds elevate results from the formation of the median hinge point (MHP) (Figure 2.1B). Here, the neural folds remain straight

forming a slit-like lumen of the neural tube (Shum & Copp, 1996).

In Mode 2 neurulation, dorsolateral hinge points (DHLPs) form at more caudal levels of the spinal neuraxis (Figure 2.1C). The formation of DHLPs enhance the convergence of the apposing tips of the neural folds towards the dorsal midline (Shum & Copp, 1996). In this mode, MHP is also present whereas the remaining parts of the neuroepithelium do not bend. Mode 2 generates a diamond-shaped lumen which takes place during 12-15 somite stage embryos. Later at 17-27 somite stage, Mode 3 occurs whereby all parts of the neural plate appear to bend causing the MHP to not be easily discernible whereas the DLHPs are retained (Figure 2.1D). Mode 3 produces an almost circular lumen (Copp *et al.*, 1990; Ybot-Gonzalez & Copp, 1999).

The final event of primary neurulation is the adhesion and fusion of the apposing tips of the neural folds in the dorsal midline (Figure 2.1E) (Copp *et al.*, 2003). Prior to the fusion, cellular specialisations comprising of cell surface protrusions and lamellipodia projected are found at the apposing tips of the neural folds and the point of adhesion (Copp *et al.*, 1990). Then, the cell surface projections interdigitate leading to eventual adhesion and then fusing together to form a neural tube. Subsequently, the newly formed neural tube remodels via apoptosis to create two continuous epithelial layers; the surface ectoderm and the neural tube (Figure 2.1F) (Yamaguchi *et al.*, 2011). Primary neurulation generates the brain and most of the spinal cord (Copp *et al.*, 2003).

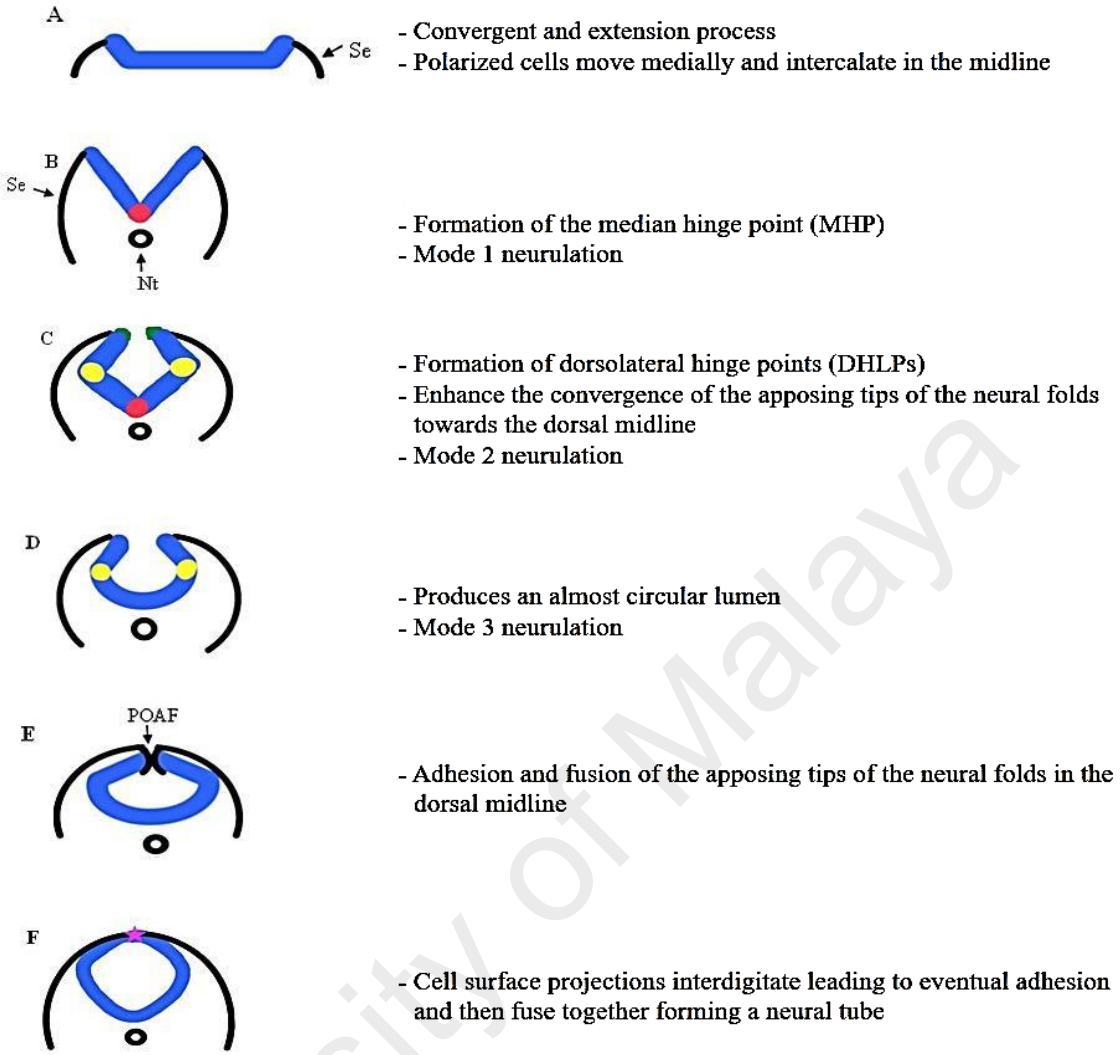


Figure 2.1: Diagrammatic transverse section of the mouse spinal neural tube (Modified from Mohd-Zin *et al.* (2017)) (A) Thickening and converging of neuroepithelium; (B) formation of bilateral neural folds which are elevated at median hinge point (MHP) representing Mode 1 neurulation; (C) apposing tips of neural folds assisted by bending at the dorsolateral hinge points (DLHP) of the bilateral neural folds representing Mode 2 neurulation; (D) Mode 3 neurulation which involved the formation of almost circular lumen; (E) adhesion and fusion of the apposing tips of neural folds; (F) remodeling of the neural tube; Se, surface ectoderm; POAF, point of adhesion and fusion; Nt: notochord.

Secondary neurulation occurs without the formation of neural folds (Copp *et al.*, 1990). It involves the condensation and epithelialization of mesenchymal cells in the tail bud forming a solid mass called the medullary cord. Eventually, the medullary cord undergoes canalisation to form the neural tube (Copp *et al.*, 1990). This process creates the lowest portion of spinal cord including sacrum and all of the coccygeal regions (Copp *et al.*, 2003).

The mammalian neural tube closure takes place sequentially at different levels of body axis. The sequence in mouse that occurs from embryonic day E8.5 until day E10.5 has been most extensively studied and divided into three points of *de novo* closure; Closure 1, 2, and 3 (Figure 2.2H) (Copp *et al.*, 1990; Copp *et al.*, 2003). Closure 1 is the initiation event of neurulation that occurs near the third somite in embryos with 6 to 7 somites and spreads bidirectionally towards the rostral and caudal extremities (Copp *et al.*, 2013). Spread of closure 1 along the spinal axis is completed by closure of the posterior neuropore (PNP) at the 29 to 30 somite stage of development marking the end of primary neurulation. Closure 2 takes place at 10-somite stage between midbrain-forebrain boundary and progress caudally while Closure 3 at the rostral extremity of the forebrain soon after closure 2 (Greene and Copp, 2009). Bidirectional spread of closure 1, 2 and 3 at the cranial region initiates the formation of anterior neuropore (ANP) and hindbrain neuropore (HNP) at 18 to 20 somite stage (Figure 2.2H).

Neural tube closure in human embryos occurs between days 16 to 32 of conception (Sadler & Langman, 2012). Neurulation events that occur in the human embryos are similar as closure 1 and 3 in mice. The brain formation in humans is achieved by rostral spreading of closure 1 and caudally from closure 3 which is completed by the ANP closure (Figure 2.2A). Meanwhile, the closure of PNP is achieved by caudal

spreading of closure 1 that indicates the completion of primary neurulation (Copp, 2005).

2.2 Neural tube defects (NTDs)

Failure of the morphogenetic process of neural tube closure leads to NTDs. NTDs are the most severe central nervous system disorder of the developing embryos and foetus and the second most common birth defect after the congenital heart defects (Detrait *et al.*, 2005). There is a wide range of NTD phenotypes whereby the severity and type of defects vary depending on the level of the affected site of closure (Greene & Copp, 2014; Mohd-Zin *et al.*, 2017). The phenotypes of NTDs include craniorachischisis, anencephaly and spina bifida. The explanation of each phenotype is in the next section. These phenotypes are shown pictorially in Figure 2.2.

2.2.1 Craniorachischisis

Craniorachischisis is the most severe form of NTD with very few reported cases. Craniorachischisis patients are not able to survive as almost the entire neural tube remains open (Copp *et al.*, 2003) (Figure 2.2 B & E), and is due to the failure at closure 1.

2.2.2 Anencephaly

Anencephaly is the outcome of failure of neural tube closure at the cranial region with the absence of forebrain, midbrain and skull vault (Seller, 1995; Greene & Copp, 2014). In the mouse embryo, this phenotype occurs at closure 2 between midbrain-forebrain boundaries. In human embryos, anencephaly occurs due to failure of the cranial closure of the ANP (Copp & Greene, 2010). Most of anencephalic cases are stillborn. Those who survive normally will die shortly after birth due to complications including sepsis, pneumonia or atelectasis (Jaquier *et al.*, 2006) (Figure 2.2 C & F).

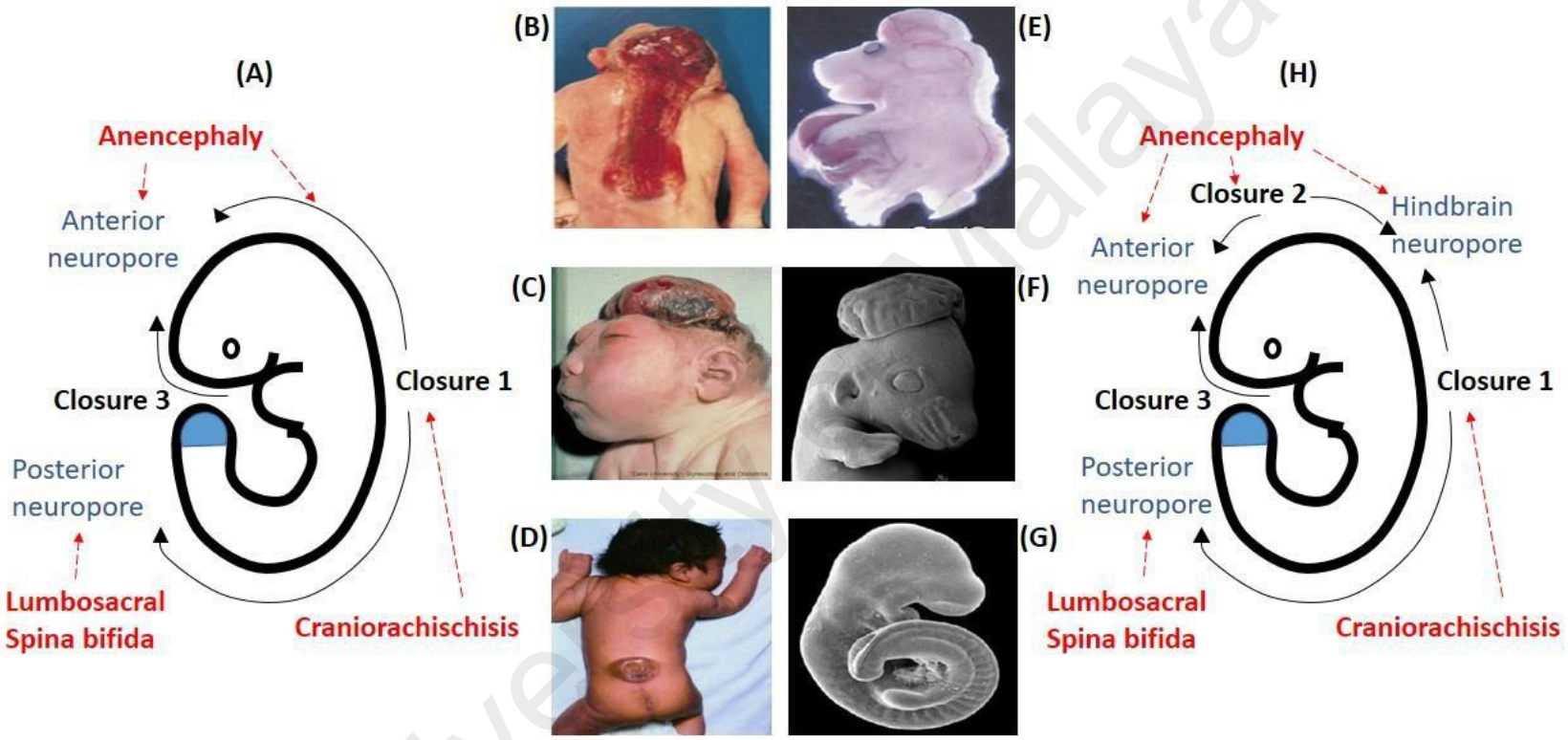


Figure 2.2: Schematic diagram of neural tube closure sites and phenotype of NTDs (Modified from Greene & Copp (2009); Copp *et al.* (2013); Sadler and Langman (2012)). (A) Pattern of human neural tube whereby failure at closure 1 results in craniorachischisis, anencephaly results in failure at the cranial closure of anterior neuropore, and spina bifida due to the failure at the caudal end of the posterior neuropore. (B) Craniorachischis observed in human; (C) anencephaly of the human newborn; (D) spina bifida in human infant; (E) appearance of craniorachischisis in mouse embryo; (F) exencephaly phenotype which is an early stage of anencephaly found in mouse embryo; (G) spina bifida observed in mouse embryo. (H) Pattern of neural tube closure in mouse whereby craniorachischisis due to failure at closure 1 as in human, anencephaly occurs at closure 2 between midbrain-forebrain boundary and spina bifida as the caudal closure.

2.2.3 Spina bifida

Spina bifida is also referred to as the caudal lesion of the spinal cord affecting the vertebrae and skin (Mitchell *et al.*, 2004) (Figure 2.2 D & G). In general, this defect can be classified into two categories; the closed spina bifida known as spina bifida occulta and the open type, spina bifida aperta. Spina bifida occulta is the bony defect at the back of the body covered by skin and is thought to be without neurological deficit (Copp *et al.*, 2015). As for spina bifida aperta, it occurs when there is failure of vertebral arches to form, the neural tube fails to close and often, neural tissues are exposed to the surrounding environment without skin covering (Botto *et al.*, 1999; Sadler & Langman, 2012). Nevertheless, the severity of spina bifida does not solely depend on the type of defect, but it is also governed by the level of lesion (Greene & Copp, 2014).

Spina bifida can be further divided into few more subphenotypes: myelomeningocele, meningocele, lipomyelomeningocele and lipomeningocele depending on the pathological findings (Figure 2.3). Myelomeningocele is a severe form of spina bifida aperta whereby the spinal cord protrudes out of the spinal canal and is exposed to its surrounding environment (Figure 2.3A) (Botto *et al.*, 1999; Sadler & Langman, 2012; Copp *et al.*, 2015; Mohd-Zin *et al.*, 2017). Meningocele is a less severe phenotype compared to myelomeningocele where the spinal cord is not present in the herniated sac (Mohd-Zin *et al.*, 2017). However, this phenotype is either aperta or occulta as the status is still under argument (Figure 2.3B) (Mohd-Zin *et al.*, 2017). Lipomeningocele differs from meningocele where it is considered as spina bifida occulta with the presence of lipid globules but the spinal cord remains in the spinal canal (Figure 2.3D) (Vogel *et al.*, 2003; Mohd-Zin *et al.*, 2017). Lipomyelomeningocele is also a form of spina bifida occulta with the presence of intermeshed lipid globules and the spinal cord (Figure 2.3C) (Sarris *et al.*, 2012; Mohd-Zin *et al.*, 2017).

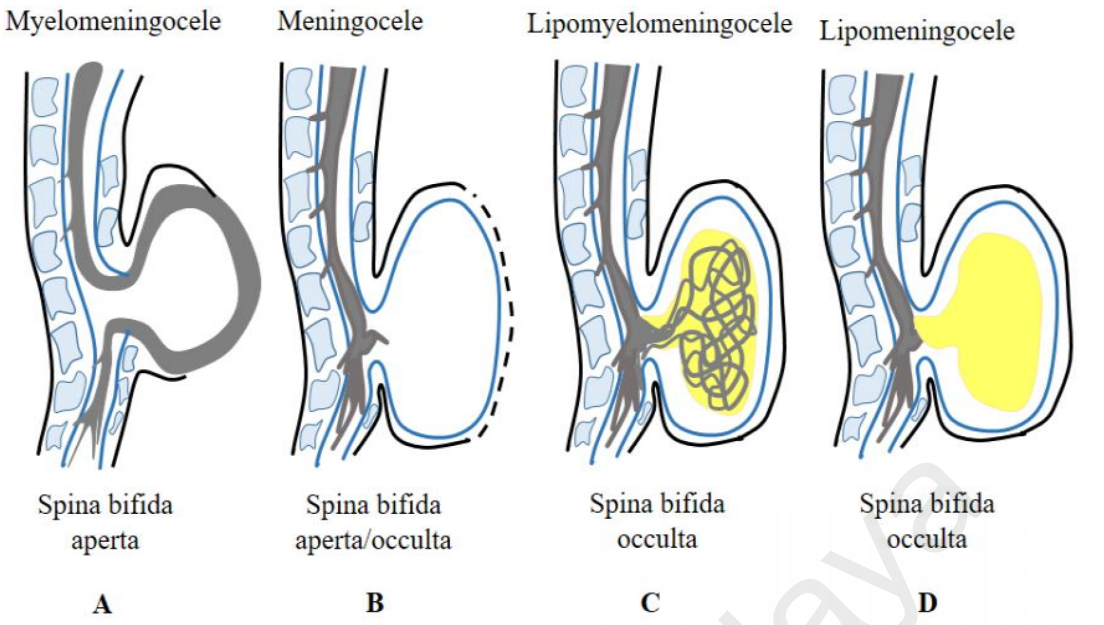


Figure 2.3: Schematic representation of different spina bifida subphenotypes (Sahmat *et al,* 2017). (A) Myelomeningocele is shown whereby the spinal cord lies outside the spinal canal. This phenotype represents the severe form of spina bifida aperta. (B) Meningocele is shown whereby the spinal cord does not lie outside the spinal canal. This phenotype represents spina bifida occulta or spina bifida aperta depending on the presence or absence of neural matter in its herniated sac. (C) Lipomyelomeningocele that is the spina bifida occulta type is shown with the presence of intermeshed lipid globules (in yellow) and spinal cord. (D) Lipomeningocele that represents spina bifida occulta is shown mimicking the meningocele but with the presence of lipid globules.

2.3 Syndromic and non-syndromic NTDs

A small proportion of the reported NTDs in life born infants are syndromic meaning the NTD is associated with other defects as opposed to non-syndromic forms, where failure of the neural tube to close completely is the only obvious primary defect (Padmanabhan, 2006; Greene & Copp, 2014; Copp *et al.*, 2015). Syndromic NTDs means that the associated defects that coexist with NTDs can be either chromosomal or non-chromosomal conditions which include trisomy 13 or 18 (Sepulveda *et al.*, 2004), Marfan syndrome (Voyvodic *et al.*, 1999), and X-linked heterotaxy (Gebbia *et al.*, 1997). The more severe the level of NTDs, the more likely they are to be associated anomalies (Seaver & Stevenson, 2006). Craniorachischisis has the highest rate of syndromic association with other anomalies. On the other hand, the incidence rate of syndromic association with spina bifida is higher than those with anencephaly (Table 2.1).

Table 2.1: Frequency of various types of NTDs with associated congenital anomalies (Modified from Seaver & Stevenson (2006))

Reference and number of patients	Series	Craniorachishisis	Anencephaly	Upper Spina Bifida	Lower Spina Bifida	All
(Kallen *et al.*, 1998)	French (1976-1994), Swedish	-	25%	35%	20%	23%
n=2955 infants	(1973-1993) and California	-	26%	-	-	25%
n=734 infants	Birth Defects Registries (1983- 1992)					
(Stevenson *et al.*, 2004)	South Carolina(1992-2002)	-	41%	31%		17%
n= 564 infants						
(Hall *et al.*, 1988)	British Columbia (1958-1984)	62%	15%	19.5%	6.7%	14%
n=512 infants						
(Stoll *et al*., 2011)	Strasbourg, France (1979-2008)	-	11.5%	23.8%	-	20.4%
n= 441 infants						
(Nielsen *et al.*, 2006)	Copenhagen, Denmark (1989-2004)	19.7%	29.5%	50.8%		63%
n=97 infants						
(Toru *et al.*, 2016)	Akdeniz University, Turkey (2006-2012)	-	9.7%	14.7	-	32.4
n=62 infants						

2.4 Epidemiology

The worldwide incidence of NTDs ranges from 0.5 to more than 10 cases per 1000 births (Greene & Copp, 2014). The highest prevalence of NTDs was reported at the Shanxi province of Northern China (Chen *et al.*, 2009). Minimal data reporting the prevalence rate of NTDs were available among the South-East Asian region which only includes Vietnam, Thailand, Singapore and Malaysia (Zaganjor *et al.*, 2016). The worldwide prevalence rate of NTDs are as presented in Table 2.2.

Table 2.2: Prevalence rate of NTDs per 1000 births estimated worldwide

Country	Location	Prevalence rate per 1000 births	Reference
China	Shanxi province	19.94	(Chen *et al.*, 2009)
India	Delhi	6.62	(Sood *et al.*, 1991)
	Sevagram, Wardha	0.75	(Taksande *et al.*, 2010)
Africa	Algeria	7.54	(Houcher B *et al.*, 2012)
	Nigeria	0.52	(Ekanem *et al.*, 2008)
Pakistan	Swat	12.41	(Khattak *et al.*, 2010)
United Arab Emirates	-	0.21	(Al Hosani *et al.*, 2005)
Europe	-	0.94	(Dolk *et al.*, 2010; EUROCAT, 2012)
United States of America	-	0.53	(Canfield *et al.*, 2014)
Vietnam	-	0.43	(Hoang *et al.*, 2013)
Thailand	-	0.19	(Jaruratanasirikul *et al.*, 2014)
Singapore	-	0.12	(Shi *et al.*, 2002)
Malaysia	Kinta District	0.73	(Thong *et al.*, 2005)
	-	0.42	(Boo *et al.*, 2013)

2.5 NTDs risk factors

In non-syndromic NTDs cases, both environmental and genetic factors are believed to be involved (Copp & Greene, 2010). Meanwhile, syndromic NTDs have more well-defined genetic causative components.

2.5.1 Environmental factors

Diminished folate status is the best known NTD influencing factor. Over the past 40 years, researchers have found mildly deficient levels of folate in women who had a pregnancy affected by NTD (Smithells *et al.*, 1976). Studies involving 33 centers around the world by the Medical Research Council Vitamin Trial suggested folic acid supplements in the periconceptional period could prevent the recurrence of NTD cases for up to 72% (MRC-Vitamin-Study-Research-Group, 1991). In addition, maternal periconceptional exposure to folic acid antagonists for example the intake of valproic acid (Lloyd, 2013), trimethoprim (Hernández-Díaz *et al.*, 2001), fumonisin (Missmer *et al.*, 2006), phenytoin (Hernández-Díaz *et al.*, 2001; Hill *et al.*, 2010), nitrofurantoin (Ailes *et al.*, 2016), thalidomide (Vargesson, 2015), carbamazepine (Hernández-Díaz *et al.*, 2001; Matlow & Koren, 2012) and methotrexate (Hernández-Díaz *et al.*, 2001) have been shown to increase the risk of NTDs.

Other environmental factors associated with increased risk of NTDs include maternal smoking (Kyrklund-Blomberg *et al.*, 2005; Hackshaw *et al.*, 2011; Mund *et al.*, 2013), prepregnancy obesity (Shaw *et al.*, 1996; Werler *et al.*, 1996), and maternal illnesses during pregnancy such as hyperthermia (Moretti *et al.*, 2005) and diabetes (Salbaum & Kappen, 2010). The occupation of parents in a high-risk environment such as mining have also been implicated (Liao *et al.*, 2010; Ahern *et al.*, 2011). Other factor that leads to the incidence of NTDs include exposure to toxic agents such as Agent Orange and uranium (Hindin *et al.*, 2005; Ngo *et al.*, 2010; Noel *et al.*, 2016). Dietary factors such as simple sugar intake (Shaw *et al.*, 2003), trace elements and other micronutrients deficiency (Cengiz *et al.*, 2004; Martin *et al.*, 2004; Ulman *et al.*, 2005), and accidental intake of agricultural compounds in drinking water like nitrate, atrazine, and arsenic have been associated with prenatal exposure and birth defects (Brender & Weyer, 2016). Table

2.3 summaries the potential environmental factors for NTDs.

Table 2.3: The list of potential risk factors for neural tube defects (Modified from Copp *et al.* (2015))

Maternal nutrition	Other maternal factors	Environmental factors
Low folate intake (Pitkin, 2007)	Smoking (Hackshaw *et al.*, 2011)	Ambient air pollution (Padula *et al.*, 2013)
Alcohol use (Grewal *et al.*, 2008)	Hyperthermia (Moretti *et al.*, 2005)	Disinfectant by-products in drinking water (Brender & Weyer, 2016)
High caffeine use (Schmidt, 2007)	Low-socio-economic status (Wasserman *et al.*, 1998)	Organic solvents (Cordier *et al.*, 1997)
Low dietary quality (Cengiz *et al.*, 2004)	Maternal illnesses and infections (Shaw *et al.*, 1998)	Heavy metals and herbicide (Hindin *et al.*, 2005; Ngo *et al.*, 2010)
Elevated glycaemic load (Shaw *et al.*, 2003)	Pregestational insulin-dependent diabetes (Salbaum & Kappen, 2010)	High risk occupation (Ahern *et al.*, 2011)
Low zinc intake (Ulman *et al.*, 2005)	Pregestational obesity (Rasmussen *et al.*, 2008)	
Low vitamin levels (Smithells *et al.*, 1976)	Folic acid antagonists use (Hernández-Díaz *et al.*, 2001)	
Low serum choline (Shaw *et al.*, 2004)		
Low methionine intake		

2.5.2 Genetic factors

To date, more than 240 mouse mutants that mimic human NTDs have been created with 205 genes implicated, another 30 implicating unidentified genes and 9 multiple genes implicated (Harris & Juriloff, 2010). The extensive genetic involvement in cranial neurulation is reflected to additional complexity and greater sensitivity to disruption compared with spinal neurulation (Copp *et al.*, 1990; Harris & Juriloff, 2010; Greene & Copp, 2014). Despite the large list of mouse mutants with NTDs, the genetic basis of NTDs in humans remains not well understood (Greene & Copp, 2014).

The genetics of human NTDs appear to be more complex (Bassuk & Kibar, 2009; Greene *et al.*, 2009). The study of genetic variants is important for the identification of causal, risk or associated factors which contribute to the aetiology of NTDs. To date, the folate-related gene analysis, candidate gene approach in human and animal studies are among the current focus of genetic studies for human NTDs (Bassuk & Kibar, 2009; Copp *et al.*, 2013; Copp *et al.*, 2015).

The most studied group of NTD-associated genes in humans are those involved in folate one-carbon metabolism particularly the methylenetetrahydrofolate reductase (*MTHFR*) (Amorim *et al.*, 2007; Yang *et al.*, 2015). *MTHFR* is an enzyme responsible for generating the 5-methyltetrahydrofolate which is essential for the conversion of homocysteine to methionine (de Franchis *et al.*, 1998). The C677T variant of the *MTHFR* gene creates an amino acid change from alanine to valine at position 222 of the protein. Homozygosity of the C677T variant results in a thermolabile enzyme which reduces the enzyme activity and elevates homocysteine concentrations (Blom *et al.*, 2006). This thermolabile genotype is more frequent in mothers and patients with NTD compared to the healthy subjects (Gonzalez-Herrera *et al.*, 2002). The homozygous genotype was found to be the NTD risk factor in non-Latin populations (Amorim *et al.*, 2007). This variant has also been associated with increased NTD risk in Irish, mixed USA, mixed UK, and Italian populations but not in many other populations across the world such as Dutch and Mexicans (Table 2.4). Another *MTHFR* variant, A1298C does not appear to confer any significant risk of NTDs in a Chinese cohort (Zhang *et al.*, 2013), as well as in Mexican (Yucatan), Italian, and Dutch populations (Table 2.4). Regardless of some indication that *MTHFR* variant is associated with spina bifida in particular, however, the variant is not likely a major contributor in NTD globally as it is not well replicated in many other worldwide populations.

Furthermore, the planar cell polarity (PCP) genes that include *VANGL1*, *CESLR1* and *SCRIB* have been implicated as spina bifida risk gene among the Italians, Americans and French (Table 2.4). Other genes including those that play a role in folate transport (*CUBN* and *SLCA19A1)*, DNA repair and DNA methylation (*APE1*, *XPD* and *SOX18*), and glucose metabolism (*GLUT1*, *HK1*, *LEP* and *LEPR*) have also been studied to be NTDs related genes or risk factor for spina bifida (Table 2.4). In addition, mutations in tumor suppressor genes such as *BRCA1* and *PAX3 and ZIC3* (implicated in mouse models) are shown to increase the spina bifida risk in certain populations (Table 2.4).

Table 2.4: Comprehensive list of spina bifida genes (as reviewed in Greene *et al.* (2009))

No	Gene	Location	Population			
			Association/ risk factor	Reference	No association	Reference
	Folate metabolism					
1	*CBS*	Chr21, NC_000021.9	Mixed USA (259 cases)	(Shaw *et al.*, 2009)	Dutch (180 patients)	(Franke *et al.*, 2009)
2.	*DHFR*	Chr 5, NC_000005.10	Irish (283 cases)	(Parle-McDermott *et al*., 2007)	Mixed USA (259 cases)	(Shaw *et al.*, 2009)
			Mixed USA (61 cases, multi-affected families)	(Johnson *et al*., 2004)	Mixed UK (229 patients)	(Doudney *et al.*, 2009)
					Dutch (180 patients)	(Franke *et al.*, 2009)
					Dutch (109 patients)	(van der Linden *et al*., 2007)
3.	*MTHFD1*	Chr 14, NC_000014.9	Irish (509 mixed cases)	(Carroll *et al*., 2009)	Mixed UK (229 patients)	(Doudney *et al.*, 2009)
			Irish (176 mixed cases)	(Parle-McDermott *et al*., 2006)	Dutch (103 cases)	(van der Linden *et al*., 2007)
			Italian (142 cases)	(De Marco *et al*., 2006)	Dutch (180 patients)	
			Mixed USA (259 cases)	(Shaw *et al*., 2009)		(Franke *et al*., 2009)
4.	*MTHFR*	Chr 1, NC_000001.11	Irish (471 cases)	(O'Leary *et al*., 2005)	Dutch (180 patients)	(Franke *et al.*, 2009)
			Mixed USA (259 cases)	(Shaw *et al.*, 2009)	Mexican (Yucatan) (97 cases)	(Gonzalez-Herrera *et al*., 2007)
			Mixed UK (229 patients)	(Doudney *et al*., 2009)		
			Italian (15 cases)	(Grandone *et al*., 2006)		
5.	*TYMS*	Chr 18, NC_000018.10	Non-Hispanic white USA (264 cases)	(Volcik *et al*., 2003)	Dutch (180 patients)	(Franke *et al*., 2009)
			Mixed USA (259 cases)	(Shaw *et al*., 2009)		
	One carbon metabolism (including homocysteine remethylation)					
6.	*ALDH1L1*	Chr 3, NC_000003.12	Dutch (180 patients)	(Franke *et al.*, 2009)		
7.	*BHMT*	Chr 5, NC_000005.10	Mixed USA (259 cases)	(Shaw *et al.*, 2009)	Mixed USA (252 cases)	(Zhu *et al.*, 2005)
					Dutch (180 patients)	(Franke *et al*., 2009)
8.	*CHKA*	Chr 11, NC_000011.10	Mixed USA (103 cases)	(Enaw *et al*., 2006)	No study	
9.	*MTRR*	Chr 5, NC_000005.10	Mixed UK (229 patients)	(Doudney *et al*., 2009)	Irish (575 mixed families)	(O'Leary *et al*., 2005)
			Dutch (109 cases)	(van der Linden *et al*., 2006)	Dutch (180 patients) Mixed USA (259 cases)	(Franke *et al*., 2009) (Shaw *et al*., 2009)
			Mixed USA (259 cases)	(Shaw *et al*., 2009)		
10.	*NOS3*	Chr 7, NC_000007.14	Mixed USA (301 families)	(Brown *et al*., 2004)	Mixed USA (259 cases)	(Shaw *et al*., 2009)
			Dutch (109 cases)	(van der Linden *et al*., 2007)	Dutch (180 patients)	(Franke *et al*., 2009)
			Caucasian (578 patients)	(Soldano *et al*., 2013)		
11.	*PCYT1A*	Chr 3, NC_000003.12	Mixed USA (103 cases)	(Enaw *et al*., 2006)		

Table 2.4, continued

12.	*SARDH*	Chr 9, NC_000009.12	Dutch (180 patients)	(Franke *et al.*, 2009)		
13.	*TRDMT1*	Chr 10, NC_000010.11	Dutch (180 cases)	(Franke *et al*., 2009)		
	Glucose metabolism					
14.	*GLUT1*	Chr 1, NC_000001.11	Mixed USA (507 cases)	(Davidson *et al*., 2008)		
15.	*HK1*	Chr 10, NC_000010.11	Mixed USA (507 cases)	(Davidson *et al*., 2008)		
16.	*LEP*	Chr 7, NC_000007.14	Mixed USA (507 cases)	(Davidson *et al*., 2008)		
17.	*LEPR*	Chr 1, NC_000001.11	Mixed USA (507 cases)	(Davidson *et al*., 2008)		
	DNA repair, and DNA methylation					
18.	*APE1*	Chr 14, NC_000014.9	Mixed USA (380 patients)	(Olshan *et al.*, 2005)		
19.	*XPD*	Chr 19, NC_000009.10	Mixed USA (380 patients)	(Olshan *et al.*, 2005)		
20.	*SOX18*	Chr 20, NC_000020.11	Belgium (83 patients)	(Rochtus *et al*., 2016)		
	Folate transport					
21.	*CUBN*	Chr 10, NC_000010.11	Dutch (179 patients)	(Franke *et al.*, 2009)		
22.	*SLCA19A1*, *RFC-1*	Chr 4, NC_000004.12	Dutch (180 patients)	(Franke *et al.*, 2009)	Mixed USA (259 cases) Mixed UK (229 patients)	(Shaw *et al.*, 2009) (Doudney *et al.*, 2009)
	Planar cell polarity genes					
23.	*VANGL1*	Chr 1, NC_000001.11	Italian and mixed USA (658 patients) Italian and French (102 patients)	(Kibar *et al*., 2009) (Kibar *et al*., 2007)	Mixed UK and USA (24 patients)	(Doudney & Stanier, 2005)
24.	*CELSR1*	Chr 22, NC_000022.11	California (192 patients)	(Lei *et al*., 2014)		
25.	*SCRIB*	Chr 8, NC_000008.11	California (192 patients)	(Lei *et al*., 2013)		
26	*DVL1*	Chr 1, NC_000001.11	Han Chinese cohort (20 cases)	(Chen *et al.*, 2016)		
	Retinol metabolism					
27.	*ALDH1A2*	Chr 15, NC_000015.10	Mixed USA (318 families)	(Deak, Dickerson, *et al.*, 2005b)		
	Methylation reactions					
28.	*PCMT1*	Chr 6, NC_000006.12	Mixed USA (152 cases)	(Zhu *et al.*, 2006)	Dutch (180 patients)	(Franke *et al.*, 2009)
	Oxidative stress					
29.	*SOD1*	Chr 21, NC_000021.9	Mixed USA (610 trios or duos)	(Kase *et al.*, 2012)		
30.	*SOD2*	Chr 6, NC_000006.12	Mixed USA (610 trios or duos)	(Kase *et al.*, 2012)		
	Intermediate filament protein					
31.	*LMNB1*	Chr 5, NC_000005.10	Mixed UK, USA and Swedish (233 patients)	(Robinson *et al*., 2013)		

Table 2.4, continued

	Cell adhesion molecules					
32.	*NCAM1*	Chr 11, NC_000011.10	USA (204 patients)	(Deak, Boyles, *et al.*, 2005a)		
	Axial development in mouse					
33.	*T (Brachyury)*	Chr 6, NC_000006.12	Mixed USA (316 cases)	(Jensen *et al*., 2004)		
	NTDs in mouse mutant					
34.	*BRCA1*	Chr 17, NC_000017.11	Mixed USA (268 patients and parents)	(King *et al*., 2007)		
35.	*CFL1*	Chr 11, NC_000011.10	Mixed USA (246 cases)	(Zhu *et al*., 2007)		
36.	*PAX3*	Chr 2, NC_000002.12	USA (74 cases)	(Lu *et al*., 2007)		
37.	*PDGFRA*	Chr 4, NC_000004.12	Dutch (88 cases and 56 mothers)	(Toepoel *et al*., 2009	Mixed USA (407 triads)	(Au *et al*., 2005)
38.	*TXN2*	Chr 22, NC_000022.11	Mixed USA (48 cases)	(Wen *et al*., 2009)		
39.	*ZIC2*	Chr 13, NC_000013.12	Dutch (117 mixed patients)	(Klootwijk *et al*., 2004)		
40.	*ZIC3*	Chr X, NC_000023.11	Dutch (117 mixed patients)	(Klootwijk *et al*., 2004)		

2.6 Whole-Exome Sequencing (WES)

WES is a method used to sequence only the exome (all exons in all genes) that is the coding regions of a genome (van Dijk *et al.*, 2014). Although these coding regions constitute less than 2% of the human genome, it contains almost 85% of known disease-causing variants, which make WES as a feasible and cost-effective alternative to whole-genome sequencing (Choi *et al.*, 2009). The use of WES permits the identification of the single nucleotide variants (SNV) as well as small insertions or deletions (InDel) that is responsible for Mendelian diseases (Isakov *et al.*, 2013). This method also allows the detection of rare and common genetic variants in humans that describe the heritability of complex diseases (Isakov *et al.*, 2013; Nayarisseri *et al.*, 2013). Example of the use of WES in identifying causal variants include Parkinson's disease (*TNK2:* p.Arg877His), Crohn disease (*XIAP*: p.Cys231Tyr), and epileptic encephalopathies (*DYNC1H1*:p.Met3392Val) (Worthey *et al.*, 2011; Farlow *et al.*, 2016; Lin *et al.*, 2017).

CHAPTER 3: MATERIALS AND METHODS

This chapter outlines the common materials and methods used to obtain data for the genetics studies. Specific methods for determining the prevalence and distribution of neural tube defects (NTDs), and whole embryo culture using the antisense oligonucleotide phosphorothioate technology are explained in detail in their respective chapters (Chapter 4 and 6).

In the genetic aspects, this chapter covers the selection of patients based on their clinical phenotype of having three different phenotypes of spina bifida; (1) syndromic spina bifida aperta, (2) non-syndromic spina bifida aperta, and (3) non-syndromic spina bifida occulta. The DNA samples were isolated for whole-exome sequencing (WES). PCR amplification and Sanger sequencing were then used to verify the candidate variants. The outline of this chapter is as shown in the flow chart (Figure 3.1).

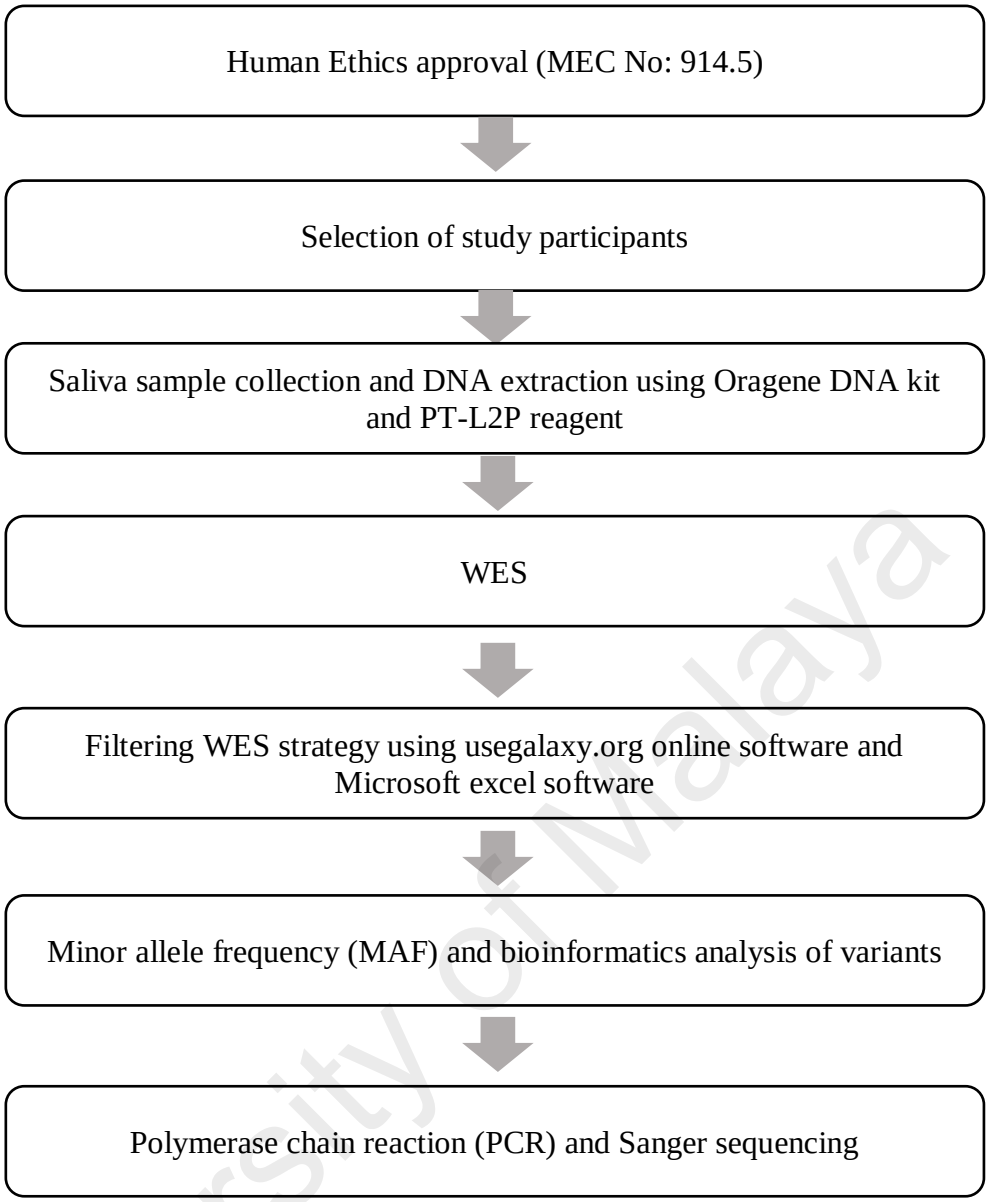


Figure 3.1: Flowchart of methods for genetic studies used in Chapter 5 and Chapter 6

3.1 Ethics approval

Ethical approval involving the collection of saliva samples from the patients and families and to retrieve their respective medical records was obtained from the Medical Ethics Committee (MEC) The University of Malaya Medical Centre (UMMC). The approved MEC reference number is 914.5.

3.2 Patient selection for WES

Three categories of spina bifida patients were chosen for WES involving triads (trios) for the finding of the possible pathogenic candidate variants in spina bifida patients. These patients represent (1) syndromic spina bifida aperta, (2) non-syndromic spina bifida aperta and (3) non-syndromic spina bifida occulta. The selection of patients was according to the diagnosis of the patients with varying degree of spina bifida and the availability of DNA samples within our patient cohort. A total of three families and three unrelated controls were selected for this study. Written consent was obtained from the patients or guardians (parents) prior to the sample collection. The patients' medical records and information during pregnancy, family history of any inherited diseases and the management of spina bifida were also acquired.

3.3 Saliva sample collection and DNA extraction

Saliva samples of the patients and parents were collected using Oragene DNA OG-500 kit and Oragene DNA OG-575 kit (DNA Genotek, Canada). The sample was incubated overnight at 50˚C to ensure adequate DNA was released and to inactivate the nucleases. A total of 500 µl of sample was transferred into 1.5ml microfuge tube and 20µl of PT-L2P reagent (DNA Genotek, Canada) was added into the tube. The mixture was then incubated on ice for 10 minutes and centrifuged for 15 minutes at 15,000 xg at room temperature to reduce the turbidity of the DNA solution. Then, the clear supernatant was placed into a fresh microfuge tube and added with 600 µl of 100% ethanol. The mixture was gently mixed by inversion 10 times. At this point, clot of DNA fibers might be appeared. However, this occurrence depends on the amount of DNA in the sample. The mixture was then mixed gently and left for 10 minutes at room temperature followed by centrifugation at 15,000 xg for 2 minutes. The supernatant with impurities was discarded while the DNA pellet was washed with 250 µl of 70% ethanol and allowed to stand at

room temperature for 1 minute before centrifuge for 5 minutes at 15,000 xg. After that, the ethanol was completely removed without disturbing the DNA pellet and added with 25 µl of MilliQ water. The method of extraction was performed according to the manufacturer's protocol. The extracted DNA samples were measured using spectrophotometer (Nanodrop) (Thermo Scientific) to identify the DNA concentration and purity. Absorbance ratio at 260 nm and 280 nm was used to measure the DNA purity whereby ratio of ~1.8 is considered as "pure" for DNA.

3.4 Whole-exome sequencing (WES)

A total of 20 to 40 µl of high quality genomic DNA of index family members was further evaluated using the WES technique. The sequencing libraries were analysed using Illumina TruSeq kit and reads on the HighSeq 2000 Sequencer platform. This platform generated an average read length of 100bp and a median of 50X coverage. The sequencing reads were mapped to the Human Genome February 2009 (GRCh37/hg19) reference exome using the Burrows-Wheeler Aligner (BWA) (v0.7.8). BWA was run with default parameters. Variants (single nucleotide polymorphisms and indels) were identified using SAMtools and annotated with dbSNP, the 1000 Genomes databases.

3.5 Filtering WES strategy

Data filtering of the WES results were performed using tools from usegalaxy.org to check for more common variants (Afgan *et al.*, 2016). These variants were then filtered by comparing them to the reported single nucleotide variants (SNVs) databases and the in-house polymorphism databases to eliminate known/common SNVs that were unlikely to be pathogenic. The triads were then analysed using *de novo* mutation approach as only the patient was diagnosed to be affected while parents were coded as unaffected. The *de novo* list obtained were then aligned with the public databases (1000 Genome Project at

http:11www.1000genomes.org/home) was selected by taking into account the MAF<0.1 and non-synonymous exonic SNV. The list was further reduced by reviewing the literature for the genes involvement in NTDs. Potential new variants which may be pathogenic were selected, and validated by testing against the presence of variants in other affected family members and unaffected controls.

3.6 Minor allele frequency (MAF) and bioinformatics analysis of variants

Prior to the study**,** the MAF of the variants were queried in the 1000 Genomes database (1000g2011_oct all), 1000 Genomes UCSC (University of California Santa Cruz) Browser**,** Go-Exome Sequencing Project (GO-ESP), Exome Aggregation Consortium (ExAC) (Karczewski *et al.*, 2016) and in the Singapore Genome Variant Project (SGVP) (Teo *et al.*, 2009). The deleterious scores of the variants were analysed by looking at the functional protein prediction scores. The amino acid substitutions of the variants were evaluated using the Sorting Tolerant from Intolerant (SIFT) (Kumar *et al.*, 2009), Provean (Choi & Chan, 2015) and Polyphen 2 (Adzhubei *et al.*, 2010).

3.7 Polymerase chain reaction (PCR) and Sanger sequencing

The selected potential variants were further analysed and validated using PCR and Sanger sequencing. Primer sequences meant for validation were designed using Primer-BLAST, NCBI (Table 3.1). All PCRs (20 µl) were performed in a SuperCycler thermal cycler (Kyratec, Australia) by adding 1µl DNA template, 1.5 µl (10µM) of each primer, 6 µL sterile MilliQ water, and 10 µl 2X ExPrime TM Taq Premix (GeNet Bio, Korea) (Table 3.2). PCR amplification conditions include 1 minute initial denaturation at 95ºCfollowed by 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 52ºC to 58ºC for 30 seconds, extension at 72ºC for 1 minute. A final extension at 72ºC for 10 minutes was then performed and the reactions were held at 4ºC (Table 3.3). All PCRs

were duplicated to verify the results. The amplified PCR products were analysed by agarose gel electrophoresis. A total of 4 µl of 100 bp DNA ladder (GeneDirex, Taiwan) was electrophoresised in parallel with 4 µl PCR products in 1.5% agarose gel with TrisAcetate- EDTA (TAE) buffer and stained with ethidium bromide. Upon completion, gels were visualized using a UV transluminator (Nyx Technik Inc, Taiwan). The presence of amplified products with expected band sized were considered as positive amplification results. The PCR products were then sent for Sanger sequencing sequenced to MyTACG Bioscience Enterprise (Selangor, Malaysia) using forward or reverse primers of the respective amplification reaction. Sequence electropherograms were analysed using BioEdit Sequence Allignment Editor and verified for the presence of the variants using BLAST to compare the sequences with the human reference sequence.

Table 3.1: Primers used in this study for screening and validation of human variants

No.	Genes	Accession ID	Primers	Expected size (bp)
1	*MTHFR*	NC_000001.11	Forward: 5' CGGAAGAATGTGTCAGCCTCA 3' Reverse: 5' TTCATCCCTCGCCTTGAACA 3'	188 bp
2	*MTCH2*	NC_000011.10	Forward: 5' TCAAGAGGAAGAAAGGACAGGG 3' Reverse: 5' TGAGGACTGTGATAGGCAGG 3'	353 bp
3	*RORA*	NC_000015.10	Forward: 5' AAAGATGTGCAGTGTGTGGCC 3' Reverse: 5' GATGCTTATGCCTGTTCTTGC 3'	339 bp
4	*HOXA10*	NC_000007.14	Forward: 5' AGCGCTCGGGAAGTGAAAAA 3' Reverse: 5' GTGTGGCCTCGACTTAATCATC 3'	363 bp
5	*SEC63*	NC_000006.12	Forward: 5' GAAGCTGTACACGTAAGACTTGA 3' Reverse: 5' AAGGACCCAAGAAAACTGCT 3'	203 bp
6	*ZIC2 (SET 1)*	NC_000013.11	Forward: 5' GGAACATTTCTGGGGGTGC 3' Reverse: 5' CAACATGATCACAAGGTGCCC 3'	552 bp
7	*ZIC2 (SET 2)*	NC_000013.11	Forward: 5' AGCACATGAAGGTACCACCG 3' Reverse: 5' ACATGATCACAAGGTGCCCTC 3'	673 bp
8	*ZIC2 (SET 3)*	NC_000013.11	Forward: 5' GGCTTTTGTCTTGCAGGTCCAT 3' Reverse: 5' CAACATGATCACAAGGTGCCC 3'	467 bp
9	*ZIC2 (SET 4)*	NC_000013.11	Forward: 5' GGCTTTTGTCTTGCAGGTCCAT 3' Reverse: 5' ACATGATCACAAGGTGCCCTC 3'	465 bp
10	*ZIC2 (SET 5)*	NC_000013.11	Forward: 5' GGAACATTTCTGGGGGTGC 3' Reverse: 5' ACATGATCACAAGGTGCCCTC 3'	550 bp

Table 3.2: PCR reaction mixture used for PCR amplification of the DNA samples

Components	Concentration	Volume (µl per reaction)
DNA template	~50 ng/µl	1
ExPrime TM Taq Premix	2X	10
Forward primer	10 µM	1.5
Reverse primer	10 µM	1.5
Sterile MilliQ water		6
Total		20

Table 3.3: PCR cycling parameters

Cycle step	Temperature (˚C)	Time	Number of cycles
Initial denaturation	95	1 minute	1
Denaturation	95	30 seconds	
Annealing	52-60	30 seconds	35
Extension	72	1 minute	
Post cycling extension	72	10 minutes	1

CHAPTER 4: A PREVALENCE AND DISTRIBUTION OF NEURAL TUBE DEFECTS IN UNIVERSITY OF MALAYA MEDICAL CENTRE (UMMC)

4.1 Introduction

The prevalence rate of neural tube defects (NTDs) varies among countries and in different geographic locations within countries. For instant, both highest and lowest prevalence rate were reported in different regions of China; Luliang, Shanxi Province (19.4 per 1000 births) and Beijing (0.03 per 1000 births) (Chen *et al.*, 2009, Zaganjor *et al.*, 2016). The latest prevalence study of NTDs in Malaysia was reported in 2013 using data from the Malaysian National Neonatal Registry (0.42 per 1000 live births) (Boo *et al.,* 2013) and there was a report of birth defects prevalence using data from a small population in the Kinta district back in 2005 (0.73 per 1000 live births) (Thong *et al.*, 2005). Using the data captured from the University of Malaya Medical Centre (UMMC), which serve as a single major referral center in Malaysia, this chapter focusses on the occurrence and follow-up of NTDs cases from 2003 until 2016. The data collected also comprise of basic patients' and maternal parents' information including gender predisposition, ethnicity, birth weight, maternal age, details of the defects and associated conditions. Patients' ambulation and education level were also measured. This study is significant due to the lack of publications on NTDs in Malaysia.

4.2 Materials and methods

4.2.1 Data collection

UMMC is part of University of Malaya and it is a semi-government-funded medical institution situated in Kuala Lumpur, Malaysia. It serves as a tertiary referral medical center in Malaysia. Data for this prevalence and distribution study were obtained from the UMMC Department of Patient Information. Records obtained were from patients

diagnosed with NTDs according to ICD10: Q00 (Anencephaly), Q001 (Craniorachischisis) and Q05 (Spina bifida) in the period of 14 years (2003 to 2016). A total of 86 cases of spina bifida and 20 cases of anencephaly were recorded. Data captured also included (a) demographic details of patient ethnicity, gender, year of birth, birth weight, birth term (full term/ premature/ termination of pregnancy), mother's age and mode of delivery (spontaneous vaginal delivery /caesarean); (b) details of defects on diagnosis, open or closed lesion, level of lesion, syndromic or non-syndromic; (c) presence of other conditions associated with spina bifida such as hydrocephalus including any insertion of the ventriculo-peritoneal (VP) shunt; (d) patients' ambulation and education. This study is crucial because obtaining in-depth data on NTDs is a multi-pronged effort. Over three years, in-depth analytical assessment was done in triplicates in order to filter the data carefully and to prevent loss of data due to poor reporting.

4.2.2 Statistical analysis

The data was analysed using the Statistical Program for the Social Sciences (SPSS, version 22.0, 2013, IBM corp). A contingency table was used to display frequency distribution of the ethnicity and genders based on the types of diagnosis and tested using Chi-square. Significant difference ($p<0.05$) indicates that there is a relationship between the variables. GraphPad Prism 5 was used to generate graphs.

4.3 Results

4.3.1 Data analysis of craniorachischisis

No single incidence of craniorachischisis was reported. Therefore the prevalence and distribution of craniorachisis was excluded in this study

4.3.2 Data analysis of anencephaly

Eighteen cases and a single case of twins born with anencephaly were obtained from the Patient Records Department and further analysed. From the 20 patients with anencephaly, the estimated number in UMMC alone is 2. Using a total of 5262 as the annual average number of live births based on the Department of Patient Information UMMC, the estimated prevalence rate of anencephaly in this study sample is 0.38 per 1000 live births.

Calculation method 1:

$$\left(\frac{\text{Reported anencephaly case in UMMC per year}}{\text{UMMC average number of live births}}\right) \text{ X } 1000$$

$$= \left(\frac{2}{5262}\right) \text{ X } 1000$$

= 0.38 per 1000 live births

4.3.2.1 Proportion of termination of pregnancy (TOP) in anencephalic cases

However, another issue that may potentially increase the number of occurrence is the inclusion of TOP, which is not accounted for as live births. From the 20 number of infants, 5 cases were reported as TOP. All cases were premature birth with less than 23 weeks of gestation and had birth weight less than 500 grams. A second calculation was performed to calculate the proportion of TOP. In overall, 25% of pregnancy with anencephalic foetuses were terminated due to their inability to survive in prenatal life.

Calculation method 2:

$$\left(\frac{\text{Number of TOP}}{\text{Total number of anencephalic patients}}\right) \text{ X } 100$$

$$= \left(\frac{5}{20}\right) \text{ X } 100$$

= 25%

4.3.2.2 Demographic data analysis of anencephay

Data retrieved in UMMC spanning 14 years study recorded four cases of anencephaly in year 2011 which marked the highest number. No record were found in year 2014 and year 2015 (Figure 4.1A). In term of ethnicity, Malays dominated majority of the cases (n=13) followed by Chinese (n=5) and Indians (n=2) (Figure 4.1B). In addition, almost equal numbers of males and females were reported with one unidentified gender (Figure 4.1C). The maternal age during childbirth ranged from 24 to 40 years old and the most affected age were between 26 to 35 years old (Figure 4.1D). As with most premature cases, babies born were below the normal birth weight (Figure 4.1E). Excluding the cases of TOP, majority of the anencephaly patients were premature births (n=9, 60%) and the rest were full term birth (n=6, 40%) (Figure 4.1F).

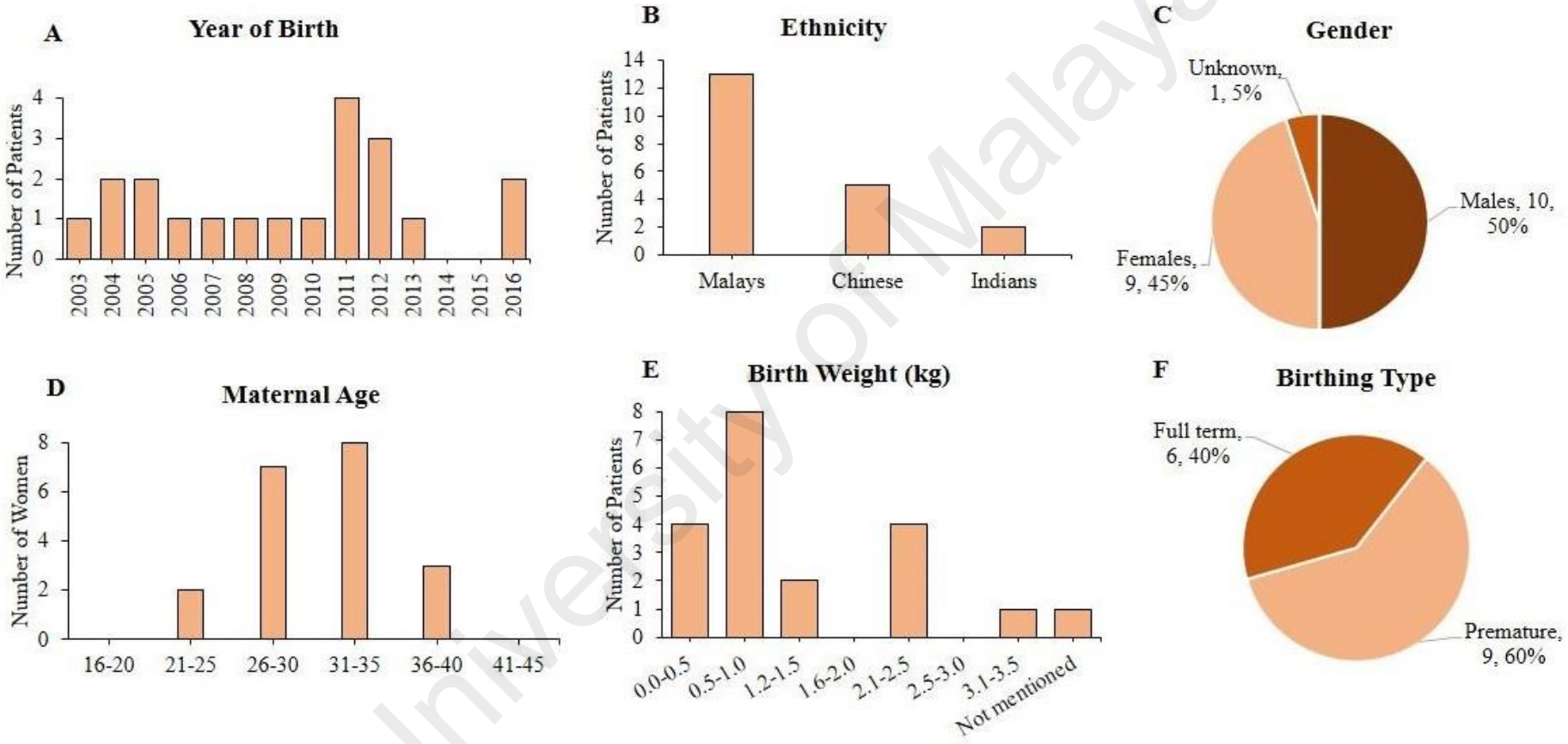


Figure 4.1: Demographics of anencephaly in the University of Malaya Medical Centre between the years 2003 until 2016**. (**A) Bar chart showing year of birth for patient cohort. (B) Ethnicity of anencephaly patients. (C) Gender preponderance of anencephaly patients. (D) Maternal age with highest peak at 31 to 35 years old. (E) Birth weight where majority were 0.5 to 1.0 kg. (F) Birthing type of anencephaly patients.

4.3.3 Data analysis of spina bifida

A total of 206 cases from the Department of Patient Information were obtained, which was, classified ICD10: Q05. Eighty-six patient records were confirmed as spina bifida. From the 86 patients, the calculated number of spina bifida in-house and referred cases annually in UMMC alone is 7. Using the same formula in calculation 1 on the data analysis of anencephaly section, the estimated prevalence rate of spina bifida in this study sample number is 1.33 per 1000 live births.

Calculation method 1:

$$\left(\frac{\text{Reported spina bifida case in UMMC per year}}{\text{UMMC average number of live births}}\right) \text{X } 1000$$

$$= \left(\frac{7}{5262}\right) \text{X } 1000$$

= 1.33 per 1000 live births

Taking into account that UMMC is a referral centre for high risk pregnancies, this may explain why UMMC records higher numbers compared to data by Boo and co-workers (2013) and Thong and co-workers (2005). However, other issues, which potentially decrease the number of occurrences, will include termination of pregnancies of spina bifida, miscarriage of spina bifida, and cases of spina bifida which go unreported. It is not possible to distinguish spina bifida cases born and referred to UMMC. It is not known which among the 86 live births occurred in UMMC and which were referred. In this study, only 86 cases were confirmed as mentioned above and as a result, as many as 120 cases listed as spina bifida under the ICD10: Q05 were confirmed to be incomplete. Therefore, weighting adjustment was performed accordingly (Howards *et al.*, 2015) by which weighting of 6.4 was obtained based on this calculation.

Calculation method 2:

$$\text{Weighting adjustment} = \frac{\%\ \text{stratum of population}}{\%\ \text{stratum of sample}}$$

$$\%\ \text{stratum of population} = \frac{206}{100} \times 0.13 \times 100$$
$$= 26.78\%$$

$$\%\ \text{stratum of sample} = \frac{86}{206} \times 100$$
$$= 41.75\%$$

$$\text{Weighting adjustment} = \frac{26.78\%}{41.75\%}$$
$$= 0.64$$

$$= 6.4 \text{ per } 1000 \text{ live births}$$

4.3.3.1 Demographic data analysis of spina bifida

The number of spina bifida cases between 2003 until 2016 is as shown in Figure 4.2A. A total of 35% (n= 30) patients were born through spontaneous vaginal delivery (SVD) while 42% (n= 36) were born by lower segment Caesarean section (LSCS). The other 20 cases were not accounted for in terms of mode of delivery. The maternal age during childbirth ranged from 17 to 42 years old and the most affected age were below than 35 years old (Figure 4.2B). The common birth weight were at 3.1 to 3.5 kg and it ranged between 1.3 to 4.6 kg (Figure 4.2C). Data for birth term was retrieved from 81% of cases, where 75% were full term babies and 6% were premature babies. Pregnancy age less than 37 weeks were considered as premature while 37 weeks and above as full term. In this cohort, 59% were males and 41% females (Figure 4.2D). Majority of cases in the database were of Malay ethnicity (41.86%; n= 36), followed by an equal number of Chinese and Indians at 27.91% (n= 24 each). There was only a single case each of ethnic minorities, a Kadazan and a Punjabi child, which registered at 1.16% (n= 1 each) (Figure 4.2E).

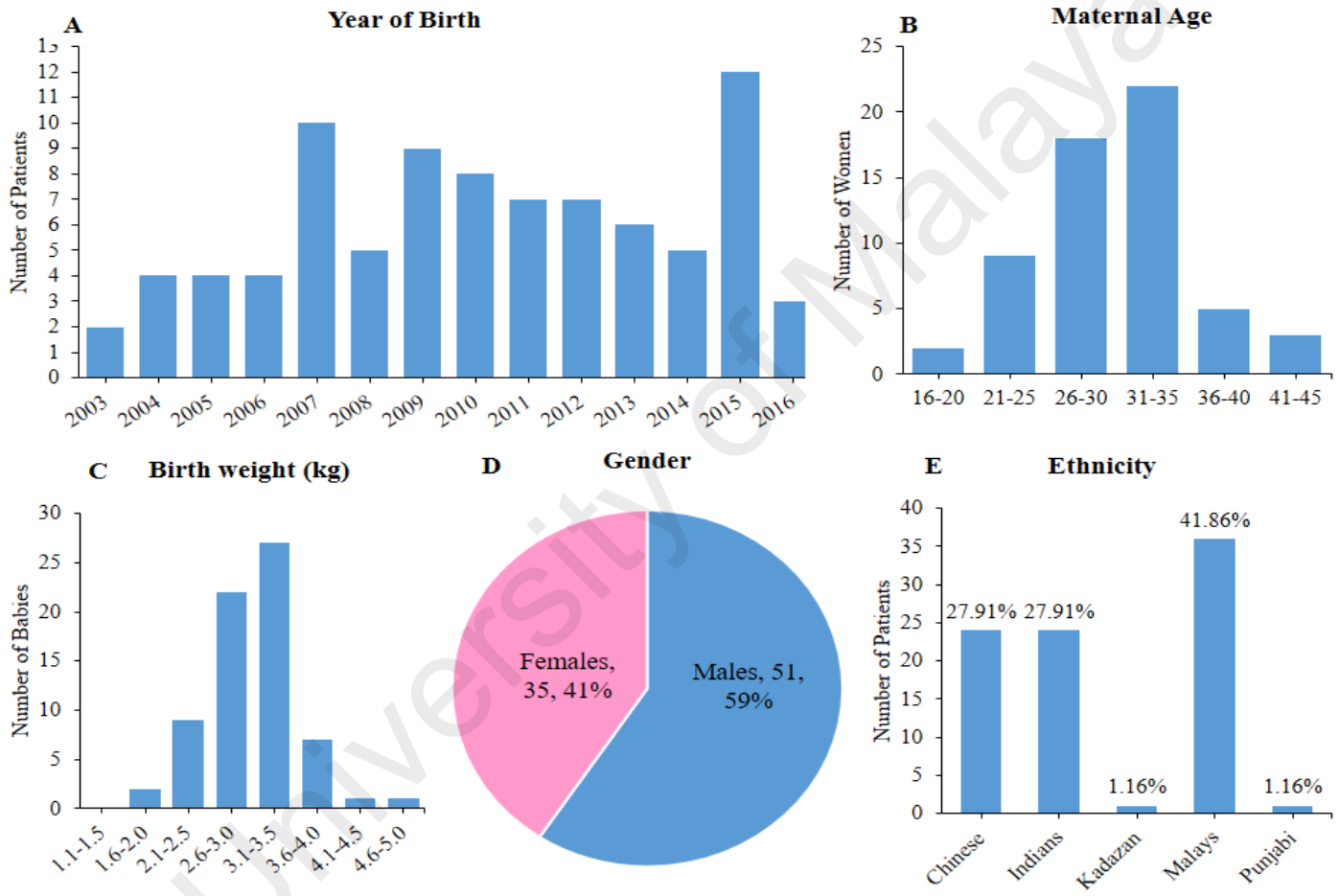


Figure 4.2: Demographics of spina bifida in the University of Malaya Medical Centre between the years 2003 until 2016. (A) Bar chart showing year of birth for patient cohort. (B) Maternal age with highest peak at 31 to 35 years old. (C) Birth weight where majority at 3.1-3.5kg. (D) Gender preponderance of spina bifida patients. (E) Ethnicity of spina bifida patients.

4.3.3.2 Types of Defects

The most commonly reported NTD type was spina bifida with myelomeningocele (45.35%, n= 39) (Table 4.1). There was a single case of encephalocele with meningocele (1.16%) and 11 cases of lipomyelomeningocele (12.79%) (Table 4.1). The most commonly reported level of of spina bifida lesion was at the lumbar region (26.7%, n= 23) (Table 4.2). Non-syndromic spina bifida represented the majority of the cases (91%). In this study, 37% (n= 32) of spina bifida patients also had hydrocephalus, which is considered an associated NTD, 40% (n=34) were noted to be without hydrocephalus and there were no specific record for 23% of the cases. Surgery to insert VP shunts had been performed for 97% of patients with hydrocephalus.

Table 4.1: Number and percentage of patients with types of spina bifida recorded

Diagnosis	**Number of patients**	**Percentage (%)**
Spina bifida only	12	13.95
Myelomeningocele	**39**	**45.35**
Meningocele	13	15.12
Encephalocele with meningocele	1	1.16
Lipomyelomeningocele	11	12.79
Lipomeningocele	10	11.63
Total	86	100

Bold font represents the highest percentage of the diagnosis

Table 4.2: Number and percentage of patients with spina bifida and level of lesion

Level of lesion	Number of patients	Percentage (%)
Thoracic	3	3.49
Thoracolumbar	6	6.98
Lumbar	**23**	**26.74**
Lumbosacral	18	20.93
Sacral	16	18.60
Sacrococcygeal	1	1.16
Not available	19	22.09
Total	86	100

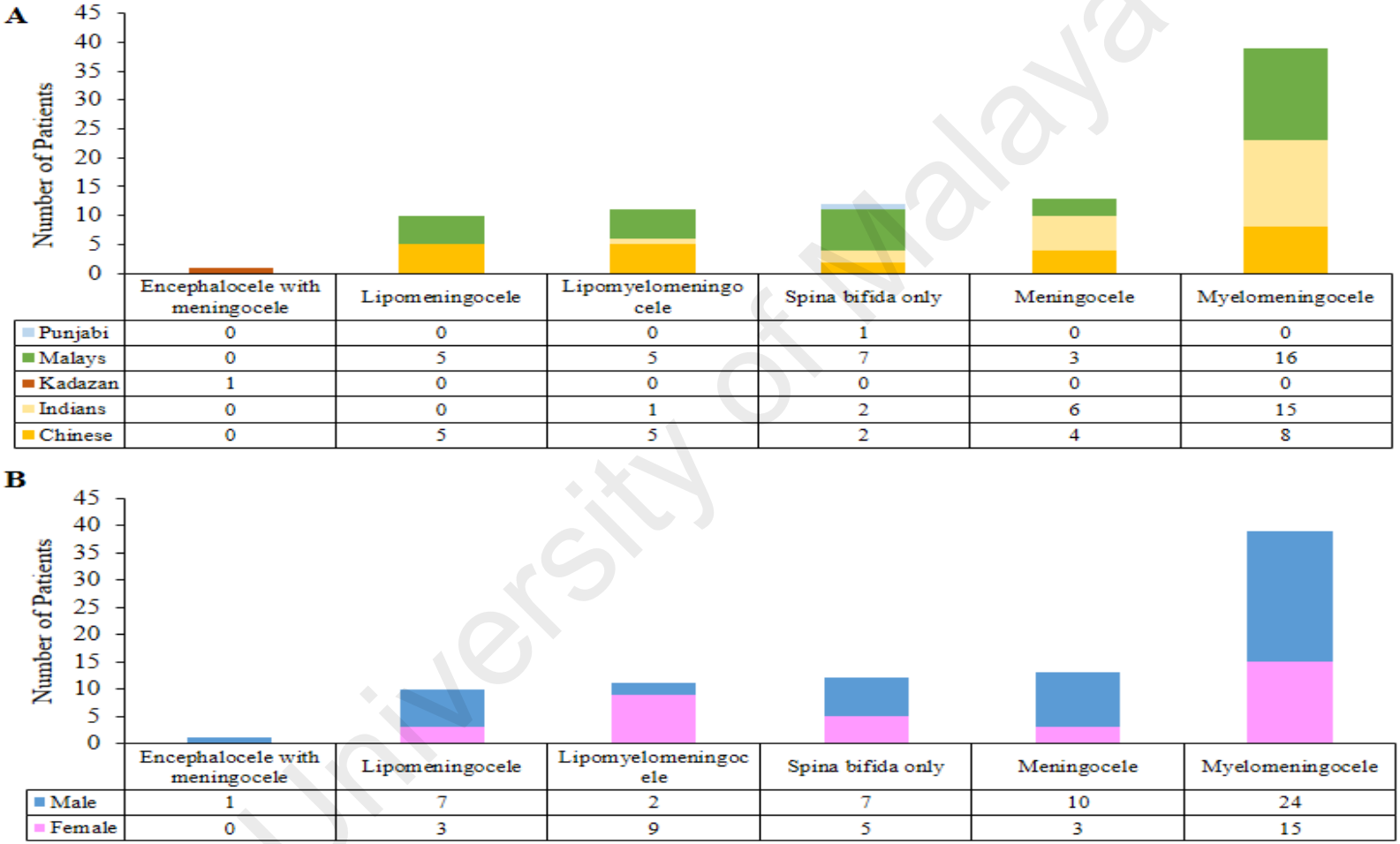
Bold font represents the highest percentage of the level of lesion

4.3.3.3 Mobility and Education

Out of the 86 patients in our cohort, 22 over 67 (32.84%) of patients between age 4 to 16 years old were captured in terms of mobility where they were able to ambulate independently using aids without having to depend on others. The rest of the data was not captured. A total of 22 out of 61 patients (36.07%) ranging from 5 to 16 years old have varying levels of education. Two patients (3.28%) age 7 and 14 years old were not attending school. The other 60.66% (n=37) patients were unaccounted for in terms of education.

4.3.3.4 Analysis of Diagnosis

There was no significant relationship ($p>0.05$) between the genders in comparison to the types of diagnosis. However, there was an association between ethnicity and the types of diagnosis ($p<0.05$) (Figure 4.3). No statistical correction analysis was performed in the present study. Nevertheless, it is highly suggested that the used of Bonferroni correction should be carried out for this small number of samples.



	Encephalocele with meningocele	Lipomeningocele	Lipomyelomeningocele	Spina bifida only	Meningocele	Myelomeningocele
Punjabi	0	0	0	1	0	0
Malays	0	5	5	7	3	16
Kadazan	1	0	0	0	0	0
Indians	0	0	1	2	6	15
Chinese	0	5	5	2	4	8

	Encephalocele with meningocele	Lipomeningocele	Lipomyelomeningocele	Spina bifida only	Meningocele	Myelomeningocele
Male	1	7	2	7	10	24
Female	0	3	9	5	3	15

Figure 4.3: Analysis of diagnosis. (A) Comparison between ethnicity and type of diagnosis. (B) Comparison between genders and type of diagnosis.

4.4 Discussion

This study aims to carefully navigate data obtained from patient records by unbiased filtering, followed by extrapolation of data to produce coherent and potentially revealing information which can be used fruitfully for the betterment of the quality of life of anencephaly and spina bifida patients. This study revealed the rate of prevalence of anencephaly and spina bifida in a major referral centre in Kuala Lumpur, leading ethnic group, maternal age, method of delivery, birth weight, birth term, gender, type of spina bifida, incidence of hydrocephalus and VP shunt insertion with spina bifida, types of defect and the level of lesion of spina bifida, including mobility and the level of education.

It is the nature of a retrospective study to extract data from records and run analysis on the available data. However, data retrieved was incomplete due to (a) information and clinical examinations provided by physician in the records are based on the urgency in attending treatments or procedure instead of diagnosis or cause of anencephaly and spina bifida (b) the 14 years records of patients referred to UMMC covers only the period of time the spina bifida patients were admitted at a later age and so, information regarding him or her during birth is unknown and; (c) discrepancy of patients in providing sufficient information to their physician. Nevertheless, the discussions will be based on the captured data from our records and any discrepancy has been mentioned and accounted for. Hence, we suggest a more standardized form of tabulating information for patient records including validation of imaging. Also, online links should be made from the patients' records to the medical imaging repository to ensure the information can be verified.

4.4.1 Prevalence of anencephaly and spina bifida in UMMC as an indicator for urban Malaysia

Data captured from the 20 patients of anencephaly (Figure 4.1A) revealed prevalence rate of 0.38 per 1000 live births while for the 86 spina bifida patients (Figure 4.2A), the prevalence ranged from 1.33 – 6.4 per 1000 live births that revealed a much higher prevalence rate than that which was previously recorded. However, in the case of anencephaly, the inclusion of TOP may potentially increase the number of occurrence. In this study, 25% of the cases were set for TOP as some of the anencephalic fetuses failed to survive in prenatal life. The prevalence rate obtained was unsurprisingly high owing to the fact that the data were retrieved from UMMC, which serve as a major referral hospital in Malaysia. A prospective cohort study of neonates with anencephaly and spina bifida using the data from the Malaysian National Neonatal Registry has shown a prevalence rate of 0.19 per 1000 births for anencephaly and 0.11 per live 1000 births for spina bifida (Boo *et al.*, 2013). Data captured from that study include patients born in 2009 from 32 Malaysian hospitals. In another study of a small population in the Kinta district, Malaysia reported an incidence of NTDs at 0.73 per 1000 births but does not specify the NTD phenotype (Thong *et al.*, 2005). The EUROCAT (European Surveillance of Congenital Anomalies) estimates the rate of anencephaly in Europe at 0.32 per 1000 births and spina bifida at 0.51 per 1000 live births from year 2003 to 2007 (Dolk *et al.*, 2010; Copp *et al.*, 2015). The frequency is found to be higher in the United States of America and United Kingdom (Lawrenson *et al.*, 2000; Parker *et al.*, 2010; Mohd-Zin *et al.*, 2017) but lower for the case of anencephaly (Boulet *et al.*, 2008; Canfield *et al.*, 2014). Meanwhile, certain region in China such as Shanxi Province has a much higher preponderance to these conditions than the other parts of the world (Chen *et al.*, 2009; Jin *et al.*, 2013; Zaganjor *et al.*, 2016). Since the Boo *et al.* (2013) study, there have not been any other studies on anencephaly and spina bifida in Malaysia. Therefore,

this study aimed at garnering current relevant data on the state of both types of NTDs in Malaysia. Result from this study revealed the prevalence rate of anencephaly and spina bifida to be similar according to that cited internationally which is 0.5 to 10 per 1000 live births for NTDs cases (Greene & Copp, 2014).

4.4.2 Distribution of anencephaly and spina bifida

Records show that maternal age during childbirth mostly affected those age below 35 years old (Figure 4.1D & Figure 4.2B). This finding maybe quite revealing in that the typical childbearing age in Malaysia is between 20 to 35 years old (Kaur & Singh, 2011; Rashed *et al.*, 2016), so it would make sense for the highest number to be in that particular age range. Nevertheless, the presented data suggests that healthy mothers at their ideal childbearing age are also affected and this may be due to genetic or environmental triggers (Marco, 2012). One caveat of this study is the lack of information about folic acid intake by the mothers. Although the Ministry of Health Malaysia recommends periconceptional folic acid supplementation to all pregnant women to promote healthy pregnancy (Division-of-Family-Health-Development, 2013), the intake of folic acid is not mandatory, thus it may give rise to a higher risk of NTD.

In addition, data shows that full term babies born with spina bifida were within the normal birth weight (Figure 4.2C) as indicated by the Pediatric and Pregnancy Nutritional Surveillance System, Center for Disease Control and Prevention (CDC). As for anencephaly, most of the babies were born prematurely and below the normal birth weight (Figure 4.1E & Figure 4.1F). There have been other studies that suggest low birth weights were greater in NTD offspring without specifying the phenotype (Mahadevan & Bhat, 2005; Norman *et al.*, 2012). According to the CDC, newborns should weigh more than 2.5kg and less than 4kg (Centers-for-Disease-Control- and-Prevention, 2009).

As for gender, anencephalic patients had almost similar number of males and females with a single case of unknown gender due to premature birth (Figure 4.1C). In the case of spina bifida, male patients hold more than half of the overall percentage, which was 59% (Figure 4.2D). The numbers of males were also higher in majority of the spina bifida subphenotypes (Figure 4.3B). According to Boo and co-workers (2013), males dominate the incidence of anencephaly at (53.7%) but have low occurrence in the case of spina bifida (38.1%). The discrepancy might be related to the geographical factor in the general Malaysian population. In year 2014 to 2016, Malaysia has 0.9 million more males compared to females (Ho, 2016). However, this result contradicts studies from other countries stating that females are more predisposed to NTDs compared to males (Seller, 1987; Brook *et al.*, 1994). Recent reports from Bangladesh also record a higher preponderance of spina bifida among males (Mazumdar *et al.*, 2015). The United Kingdom population-based study found the number of females is lesser than males in overall risk of congenital anomalies. Regardless of the phenomenon, this pattern appears to be reversed in overall NTD cases where females have a higher risk of NTDs at birth (Sokal *et al.*, 2014).

From those findings, Malay patients record the highest number of both anencephaly (Figure 4.1B) and spina bifida cases (Figure 4.2E) particularly in myelomeningocele and spina bifida only subtypes (Figure 4.3A). In the previous records by the National Birth registry, NTDs were highest among the Sarawak indigenous people and lowest among the Chinese (Boo *et al.*, 2013).

4.4.3 Types of spina bifida and the level of lesion

This data conforms to the global scenario of myelomeningocele, reported as the most common and severe form of spina bifida (Copp *et al.*, 2015) (Table 4.1).

Myelomeningocele is commonly associated with hydrocephalus and encephalocele (Copp *et al.*, 2015). Thus, surgical intervention is required for myelomeningocele patients to cover the exposed spinal cord in order to prevent infection and insertion of VP shunt to treat hydrocephalus where necessary (Adzick, 2013). Lesions occurred mostly at the lumbar region (Table 4.2) as previously reported by 'The Spina Bifida Research Resource' (SBRR) (Mitchell, 2008). This data tallies with the United States of America.

In addition, syndromic spina bifida was reported in 9% of the total number of patients. The represented phenotypes include autism and 48XY (intra-abdominal gonads). Unfortunately, information about karyotype analysis is limited, as they were not provided in the medical records to confirm the diagnosis. Data on antenatal ultrasound to detect spina bifida was also not available in the present study. Ultrasound examination during prenatal check-ups is used in the early detection of spina bifida (Trudell & Odibo, 2014). However, it is not always accurate and sometimes fail to diagnose spina bifida especially the occulta type (Alfirevic, 2005).

4.4.4 Education, Mobility and the Issue of Management of spina bifida

This data shows that a proportion of spina bifida patients pursued education (36.07%, n=22/61 of patients ranging from 5 to 16 years old). Most of them are capable of enrolling in the national curriculum and participate in the process of learning. Despite that, two patients were not attending school due to unknown reason and more than half of the number of patients were unaccounted for in terms of education. Studies of Malaysian school-aged children with physical disability including spina bifida identified numerous restrictions in achieving education ranging from managing urinary or bowel incontinence, dependent mobility, inaccessible school facilities, and societal or

environmental barrier (Ong *et al.*, 2002; Khoo *et al.*, 2009). Mobility which was achieved by 32.84% (n=22/67) of patients ranging from 4 to 16 years old aided by wheelchairs, crutches, and ankle-foot orthosis are important to retain patients' mobility (Calhoun *et al.*, 2013). There is correlation between mobility and the level of lesion. Higher level of lesion leads to more mobility difficulties such as dependant ambulation, imbalance and use of mobility aids compared to patients with lower level of lesion (Ong *et al.*, 2002). Besides that, most of spina bifida patients are diagnosed with neurogenic bladder dysfunction and they require proper bladder management (Jong *et al.*, 2008). Although lack of data on the bladder management among the patients was obtained, the use of clean intermittent catheterization (CIC) is mainly utilized in the prevention of kidney damage (Campbell *et al.*, 2004). It is noteworthy that different spina bifida patients need different treatment and management as it depends on the level of lesion and the type of diagnosis. Front liners among the medical community for example emergency room doctors and general practitioners as well as obstetricians and gynaecologists should be continuously educated in the management of spina bifida as it is a common condition. Then only can patients and parents be trained as early as possible so that the patients' environment can be inclusive and that they are able to eventually live independently.

4.5 Conclusion

This study shows that the prevalence rate of anencephaly and spina bifida are higher compared to previously published record in Malaysia. Based on our data, we found that certain well- accepted norms such as this incidence of TOP following low survival rate of the anencephalic patients with the postnatal life. For the case of spina bifida, myelomeningocele was more common, lesion usually occurred at lumbar region, and higher occurrence of non-syndromic spina bifida compared to syndromic spina bifida are applicable to the Malaysian urban scenario. We urge a closer and deeper understanding of the etiology of anencephaly and spina bifida and suggest that the UMMC cohort may be useful for the understanding of these types of NTD. More studies involving the latest occurrence of anencephaly and spina bifida encompassing all of Malaysia are necessary and paramount to further understand these common central nervous system malformations.

CHAPTER 5: SCREENING FOR REPORTED SPINA BIFIDA RISK GENES IN THE PROBANDS

5.1 Introduction

The genetic involvement of spina bifida is complex, sporadic and was estimated to have 60% heritability (Bassuk & Kibar, 2009). However, the genetics of human spina bifida remains largely undetermined despite the identification of 74 mouse models with spina bifida (Harris & Juriloff, 2010). The study of genetics is important for the identification of causal, risk or associated factors which contribute to the aetiology of spina bifida. To date, the folate-related gene analysis, candidate gene approach and potential candidate genes based on animal studies are among the current focus of genetic studies for human NTDs (Bassuk & Kibar, 2009; Copp *et al.*, 2013; Copp *et al.*, 2015). Thus far, the most studied human spina bifida risk variant is the methylenetetrahydrofolate reductase (*MTHFR)* C677T (rs1801133). Despite that, this variant was not well replicated in many populations worldwide indicating the variant is not likely to be a major contributor of NTDs (Shields *et al.*, 1999; Gonzalez-Herrera *et al.*, 2002; Copp & Greene, 2010). Using PCR and Sanger sequencing, this chapter describes results of screening the *MTHFR* variant (c. C677T) in 11 probands (Table 5.1) to determine the frequency of this variant in our patient cohort. In addition, we also screened other variant in the *MTHFR* gene, A1298C that is the second most studied variant which has yet to confer any significant risk of NTDs (Zhang *et al.*, 2013). Screening of the c.A1298C variant was conducted using whole-exome datasets in three representative probands subset of the 11 probands (Figure 5.1). Those three probands represent (1) syndromic spina bifida aperta, (2) non-syndromic spina bifida aperta and (3) non-syndromic spina bifida occulta. All of which were selected on the availability of exome datasets.Another reported human spina bifida genetic risk factors include the

planar-cell polarity (PCP) genes such as *VANGL1*, *CELSR1*, *SCRIB* and *DVL1*, genes that regulates one-carbon metabolism, and the NTD mouse orthologous genes; *ZIC3* and *PAX3* (Gebbia *et al.*, 1997; Brown *et al.*, 2004; Robinson *et al.*, 2012; De Marco *et al.*, 2014). Following a comprehensive list of spina bifida risk genes reviewed by Greene co-workers (2009) (Table 2.4), this chapter also screened previously reported spina bifida risk genes using exome datasets of the three probands. This work serves as the primary screening process before the identification of new potential candidate variant(s) in our cohort.

5.2 Materials and Methods

5.2.1 Samples used for the screening of reported spina bifida risk genes

DNA of 11 probands were used for the screening of *MTHFR* variant (c.C677T) (Table 5.1), whereas exome datasets of three probands (SB5A, SB7A and SB17A) subset to the 11 (Figure 5.1) were screened for other previously reported spina bifida risk variants in 40 genes including the second *MTHFR* variant, A1298C.

Table 5.1: Details of the 11 probands in our cohort

Sample ID	Gender	Ethnicity	Spina bifida type (aperta/ occulta)	Syndromic/ Non-syndromic
SB1A	Female	Malay	Occulta	Non-syndromic
SB2A	Female	Malay	Occulta	Non-syndromic
SB3A	Female	Malay	Occulta	Non-syndromic
SB4A	Male	Indian	Aperta	Non-syndromic
SB5A	Female	Indian	Aperta	Non-syndromic
SB7A	Male	Malay	Occulta	Non-syndromic
SB13A	Male	Chinese	Aperta	Non-syndromic
SB17A	Female	Malay	Aperta	Syndromic
SB25A	Male	Indian	Occulta	Non-syndromic
SB27A	Male	Chinese	Aperta	Non-syndromic
SB31A	Female	Malay	Occulta	Non-syndromic

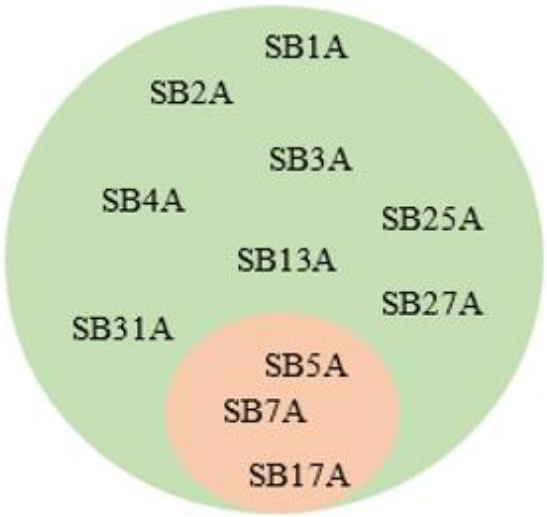


Figure 5.1: Venn diagram of three probands (SB5A, SB7A and SB17A) subset to a total number of 11 probands. DNA of the 11 probands were used for PCR and Sanger sequenced of MTHFR C677T variant while exome datasets of the three probands were used for the screening of other previously reported spina bifida risk variants.

5.2.2 Screening of the *MTHFR* variants

For c.C677T screening, we examined 11 cases whereby those cases were PCR and Sanger sequenced for verification as there were no exome datasets available from most of the probands. Primers and PCR conditions were as described in Chapter 3, Table 3.1, 3.2 and 3.3. The second *MTHFR* variant (c.A1298C) were screened using three representative probands (SB5A, SB7A and SB17A) whom have been exome sequenced as explained in Chapter 3. Filtering of the exome datasets were conducted through excel file (Appendix A) to look for the c.A1298C variant.

5.2.3 Screening for the other reported spina bifida genes in three probands

This analysis was also performed in the three representative spina bifida probands using their respective whole-exome datasets. We filtered the dataset of WES through the excel file to look for the previously reported variants in spina bifida risk genes as listed in Chapter 2, Table 2.4. In total, this screening involved 107 variants representing 40 genes. Out of the 107, only non-synonymous variants were selected and those variants

were subjected to bioinformatics analysis to determine their level of pathogenicity as described in Chapter 3.

5.3 Results

5.3.1 *MTHFR* variants C677T and A1298C are not common in our cohort

The C677T variant was only heterozygous in SB5A and SB13A where both were spina bifida aperta. This variant appeared to be wild type in the rest of the studied probands (Figure 5.2). The C677T variant was highly pathogenic predicted by all the bioinformatics analysis (Table 5.2). Meanwhile the A1298C was heterozygous in SB5A and SB7A whereas SB17A was genotypically wild type. This variant has potentially damaging effect predicted by Provean (Table 5.2).

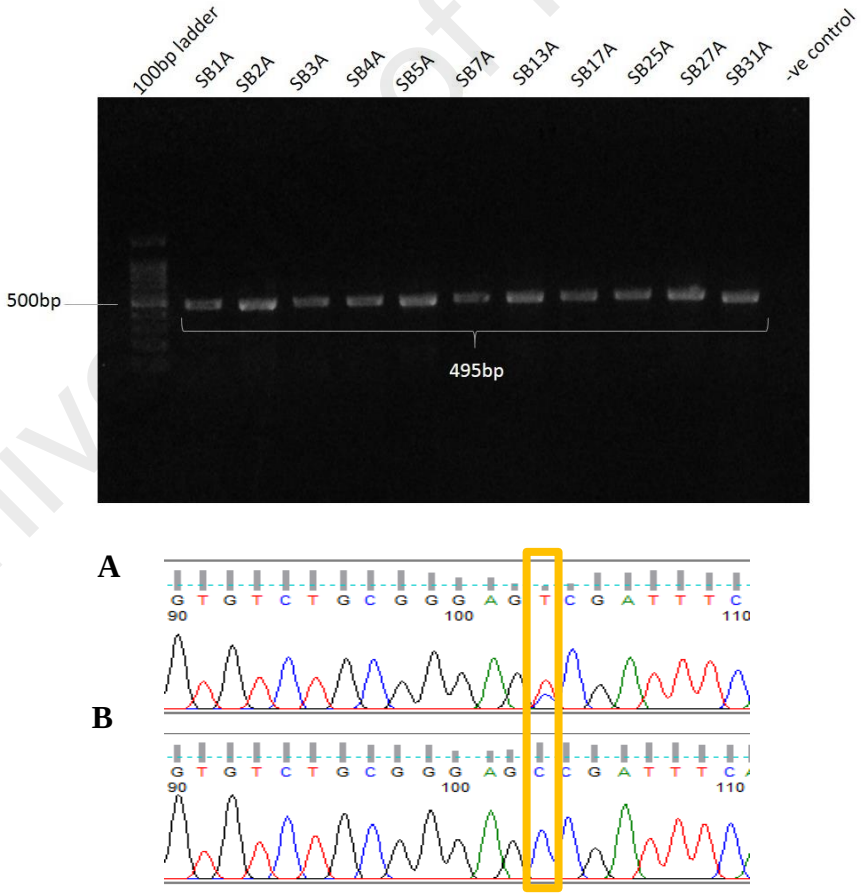


Figure 5.2: PCR and sequencing electropherograms of the C677T variant. Yellow box indicates presence of C or T at position in heterozygous and wild-type. (A) Heterozygous of the variant found in SB5A and SB13A. (B) Wild-type appearance of the variant in other probands (SB1A, SB2A, SB3A, SB4A, SB7A, SB17A SB25A, SB27A, and SB31A).

5.3.2 Identification of variants in other folate metabolism genes

In total there were seven variants screened representing four different genes of folate metabolism with the exclusion of *MTHFR* variants (Appendix B). Only one non-synonymous variant (*MTHFD1:* rs2236225; c.G1958A) was identified which was predicted to be non-deleterious.

5.3.3 Identification of variants in one-carbon metabolism genes

Overall, 28 variants in eight genes involved in one-carbon metabolism were screened (Appendix B). Nine were found to be non-synonymous variants and only three were predicted to be deleterious. The first variant (*ALDH1L1*: rs1127717; c.A2378G) was heterozygous in SB5A and SB7A. The second variant (*MTRR:* rs1801394; c.A66G) was homozygous in SB5A and SB7A. Variant of *SARDH* (rs2073817: c.G1841A) was found to be homozygous in SB17A (Table 5.2).

5.3.4 Identification of variants in glucose metabolism genes

A total of seven variants in four glucose metabolism genes were screened (Appendix B) by which one non-synonymous variant of the *XPD* gene (rs1799793; c.G934A) was predicted to be deleterious and heterozygous in SB5A. This variant is wild type in the two other probands.

5.3.5 Identification of variants in DNA repair and DNA methylation genes

A total of three variants in three genes involved in the process of DNA repair and DNA methylation were screened (Appendix B) by which two non-synonymous variants were found in the *XDP* gene. One of the variant (rs1799793; c.G934A) found in SB5A was predicted to be deleterious according to Provean.

5.3.6 Identification of variants in folate transport genes

For the folate transport genes, 19 variants were screened (Appendix B) and three possibly pathogenic non-synonymous variants were identified in the *CUBN* gene (rs2271462; c.G5518A, rs1801239; c.A8950G and rs1801232; c.C10656A). All three variants were heterozygous in one or two probands.

5.3.7 Identification of variants in PCP genes

A total of 17 variants in five PCP genes were reported as the risk factors for spina bifida (Appendix B). However, all variants were wild type in our cohort.

5.3.8 Identification of variants in other NTD-relevant genes

A total of 15 variants in seven other NTD-relevant genes were screened (Appendix B) and one non-synonymous variant of *PCMT1* (rs4816: c.G532A) was identified. However, this variant was non-deleterious as predicted by the bioinformatics analysis.

5.3.9 Identification of the reported variant in mouse mutant NTD genes

Screening of nine variants in seven mouse mutant NTD genes identified one non-synonymous variant of the *BRCA1* (rs1799966: c.A4837G) with potential damaging effect. This variant was heterozygous in SB17A.

Table 5.2: Exome analysis on non-synonymous variants associated with human spina bifida in the three probands

No	Gene	Reported SNP	cDNA Variant	Amino acid change	SB5A	SB7A	SB17A	Deleterious prediction			
								SIFT	PROVEAN	POLYPHEN 2 (HumDiv)	POLYPHEN 2 (HumVar)
	Folate metabolism										
1	*MTHFD1* (methylenetetrahydrofolate dehydrogenase 1)	rs2236225	c.G1958A	p.Arg653Gln	Wt	Wt	Het	Tolerated	Neutral	Benign	Benign
2	*MTHFR* (methylene tetrahydrofolate reductase)	rs1801133	c.C667T	p.Ala222Val	Het	Wt	Wt	Damaging	Deleterious	Probably damaging	Probably damaging
		rs1801131	c.A1298C	p.Glu429Ala	Het	Het	Wt	Tolerated	Deleterious	Benign	Benign
	One carbon metabolism										
3	*ALDH1L1* (Aldehyde Dehydrogenase 1 Family Member L1)	rs1127717	c.A2378G	p.Asp793Gly	Het	Het	Wt	Tolerated	Deleterious	Benign	Benign
4	*BHMT* (Betaine--Homocysteine S-Methyltransferase)	rs3733890	c.G716A	p.Arg239Gln	Wt	Het	Het	Tolerated	Neutral	Benign	Benign
5	*MTRR* (methionine synthase reductase)	rs1801394	c.A66G	p.Ile22Met	Homo	Wt	Homo	Damaging	Neutral	Probably damaging	Probably damaging
		rs162036	c.A1049G	p.Lys350Arg	Wt	Homo	Wt	Tolerated	Neutral	Benign	Benign
		rs10380	c.C1783T	p.His595Tyr	Wt	Homo	Wt	Tolerated	Neutral	NA	NA
		rs1532268	c.C524T	p.Ser175Leu	Het	Wt	Het	Tolerated	Neutral	Benign	Benign
6	*NOS3* (Nitric Oxide Synthase 3)	rs1799983	c.G894T	p.Glu298Asp	Homo	Homo	Homo	Tolerated	Neutral	Benign	Benign
7	*SARDH* (Sarcosine Dehydrogenase)	rs2073817	c.G1841A	p.Arg614His	Wt	Wt	Homo	Tolerated	Neutral	Probably damaging	Possibly damaging
		rs886016	c.A1942G	p.Met648Val	Wt	Wt	Homo	Tolerated	Neutral	Benign	Benign

Table 5.2, continued

No	Gene	Reported SNP	cDNA Variant	Amino acid change	SB5A	SB7A	SB17A	Deleterious prediction			
								SIFT	PROVEAN	POLYPHEN 2 (HumDiv)	POLYPHEN 2 (HumVar)
	Glucose metabolism										
8	*LEPR* (Leptin Receptor)	rs1137101	c.A668G	p.Gln223Arg	Het	Het	Homo	Tolerated	Neutral	Benign	Benign
		rs1137100	c.A326G	p.Lys109Arg	Homo	Homo	Wt	Tolerated	Neutral	Benign	Benign
	DNA repair and DNA methylation										
9	*XPD/ ERCC2* (Excision Repair Cross-Complementing Rodent Repair Deficiency2)	rs13181	c.A2251C	p.Lys751Gln	Het	Wt	Wt	Tolerated	Neutral	Benign	Benign
		rs1799793	c.G934A	p.Asp312Asn	Het	Wt	Wt	Tolerated	Deleterious	Benign	Benign
	Folate transport										
10	*CUBN* (Cubilin)	rs1801231	c.C4675T	p.Pro1559Ser	Wt	Homo	Wt	Tolerated	Neutral	Benign	Benign
		rs2271462	c.G5518A	p.Gly1840Ser	Wt	Wt	Het	Tolerated	Deleterious	Probably damaging	Benign
		rs3740168	c.C7724G	p.Pro2575Arg	Wt	Wt	Het	Tolerated	Neutral	Benign	Benign
		rs1801239	c.A8950G	p.Ile2984Val	Het	Wt	Wt	Tolerated	Neutral	Possibly damaging	Benign
		rs1801232	c.C10656A	p.Asn3552Lys	Wt	Het	Het	Tolerated	Neutral	Possibly damaging	Benign
	Other NTD-relevant gene										
11	*PCMT1* (Protein-L-Isoaspartate (D-Aspartate) O-Methyltransferase)	rs4816	c.G532A	p.Val120Ile	Het	Homo	Homo	Tolerated	Neutral	Benign	Benign
	NTDs in mouse mutant										
12	*BRCA1* (Breast Cancer 1)	rs1799966	c.A4837G	p.Ser1613Gly	Wt	Wt	Het	Damaging	Neutral	Benign	Benign

5.4 Discussion

This chapter involved screening the WES datasets of the spina bifida patients for 107 variants in 40 reported spina bifida risk genes as reviewed in Greene *et al.* (2009). The exome datasets of three probands in our cohort were used by which only non-synonymous variants were analysed. Non-synonymous variants are believed to have significant effect on phenotype as they are responsible for almost half the percentage of disease-causing mutations (Ramensky *et al.*, 2002; Stenson *et al.*, 2014). However, it is also important to differentiate the pathogenic variants from those that are benign (Gosalia *et al.*, 2017). Therefore the interpretation of spina bifida candidate risk variants in this study was conducted by first selecting the non-synonymous variants with possible pathogenic effect. From those number variants, only 23 non-synonymous variants in 12 genes were selected and 10 variants were predicted to be possibly pathogenic according to the bioinformatics analysis (Table 5.2). Thus, this section focuses on the discussion of those 10 pathogenic non-synonymous variants.

5.4.1 *MTHFR* and other variants in folate metabolism genes are not common in this spina bifida cohort

Periconceptional folic acid supplementation helps in reducing the occurrence and recurrence risk of NTD (Pitkin, 2007; Imbard *et al.*, 2013). Folic acid must be converted into tetrahydrofolate (THF) which is an important component of the methylation cycle in order to perform its function during the metabolism of folate (Zhang *et al.*, 2013). In parallel, inhibition of the folate metabolic pathway is relevant as it increases the risk of NTD. Therefore, variations of folate metabolism genes are worthy of investigation (Imbard *et al.*, 2013; Zhang *et al.*, 2013). *MTHFR* is a gene that promotes the production of methylenetetrahydrofolate reductase enzyme whereby reduced activity of this enzyme is associated with high homocysteine levels which has been shown to cause increased

NTDs risk (van der Put *et al.*, 1995; Imbard *et al.*, 2013).

The well-studied *MTHFR* variant, C677T resulting in alanine to valine substitution in the N-terminal catalytic domain of the protein causes thermal instability, which leads to a suboptimal function of this enzyme (Zhang *et al.*, 2013; Yang *et al.*, 2015). This highly pathogenic variant thus far serves as the strongest candidate for a potential worldwide spina bifida screening gene (Greene *et al.*, 2009). There exists two heterozygous mutations within the C677T candidate variant in our cohort of 11 probands, which have been subsequently PCR, and Sanger sequenced for verification. This finding suggests genetic heterogeneity exists within our spina bifida cohort. The C677T variant was previously reported among non-Latin population (Amorim *et al.*, 2007), Irish (Doudney *et al.*, 2009), British (Doudney *et al.*, 2009), mixed US (Shaw *et al.*, 2009) and Italian (de Franchis *et al.*, 2002) but not in the Dutch (Franke *et al.*, 2009), Turkish (Boduroglu *et al.*, 1999), French (Mornet *et al.*, 1997) and German populations (Koch *et al.*, 1998). Screening of this variant in the normal population was not included in the present study. Nevertheless, the frequency of the variant in our patient cohort is low when comparing the MAF of the variant in this study (18.18%) and the 1000 Genome Database (24.54 %).

The two probands with heterozygous C677T variant in our cohort were of Chinese and Indian ethnicity respectively whereby both patients were diagnosed with spina bifida aperta. Ethnicity was not considered a potential contributory factor as screening of this variant only involved a small number of patients in our cohort. The heterozygote form of this variant was more prominent in spina bifida aperta which was equivalent to most studies whom used aperta group as their patient recruitment (de Franchis *et al.*, 1998; Koch *et al.*, 1998; Kirke *et al.*, 2004). This is parallel to the strong genetic components of

spina bifida aperta compared to spina bifida occulta in mouse mutant study (Harris & Juriloff, 2007). The T allele homozygosity was actually the 'thermolabile genotype' while only heterozygous form of the variant was notified in our study. However, recent review of *MTHFR* study identified a 30.8% risk of developing NTD in an individual with heterozygous C677T variant (Yang *et al.*, 2015). This might be attributed to the 35% abnormal enzyme activity and 10% lower red blood cell level of folate in heterozygotes (CT) individuals (Molloy *et al.*, 1998; Yang *et al.*, 2015).

The second most studied *MTHFR* variant is rs1801131 (c.A1298C) but has not been determined as spina bifida risk factor (Gonzalez-Herrera *et al.*, 2002; Franke *et al.*, 2009). This variant causing amino acid substitution from glutamate to alanine is localized within the regulatory C-terminal domain and has deleterious effect according to Provean (Table 5.2) (Zhang *et al.*, 2013). Using whole-exome datasets of three selected probands, we identified heterozygous A1298C variant in SB5A. Interestingly, this patient is a compound heterozygote for both *MTHFR* variants. Apart from the C677T and A1298C, no other non-synonymous pathogenic variants were identified either in *MTHFR* or the other genes involved in folate metabolism.

5.4.2 Several variants were identified in genes involved in one-carbon metabolism

Analyses of genes involved in one-carbon metabolism have been the primary focus following the preventive action of folic acid (Copp & Greene, 2010). Folate one-carbon metabolism regulates the homocysteine, vitamin B, methionine, and also DNA methylation (Franke *et al.*, 2009). The *ALDH1L1* was the component of one-carbon metabolism pathway that converts 10-formyltetrahydrofolate to tetrahydrofolate (Krupenko *et al.*, 2010). Variant of this gene (rs1127717; c.A2378G) located at the aldehyde dehydrogenase family domain, might reduce enzyme activity via the amino acid

substitution from aspartic acid to glycine (Krupenko *et al.*, 2010). Provean revealed a deleterious effect of this variant and was found to be heterozygous in both SB5A and SB7A (Table 5.2). Previous studies linked the association of this variant with spina bifida risk in Dutch (Franke *et al.*, 2009) but not in Han Chinese population (Wu *et al.*, 2016).

Other variant was identified in *MTRR*, a key regulator enzyme that is involved in homocysteine metabolic pathway. Down regulation of this gene inhibit MTR activity which is the most important element in the methionine cycle (Olteanu & Banerjee, 2001). A non-synonymous variant of this gene (rs1801394; c.A66G) located within the flavodoxin domain of the *MTRR* (Zhang et *al.*, 2014) was predicted to be highly pathogenic according to SIFT and Polyphen 2. This variant has been reported to elevate the risk of spina bifida in Canadian (Wilson *et al.*, 1999), South Italy (Gueant-Rodriguez *et al.*, 2003) and Hispanic (Zhu *et al.*, 2003) but not in other studies that includes British (Relton *et al.*, 2004) and mixed USA population (Shaw *et al.*, 2009). Analysis in mouse identified *Mtrr* expression between days E8.5 to E10.5 which is equivalent to the period of neurulation in mouse embryo (Copp *et al.*, 2003).

Besides that, another non-synonymous variant was also identified in the *SARDH*, an enzyme that serves as one-carbon unit supplier to the folate metabolism and is involved in the breakdown of choline that was related with increased NTD risk (Shaw *et al.*, 2004; Enaw *et al.*, 2006; Franke *et al.*, 2009). Variant of *SARDH* (rs2073817: c.G1841A) was found to be homozygous in SB17A and located at one of the protein functional domain, the aminomethyltransferase folate-binding domain (Piao *et al.*, 2016). This variant has possible pathogenic effect according to Polyphen 2 and associated with increase spina bifida risk in Dutch (Franke *et al.*, 2009) and Han Chinese population (Piao *et al.*, 2016).

5.4.3 Non-synonymous variant identified in *XPD*, a gene that involved in DNA repair and DNA methylation

A heterozygous non-synonymous variant in *XPD* gene (rs1799793; c.G934A) was identified which was located at the protein functional domain, the DEAD-like helicases superfamily resulting amino acid change from aspartic acid to asparagine (Kuper *et al.*, 2014). This variant was predicted to have pathogenic effect according to Provean (Table 5.2). Theoretically, mutation of the *XPD* gene might result in fetal abnormalities including spina bifida as the gene is involved in the process of repairing damaged DNA (Olshan *et al.*, 2005). In the early embryonic development, the activity of this gene is crucial particularly during cell proliferation period to avoid abnormal cell growth, which might lead to embryological malformations (Olshan *et al.*, 2005). However, there has been no study that examined this relationship despite this biological plausibility. Previous study identified high frequency of these two variants among the non-Hispanic spina bifida patients (Olshan *et al.*, 2005).

5.4.4 Several variants identified in the *CUBN*; the folate transport gene

The *CUBN* gene is a protein receptor for intrinsic factor–vitamin B12 (Kozyraki *et al.*, 1999; Franke *et al.*, 2009). This vitamin act as a cofactor in the folate metabolism whereby low amount of this vitamin in pregnant mothers have been linked to spina bififda in children (Franke *et al.*, 2009). We identified three non-synonymous variants in *CUBN* (rs2271462; c.G5518A, rs1801239; c.A8950G and rs1801232; c.C10656A). All three variants were located at functional CUB domain 12, 22 and 27 respectively (Shaik *et al.*, 2013). Amino acid substitutions caused by these variants have possible pathogenic effect as predicted by Provean and Polyphen 2. While there were many *CUBN* variants associated with spina bifida risk, the only variant identified in Dutch population was the rs1907362 (Franke *et al.*, 2009) by which all of our patients were wild type. Two other

variants of the same occurring gene (rs7070148 and rs2273737) were observed to be significantly associated with maternal NTD risk in Irish population (Pangilinan *et al.*, 2012). Also, the three probands were wild type for these variants. Extra analysis was done in their respective mothers whereby only mother of SB5A (SB5B) was heterozygous for rs2273737 variant.

5.4.5 Variant found in *BRCA1* the mouse orthologous human spina bifida gene

The *BRCA1* gene is a tumor suppressor gene (Silver & Livingston, 2012) whereby evidence of increased risk of breast and ovarian cancer in humans exists due to mutation of the gene (Welcsh & King, 2001). We found one non-synonymous variant of this gene (rs1799966: c.A4837G) that was predicted to be deleterious by SIFT (Table 5.2). However, this variant locus is not in the protein functional domain, indicating that the variant has no effect on *BRCA1* function. This variant has been associated with spina bifida in mixed USA populations (King *et al.*, 2007). In an animal study, homozygous *Brca1* mutant embryos were presented with spina bifida and anencephaly indicating this gene was critical for the normal embryonic development and was implicated with varying degrees of NTDs (Gowen *et al.*, 1996). In another study, exencephalic phenotype was observed in all mutant *Brca1* mouse (Wang *et al.*, 2004). Besides that, another member of *Brca* gene family, *Brca2*, was determined as the NTD associated gene whereby mutation of *Brca2* was associated with abnormal neural tube closure and exencephaly (Salbaum & Kappen, 2010).

5.5 Conclusion

This chapter attempted to investigate the potential genetic cause of spina bifida through screening of the 40 reported spina bifida risk genes as reviewed in Greene *et al.* (2009) in our patient cohort. We managed to identify 10 non-synonymous variants in seven different genes with potential pathogenic effects. Two identified non-synonymous pathogenic variants were the *MTHFR* C677T and A1298C. Others were in *ALDH1L1*, *MTRR, SARDH, XPD, CUBN* and *BRCA1* genes. The identified variants might be the risk factor for spina bifida patients individually, but do not represent as common variants within the cohort. In addition, apart from those 40 genes, there may be many other genes as well as variants that may play role in the genetic etiology of spina bifida. The contribution of these variants needs to be examined further including the segregation in the triads and in larger sample size to investigate this risk factor.

CHAPTER 6: SCREENING FOR VARIANTS IN THREE REPRESENTATIVE SPINA BIFIDA PATIENTS

6.1 Introduction

Despite the extensive efforts to identify genetic causes of spina bifida, convincing data of common and large effect genes is scarce. Genes in the folic acid metabolism pathways and candidate genes from animal studies are only linked to distinct cohorts of spina bifida and are not well replicated across the world (Bassuk & Kibar, 2009; Zhang *et al.*, 2013). Clearly, using a candidate gene approach has led to limited success thus there is a need for a better screening and identification approach (Lemay *et al.*, 2015). Recently, the used of whole-exome sequencing (WES) become the most popular method in the genetics community (Nayarisseri *et al.*, 2013). WES permits the identification of *de novo* variants which play an important role in human diseases and constitute the most extreme form of rare variants (Veltman & Brunner, 2012). Studies using WES have shown that between 60 to 75% of all sporadic cases like X-linked disorders resulted from *de novo* mutations (Yang *et al.*, 2014; Acuna-Hidalgo *et al.*, 2016). In addition, *de novo* mutations approach is valuable for identifying the common cause of neurodevelopmental diseases and birth defects (Veltman & Brunner, 2012). Therefore, this approach will be useful to identify the candidate variant for sporadic cases of spina bifida.

This chapter aims to identify potential candidate genes for spina bifida in three patients diagnosed varying spina bifida clinical phenotype. Patient 1 has the most severe of spina bifida (syndromic spina bifida aperta) whereby the lesion is not covered with skin and left exposed to the environment. The spina bifida phenotype co-exists with variant Turner syndrome. Spina bifida has also been previously reported to co-exist with

other conditions such as Jarcho-Levin Syndrome, X-linked heterotaxy, and DiGeorge Syndrome (Palacios *et al.*, 1993; Fryns *et al.*, 1996; Gebbia *et al.*, 1997; Gutierrez-Angulo *et al.*, 2002; Dane *et al.*, 2007). Patient 2, with non-syndromic spina bifida aperta, has similar phenotype as in Patient 1 but with the absence of other defect or syndrome. Patient 3 has the least severe phenotype, with a closed lesion that is covered with skin and no associated syndrome.

Spina bifida is aetiologically heterogeneous which is caused by both genetic and environmental factors (Greene & Copp, 2014). It is important to make a distinction between syndromic and non-syndromic spina bifida. Noteworthy, syndromic spina bifida has more well-defined genetic causative components. Meanwhile non-syndromic spina bifida, regardless of the aperta or occulta types, are attributed to multifactorial conditions, both genetic and environmental (Figure 6.1) (Copp *et al.*, 2015).

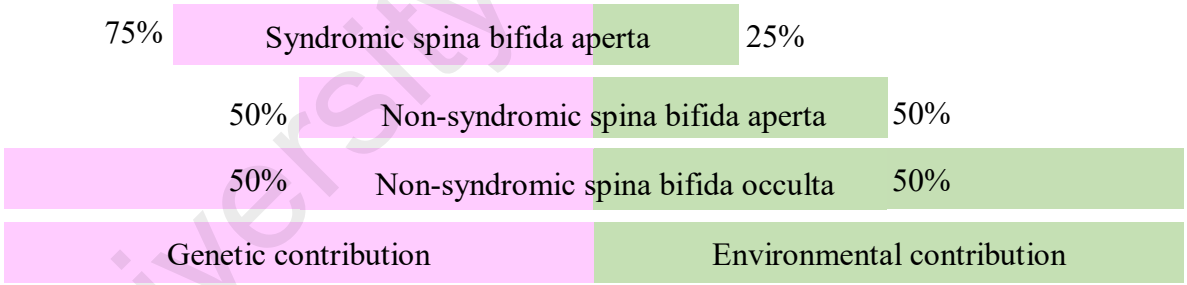


Figure 6.1: Types of spina bifida with varying degree of genetic and environmental contributions.

6.2 Materials and methods

As mentioned in Chapter 3, the strategy for this part of the work done using WES dataset is to identify possible candidate variants. Patients-parents trios (triads) were screened. Through a series of filtering steps to reduce the number of variants, only non-synonymous *de novo* variants with minor allele frequencies (MAF) <0.1 were selected for further analysis in order to identify the more likely pathogenic variants. This was

because the non-synonymous mutations causes amino acid substitutions, which is predicted to have the highest pathogenic effect on phenotype. Low MAF indicates variants are not likely to be a common polymorphism in worldwide populations. The variants were then tested for their predicted pathogenic effect on protein function using SIFT, Provean and Polyphen 2. Next, the candidate variants were amplified by PCR and Sanger sequenced for validation. However, only the variant found in Patient 2 was PCR validated in 50 unaffected Malaysian controls.

As syndromic spina bifida has more complex genetic involvement (Harris & Juriloff, 2007), all rare and highly pathogenic variants identified in Patient 1 were PCR validated. On the other hand, the selection of potential candidate variants in Patient 2 and Patient 3 were based on the reported association with NTDs. The selected variants were then analysed for their pathogenic effect using bioinformatics tools and were PCR validated.

6.2.1 Whole embryo culture using antisense oligonucleotide phosphorothioate technology for the understanding of gene identified in Patient 2

In order to further understand the potential role of *Sec63* in NTD, we opted an antisense oligonucleotide technology using whole embryo culture in mouse embryo. This method helps to study the effect of gene perturbation by the binding of the antisense nucleotide to its targeted mRNA and mediates the degradation of RNA through RNase-H activity hence inhibits the normal gene function (Kole *et al.*, 2012). This natural oligonucleotides probe however is quickly digested by nucleases in both in *vivo* and *in vitro.* Alternatively, addition of phosphorothioate to the oligonucleotide bonds help to modify the probes to be resistant to nucleases and activate RNase-H pathways (Kole *et al.*, 2012). The antisense technology has been utilized to disrupt normal expression of the

MTHFR (methylenetetrahydrofolate reductase) gene by which NTDs phenotypes were observed in the antisense-injected mouse embryos. In addition, decreased level of mRNA for *MTHFR* was identified in embryos treated with the antisense sequence suggesting the ability of the antisense sequence to decrease gene expression (Hansen *et al.*, 2001).

6.2.1.1 Methods for whole embryo culture study

Timed-mated pregnant ICR mice were maintained at Animal Bio Safety Level 2 Lab (ABSL-2) Lufter Sdn. Bhd. (Universiti Kebangsaan Malaysia, Malaysia) and were given Altromin standard diet and water ad libitum. The pregnant mice were euthanised via cervical dislocation, the gravid uteri were removed on the early embryonic day 9.5 (E9.5). The dissected embryos were free from maternal tissue and of Reichert's membrane with intact ectoplacental cone and yolk sac. The dissection was done in *Dulbecco's Modified Eagle Medium* (DMEM) solution (Nacalai Tesque, JAPAN) containing 10% fetal calf serum (Sigma, USA) under aseptic conditions at room temperature. Embryos were placed in culture tubes containing 1 ml pre-warmed rat serum at 37˚C. The culture tubes were filled with mixture of 5% oxygen, 7% carbon dioxide and 88% nitrogen and incubated at 37°C in a roller bottle incubator prior to the addition of embryos into the culture tubes as described previously (Abdul-Aziz *et al.*, 2009). After addition of the oligonucleotide solutions, the culture tubes containing injected embryos were re-gassed and placed in a rolling incubator at 30 rpm and cultured 9 hours at 37°C.

6.2.1.2 Preparation of sense and antisense solutions

Integrated DNA Technologies (IDT) Pte. Ltd., Singapore synthesized all phosphorothioate oligonucleotides. A series of 20-single-strand *Sec63* antisense oligonucleotide phosphorothioate 5’-CTTGCCACTCTGCTTCATCA-3’, a 20-single-strand *Sec63* sense oligonucleotide phosphorothioate 5’-

TGATGAAGCAGAGTGGCAAG-3' complement to the antisense sequence and phosphate buffered serum (PBS) were used as controls. A 5 pmol of sense and antisense oligonucleotide phosphorothioate diluted in PBS and PBS alone with constant volume at 5 µl per embryo were added into the culture tube (Table 6.1).

Table 6.1: List of substances added into the culture tube of E9.5 ICR mouse embryos

Substance added	Concentration (Volume in brackets)
Sec63 antisense oligonucleotide phosphorothioate primer	5pmol (5 µl)
Sec63 sense oligonucleotide phosphorothioate primer	5pmol (5 µl)
PBS	(5 µl)

6.2.1.3 Embryonic assessment after culture

Yolk sac circulation was scored as an indicator of embryo health. The score was given based on the morphological structure of the yolk sac as presented in the table below. Embryos were only included in the analysis if they had a yolk sac circulation between 2 and 3, while embryos with a score of 1 or 0 were discarded from further analysis (Table 6.2).

Table 6.2: Scoring of the yolk sac circulation

Score	Description
3	Smooth yolk sac exterior, with vigorous circulation and vascularisation throughout the yolk sac
2	Smooth yolk sac exterior, with extensive vascularisation and good circulation, albeit with slightly slower blood movement than grade 3
1	Circulation was evident only in certain regions of the yolk sac
0	Embryos without any yolk sac circulation

Embryonic age, crown rump length (CRL), and number of somite were measured as an indicator of embryo growth. Embryonic age was calculated from the day the presence of a vaginal copulation plug as embryonic day 0.5 (E0.5) to the morning of dissection at day 9 indicated as early E9.5. The CRL was quantified as the maximum distance from the cephalic pole to the caudal pole. The somite number can be used to calculate the embryonic age because their increase in number occurs in specified periodicity. In mouse, primary neurulation begins at the 6 to 7 somite stage (E8.5) and ends at 29 to 30 somite stage (E10.5). After removal of yolk sac and amnion from embryo, the assessment of anterior neuropore (ANP) and posterior neuropore (PNP) closure were performed using a high resolution stereomicroscope with digital imaging system (Brand: Leica MZ16). The length of ANP and PNP were used as measurement for neurulation. The percentage of closed ANP indicated the normal formation of the presumptive brain in embryos at E9.5 and the length of opened PNP were to study the progress of neurulation (Figure 6.2).

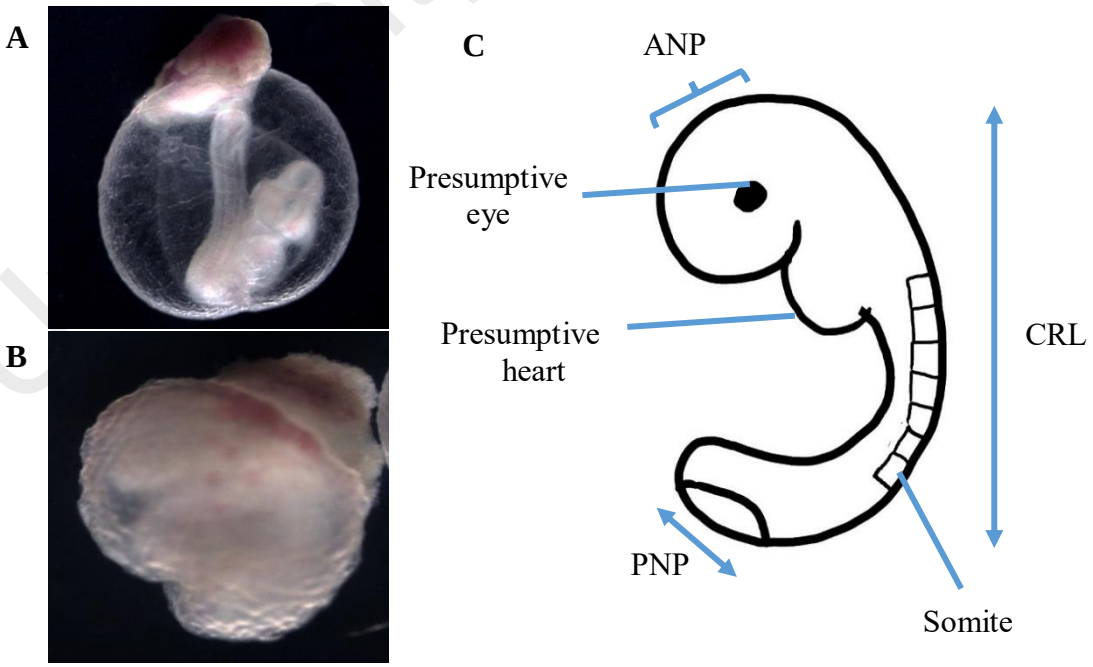


Figure 6.2: Typical embryo after culture and the schematic diagram of an E9.5 embryo. (A) Whole embryo with the presence of yolk sac circulation. (B) Embryos without yolk sac circulation. (C) The criteria of measurements for the evaluation of embryonic stage that include CRL, number of somites present, length of PNP.

6.3 Results

This section includes the clinical report and genetic data of Patient 1, followed by Patient 2 and Patient 3.

6.3.1 Analysis in Patient 1 (Syndromic spina bifida aperta)

6.3.1.1 Clinical reports of Patient 1

A 30-year old woman, primigravida at 26 weeks of pregnancy was diagnosed to have a foetus with severe hydrocephalus. Cytogenetic analysis diagnosed by amniocentesis on the 28th week revealed nineteen out of the 20 Giemsa-stained cells and a further 8 additional cells screened had an isochromosome of the long arm of the X-chromosome and a normal X-chromosome. This suggested a variant Turner syndrome with the karyotype 46, X, i(X) (q10) (Figure 6.3 A & B). A 2.1 kg premature female infant was born at 35 weeks of pregnancy and diagnosed to have the Arnold-Chiari malformation type-2 with communicating hydrocephalus, neurogenic bladder and myelomeningeocele at Lumbar 1 extending to Sacral 1 (L1-S1) region which measured 5 cm in length x 6 cm in width. The lesion was covered by reddish meninges (Figure 6.3C). She was operated on to close the spinal lesion a day after birth and a ventriculo-peritoneal (VP) shunt was also placed to drain excess cerebro-spinal fluid (CSF) on day six.

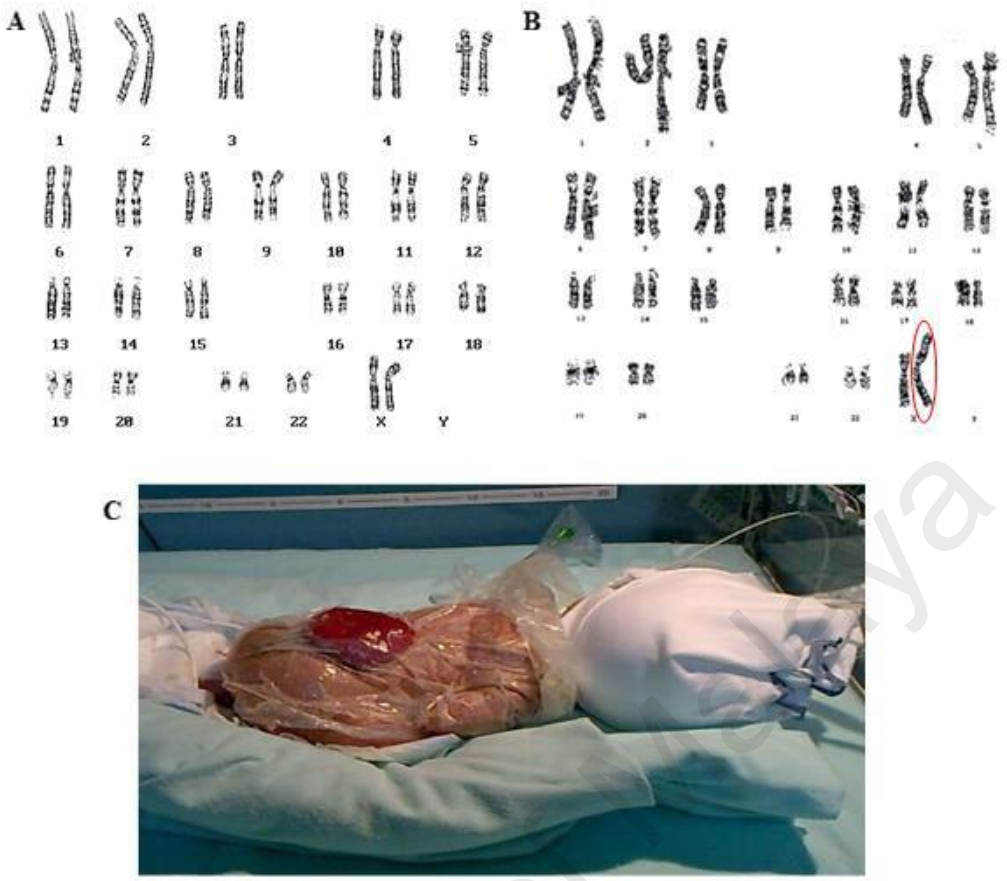


Figure 6.3: The clinical diagnosis of female infant presented a lumbosacral myelomeningocele associated with variant Turner Syndrome. (A) Normal female karyotype. (B) An isochromosome of the long arm of the X-chromosome and a normal X-chromosome of Patient 1 (Red circle). This suggested a variant Turner syndrome pattern with karyotype 46, X, i(X) (q10). (C) Lumbosacral myelomeningocele (L1-S1) region which measured 5 cm in length x 6 cm in width. The lesion was covered by reddish meninges.

6.3.1.2 Genetic data of Patient 1

The results obtained from the analysis in Patient 1 consist of the candidate *de novo* variants identified from the WES, the MAF and level of pathogenicity of the variants and the result of PCR amplification of the patient-parents trios (Figure 6.4).

6.3.1.2.1 WES and excel filtration identified 15 *de novo* variants

WES identified 15 *de novo* variants representing 10 different genes in Patient 1 (Appendix C). Most of the variants were rare and seven were predicted to have pathogenic effect (Appendix D).

6.3.1.2.2 Identification of four variant of interest in Patient 1

Among all the identified variants, four variants in 3 genes (*MTCH2*:p.Cys79Tyr, *MTCH2:*p.Cys79Arg, *RORA*: p.Leu539Phe and *HOXA10*:p.Ser372Arg) which were rare and have highly pathogenic effect were selected as the variants of interest for Patient 1.

6.3.1.2.3 False-positive *de novo* variants verified by PCR and Sanger sequencing

However, PCR and Sanger sequencing of the candidate variants performed on the patient-parents trio revealed false-positive results. All four *de novo* variants were actually wild type in the patient (Figure 6.4).

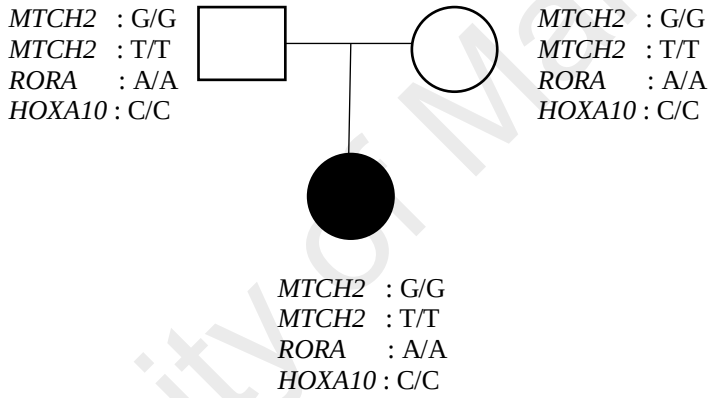


Figure 6.4: Pedigree and genotypes of Patient 1 and her parents. Four false-positive variants were identified; c.G236A:p.Cys79Tyr in *MTCH2*, c.T235C:p.Cys79Arg in *MTCH2*, c.A1617T:p.Leu539Phe in *RORA* and c.C1116A:p.Ser372Arg in *HOXA10*. Affected member is presented as filled symbols, and unaffected members as open symbols.

6.3.2 Analysis in Patient 2 (Non-syndromic spina bifida aperta)

6.3.2.1 Clinical report of Patient 2

We report a 13 year old Indian girl clinically diagnosed with lumbosacral myelomeningocele at L4 extending to S1 with tethered cord. She also presented with syringohydromyelia, congenital hydrocephalus, neurogenic bladder and bilateral Congenital Talipes Equinovarus (CTEV) also known as clubfoot. She is the fourth child in the family and is the only member that is afflicted with NTD. The mother was free from any illness, drug intake and did consume folic acid during pregnancy (Figure 6.5).

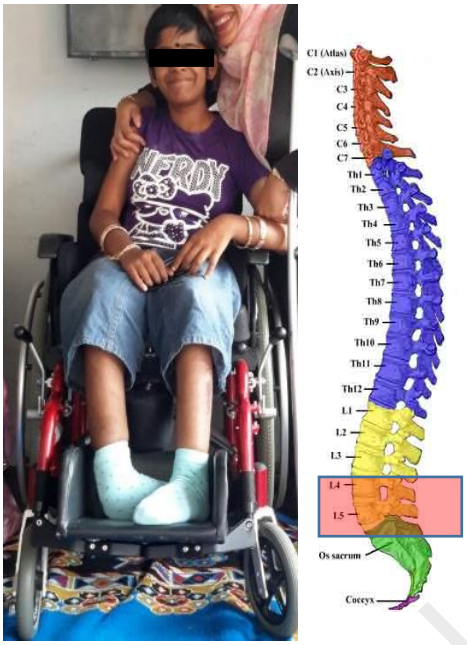


Figure 6.5: The clinical diagnosis of Patient 2. (A) Note her inability to walk with associated clubfoot. (B) Position of the affected lesion from L4-S1.

6.3.2.2 Genetic data of Patient 2

The results obtained include the identification of *de novo* variants from WES and the selection of the variant of interest for Patient 2. The MAF and pathogenicity level of the candidate variant, PCR validation of the patient, her family members and 50 unaffected controls from general Malaysian population, and the analysis of the variant were also presented.

6.3.2.2.1 WES and excel filtration identified a non-synonymous variant in the *SEC63* gene

A total of 6 non-synonymous variants with MAF<0.1 were identified in Patient 2 (Table 6.3). Of all the variants, a heterozygous variant in *SEC63* (rs17854547: c.G1666A), that changes the amino acid from valine to isoleucine was chosen as the variant of interest in this study. This was following the identification of two Sec-family genes (*Sec23a* and *Sec24b*) to cause NTD in mice studies (Wansleeben *et al.*, 2010; Zhu *et al.*, 2015).

The *SEC63* variant was considered uncommon as the global MAF of this variant was 0.0232. In addition, the variant was not reported in the Singapore Genome Variant Project (SGVP) (as of October 2017) when screening against three dominant ethnicities in Malaysia (Malay, Chinese and Indian) (Table 6.3). However, this variant was benign and non-pathogenic according to the bioinformatics analysis. SMART (Simple Modular Architecture Research Tool) prediction showed the mutation did not affect the functional domain of the SEC63 protein (Figure 6.6).

Table 6.3: MAF and the pathogenic effect of non-synonymous *de novo* variants identified in Patient 2

No	Gene	Reported SNP	Amino acid change	Minor Allele Frequency					Deleterious prediction			
				1000 Genomes NCBI	1000 Genomes UCSC Browser	Exome sequencing project (GO-ESP)	ExAC	SGVP	SIFT	PROVEAN	POLYPHEN 2 (HumDiv)	POLYPHEN 2 (HumVar)
1	*GBP7* (Guanylate Binding Protein 7)	rs79037912	p.Gln507Glu	NA	0.0137	NA	0.0137	NA	Tolerated	Neutral	Benign	Benign
2	*SEC63* (S. Cerevisiae 63)	rs17854547	p.Val556Ile	0.0232	0.0400	0.0328	0.0407	NA	Tolerated	Neutral	Benign	Benign
3	*MAP6* (Microtubule Associated Protein 6)	rs61895095	p.Val693Gly	0.0325	0.0382	0.0400	0.0395	NA	Tolerated	Neutral	Benign	Benign
4	*TAS2R19* (Taste 2 Receptor Member 19)	rs12424373	p.Lys126Gln	0.0615	0.0509	0.0058	0.0504	NA	Damaging	Neutral	Benign	Benign
5	*STARD9* (START Domain-Containing Protein 9)	rs116745790	p.Glu2083Lys	0.0443	0.0277	0.0313	0.0233	NA	Damaging	Neutral	NA	NA
6	*TNFRSF13C* (TNF Receptor Superfamily Member 13C)	rs77874543	p.Pro21Arg	0.0445	0.0589	0.0228	0.0680	NA	Tolerated	Neutral	Benign	Benign

NA=Not applicable; UCSC= University of California Santa Cruz; GO-ESP= Go exome sequencing project; ExAC= Exome Aggregation Consortium; SGVP= Singapore Genome Variation Project (National University of Singapore); SIFT= Sorting Tolerant from Intolerant

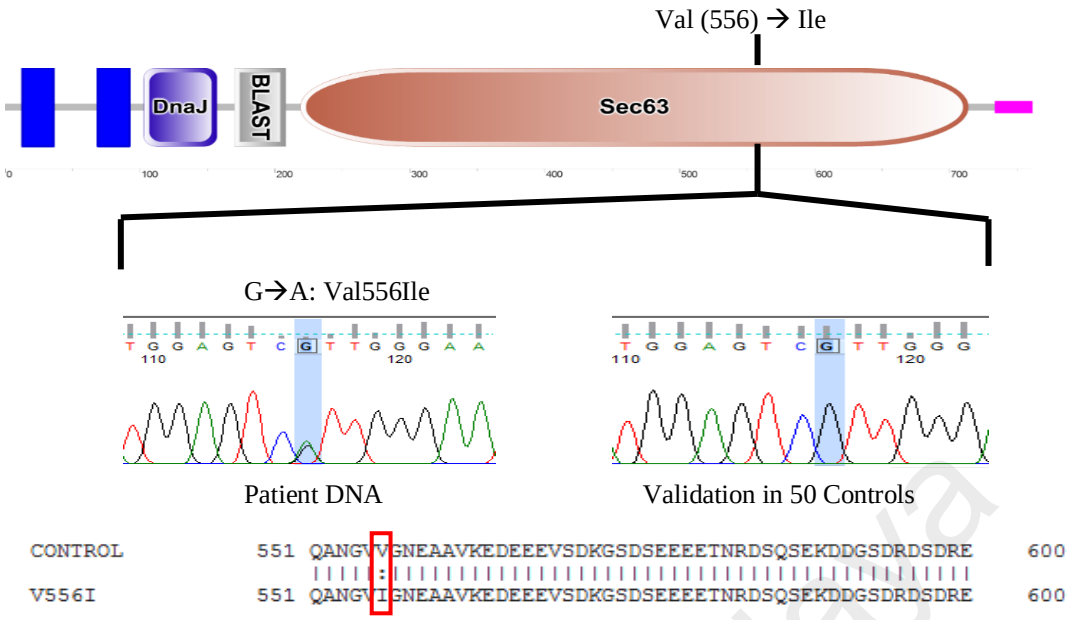


Figure 6.6: The *SEC63* variant (c.G1666A) creating a non-synonymous protein change from a conserved valine to isoleucine (V566I). A comparison of normal human control and patient *SEC63* amino acid sequence in the vicinity of the *SEC63* variant is also shown.

6.3.2.2.2 Segregation analysis of the heterozygous *SEC63* variant

The heterozygous *SEC63* variant in Patient 2 was inherited from the mother, who does not have spina bifida. Her two other unaffected siblings also have the same mutation as in the patient (Figure 6.7). Fifty unaffected control samples (2n=100 chromosomes) from the general Malaysian population were wildtype (G/G) (Figure 6.6).

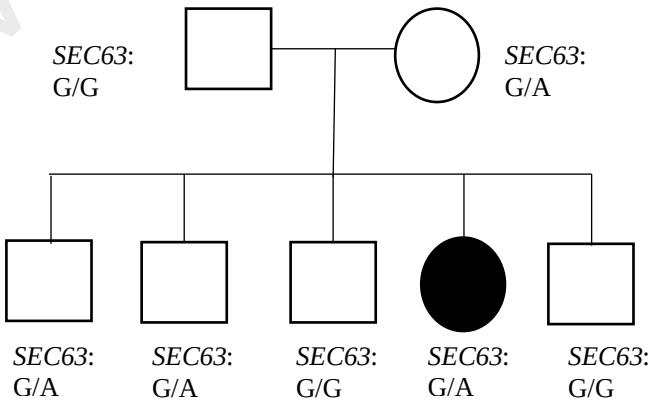


Figure 6.7: Pedigree and genotypes of Patient 2 and her family. Affected member is presented as filled symbols, and unaffected members as open symbols. Patient, the mother two other siblings are heterozygous c.1666G>A mutation.

6.3.2.2.3 Different variant and location of *SEC63* (p.Val556Ile) compared to the reported autosomal dominant polycystic liver disease variants

Mutation of the *SEC63* gene is mainly linked to autosomal dominant polycystic liver disease (ADPLD) (Davila *et al.*, 2004). Note that Patient 2 is free from ADPLD and the identified *SEC63* variant was located at different location from the reported ADPLD variants (Figure 6.8).

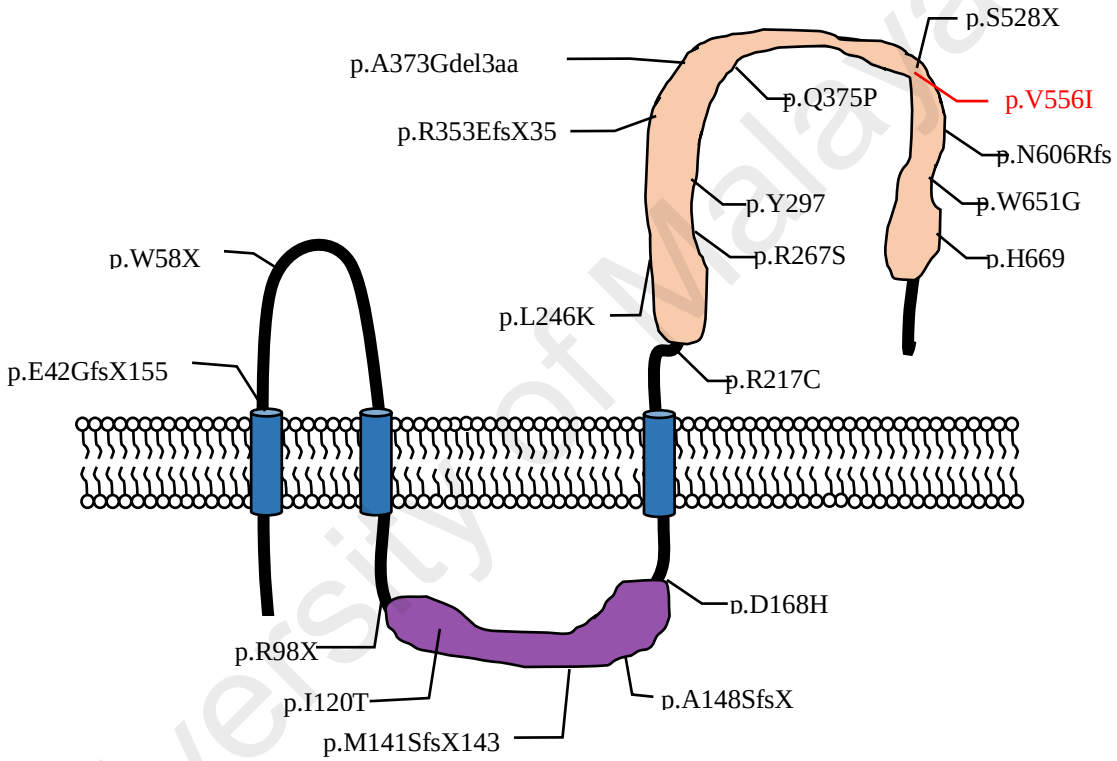


Figure 6.8: The schematic structure of Sec63p, location of the different ADPLD mutations, and the reported *SEC63* variant in Patient 2. The integral ER-membrane protein contains three trans-membrane spanning domains (blue tubes), a luminal DnaJ-like domain between trans-membrane segments 2 and 3 (purple) and a large cytoplasmic *SEC63* domain with a negatively charged C-terminus (peach). The mutation found in this study is highlighted with red font (p.V5661).

6.3.3 Analysis in Patient 3 (Non-syndromic spina bifida occulta)

6.3.3.1 Clinical report of Patient 3

The case reported involved a seven years old male patient born full term via spontaneous vaginal delivery (SVD) with an uneventful antenatal history. He is the first born child and the only affected member clinically diagnosed with lipomyelomeningocele. His Magnetic Resonance Imaging (MRI) scans confirmed that the defect is located in the posterior element from L4 with intraspinal lipoma extending to the coccyx with tethered cord. He is able to walk with sensory level L3 impaired in his lower left limb. His sensory level is intact in his lower right limb from L3 onwards, but from L4 to S5 his sensory level is absent in both lower limbs. The patient developed tendon equinovarus contracture of the left foot. He has bladder and bowel impairment that worsened after untethering of his spinal cord (Figure 6.9).

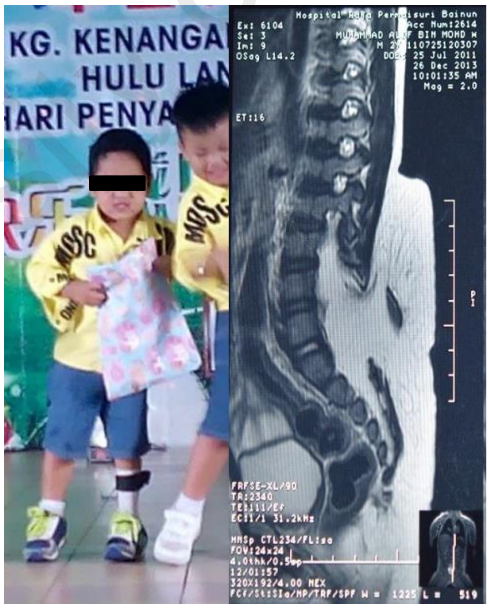


Figure 6.9: The clinical diagnosis of Patient 3. (A) The patient is able to stand and walk with designated ankle-foot orthosis**.** (B) MRI of the patient showed lesion (L4 extending to coccygeal level) and intermeshed neural matter with lipid globules indicating lipomyelomeningocele defect.

6.3.3.2 Genetic data of Patient 3

6.3.3.2.1 WES and excel filtration identified 14 *de novo* variants

WES revealed 14 *de novo* variants (Appendix C) in Patient 3. From the list, six variants (*HSPA6*:c.C890A; p.Thr297Lys, *USP49*: c.G37C; p.Ala13Pro, *AKNA*: c.A3287C; p.His1096Pro, *ZIC2*:c.C1357A; p.Leu453Met, *SYMPK*: c.C2123G; p.Ser708Cys, and *NLRP2*:c.C1151T; p.Thr384Ile) were rare and predicted to be pathogenic according to Polyphen 2 (Table 6.4). According to the WES, those identified variants were heterozygous in the patient while parents were genotypically wild type (Figure 6.10). All six variants were most likely the contributors of pathogenicity in this patient.

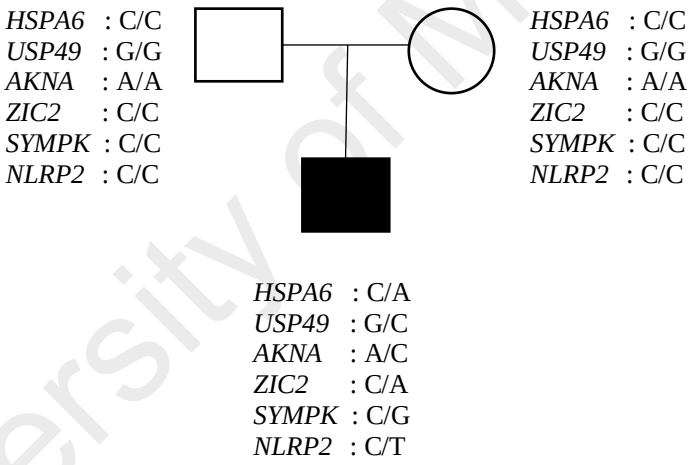


Figure 6.10: Pedigree and genotypes of the Patient 3 and his parents. The reported *de novo* variants based on WES include; *HSPA6*:c.C890A; p.Thr297Lys, *USP49*: c.G37C; p.Ala13Pro, *AKNA*: c.A3287C; p.His1096Pro, *ZIC2*:c.C1357A; p.Leu453Met, *SYMPK*: c.C2123G; p.Ser708Cys, and *NLRP2*:c.C1151T; p.Thr384Ile. Affected member is presented as filled symbols, and unaffected members as open symbols.

6.3.3.2.2 Identification of the *ZIC2* as candidate variant in Patient 3

Among the six listed pathogenic variants, the *ZIC2* variant (c.C1357A) (Figure 6.11 A & B) was selected as the candidate variant in Patient 3. This is due to the associations of the *Zic2* gene with increased spina bifida risk in mouse studies.

The *Zic2* plays a crucial role during mouse development and previously, several phenotypes of NTDs have been observed in *Zic2* mouse mutant embryos. (Nagai *et al.*, 2000; Elms *et al.*, 2003). The identified *ZIC2* variant in Patient 3 is rare as it is not reported in the Exome Variant Server (EVS) (as of October 2017). Furthermore, the global MAF of this variant is not recorded in 1000 Genome, ESP, and ExAC and SGVP databases (as of October 2017) (Table 6.4).

6.3.3.2.3 PCR amplification failure due to high GC-rich content in the *ZIC2* sequence

However, we failed to validate the *ZIC2* variant in the patient, his parents and the unaffected controls. This is due to high GC-rich content of the *ZIC2* sequence which made it difficult to amplify in the PCRs.

Table 6.4: MAF and the pathogenic effect of non-synonymous *de novo* variants identified in Patient 3

No	Gene	Reported SNP	Amino acid change	Minor Allele Frequency					Deleterious prediction			
				1000 Genomes NCBI	1000 Genomes UCSC Browser	Exome sequencing project (GO-ESP)	ExAC	SGVP	SIFT	PROVEAN	POLYPHEN 2 (HumDiv)	POLYPHEN 2 (HumVar)
1	*HSPA6* (Heat Shock Protein Family A (Hsp70) Member 6)	rs41297718	p.Thr297Lys	NA	0.01136	NA	NA	NA	Deleterious	Damaging	Probably damaging	Probably damaging
2	*USP49* (Ubiquitin Specific Peptidase 49)	.	p.Ala13Pro	NA	NA	NA	NA	NA	NA	NA	Probably damaging	Probably damaging
3	*AKNA* (AT-Hook Transcription Factor)	.	p.His1096Pro	NA	NA	NA	NA	NA	NA	NA	Probably damaging	Probably damaging
4	*ZIC2* (Zic Family Member 2)	.	p.Leu453Met	NA	NA	NA	NA	NA	NA	NA	Probably damaging	Probably damaging
5	*SYMPK* (Symplekin)	.	p.Ser708Cys	NA	NA	NA	NA	NA	NA	NA	Probably damaging	Probably damaging
6	*NLRP2* (NLR Family Pyrin Domain Containing 2)	.	p.Thr384Ile	NA	NA	NA	NA	NA	NA	NA	Probably damaging	Probably damaging

NA=Not applicable; UCSC= University of California Santa Cruz; GO-ESP= Go exome sequencing project; ExAC= Exome Aggregation Consortium; SGVP= Singapore Genome Variation Project (National University of Singapore); SIFT= Sorting Tolerant from Intolerant

6.3.3.3 Troubleshooting PCR methods to validate the *ZIC2* candidate variant in Patient 3

The selected potential candidate variant in Patient 3 (*ZIC2* variant) (Figure 6.11A) was selected to be PCR validated following the methods elaborated in Chapter 3. However, due to high GC-rich content of the *ZIC2* sequence (Figure 6.11B), five sets of *ZIC2* primers have been used throughout the validation processes but none of those primer help to yield any correct band size (Figure 6.11C). Direct sequencing of the PCR products did not yield any usable electropherograms. Alternatively, PCR amplification was also conducted using three different types of GC-rich PCR kits; (1) PCRBIO ultra polymerase (PCR Biosystems, United Kingdom), (2) nTaq-HOT (Enzynomics, Korea), and (3) Transtaq DNA Polymerase High Fidelity (HiFi) (TransGen Biotech, Beijing). Methods used for each GC-rich kit were according to the manufacturer's protocol. Unfortunately, PCR validation for this sequence was still unsuccessful. While most of the studies used the AccuPrime GC-Rich DNA Polymerase kit (Invitrogen) for the amplification of difficult-to-amplify *ZIC2* templates (Roessler *et al.*, 2009; Paulussen *et al.*, 2010), the future use of this kit might be helpful to validate the *ZIC2* variant in this patient.

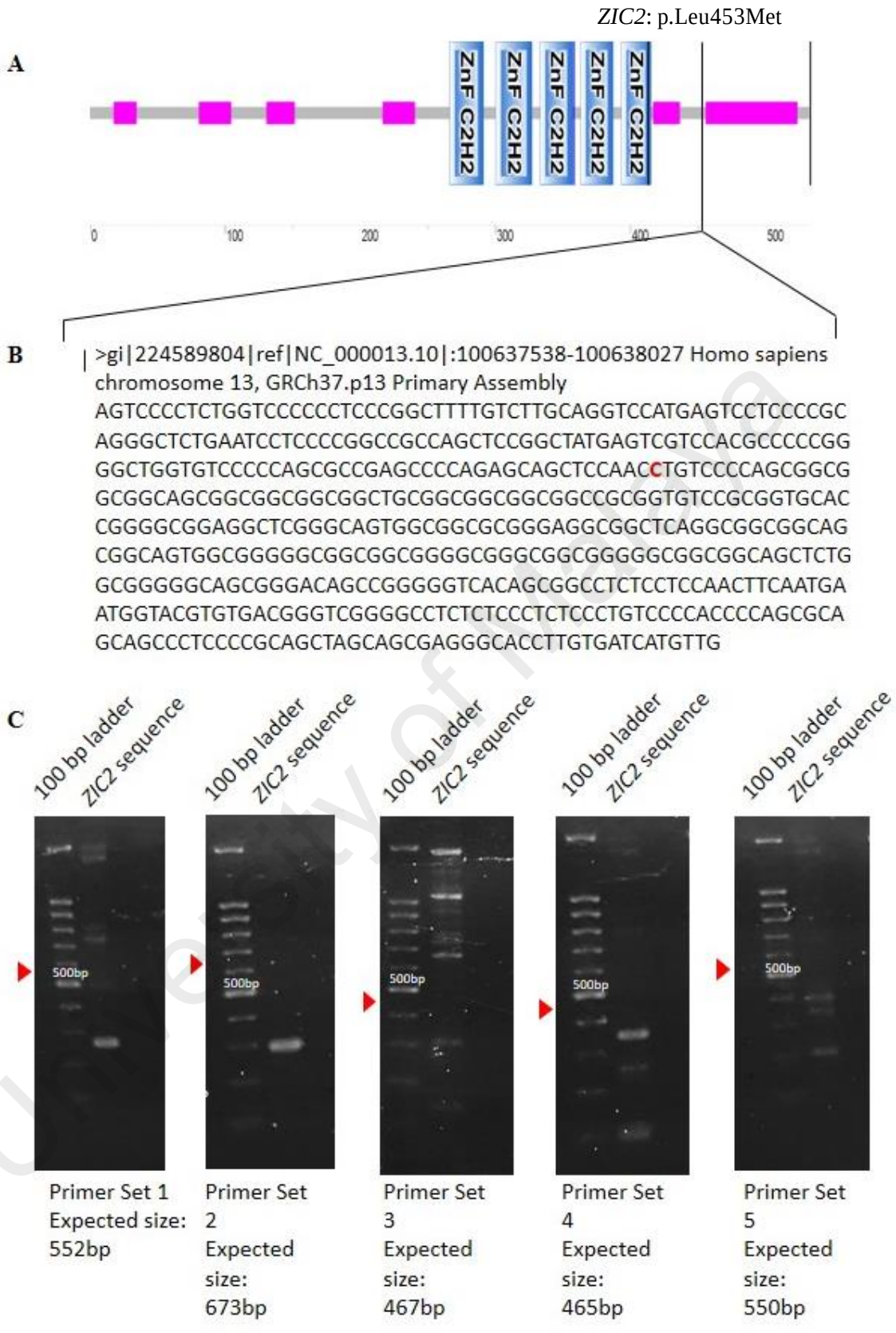


Figure 6.11: Sequence analysis of *ZIC2* variant. (A) Structure of the *ZIC2* protein comprise of five C2H2 domains from the SMART prediction analysis. (B) The targeted sequence of *ZIC2* (c.1357C>A) whereby the point of mutation as in red font. (C) Gel electrophoresis of the *ZIC2* sequence using five different sets of primers. All of the primers used failed to generate the band with the expected size. Red triangles mark the position of the expected band size.

6.4 Result of whole embryo culture study

The embryos that were assessed, scored, and finally taken into account for this analysis were those with a yolk sac circulation score of 3. The embryo treated with 5pmol of antisense oligonucleotide phosphorothioate (Figure 6.12A) had a longer PNP length in comparison to both the sense oligonucleotide phosphorothioate control (Figure 6.12B) and the PBS control (Figure 6.12C). The somite numbers of embryo A were similar to that of embryo B and embryo C. This rings true for the CRL as well (Table 6.5). This suggests that the treatment was specific to the development of the spinal neural tube. Although only 3 embryos were scored, the result is encouraging and warrants further experiments with quality rat serum used as culture medium to ensure correct interpretation of the whole embryo culture method.

(A)	**(B)**	**(C)**
Embryo treated with 5 pmol of antisense oligonucleotide phosphorothioate	Embryo treated with 5 pmol of sense oligonucleotide phosphorothioate	Embryo treated with PBS
Yolk sac score: 3	Yolk sac score: 3	Yolk sac score: 3

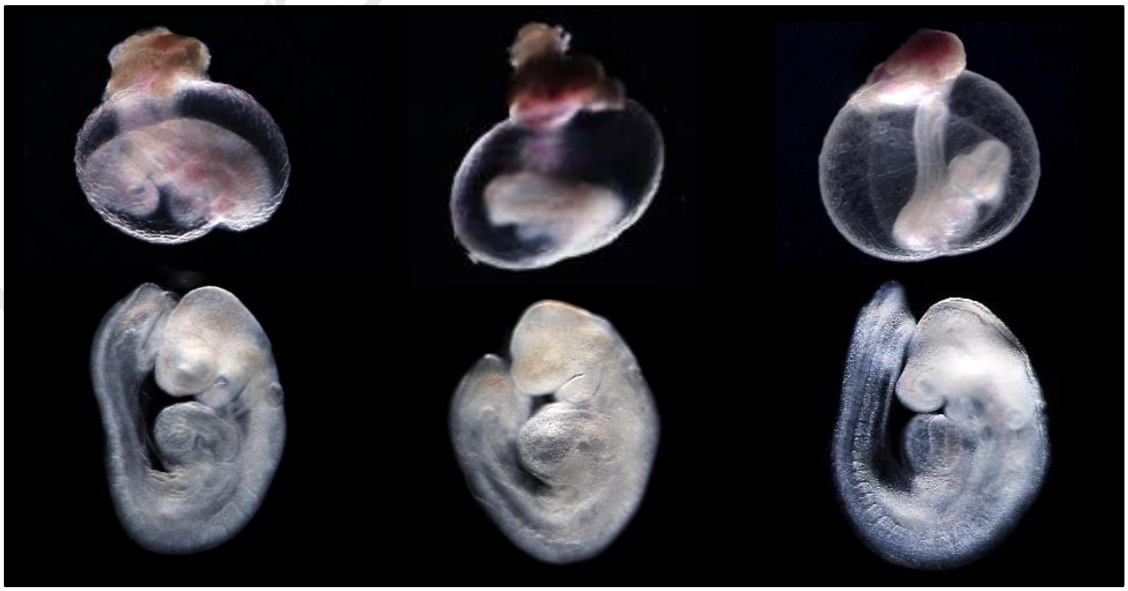


Figure 6.12: The typical representation of embryo with a yolk sac circulation score of 3. (A) Embryo treated with 5pmol of antisense oligonucleotide phosphorothioate, (B) embryo treated with 5pmol of sense oligonucleotide phosphorothioate and (C) embryo treated with PBS.

Table 6.5: Embryonic measurement after culture

Treatment	Somite number	CRL	PNP	ANP
5pmol antisense phosphorothioate oligonucleotides	22	2.27mm	0.50mm	0.18mm
5pmol sense phosphorothioate oligonucleotides	21	1.99mm	0.38mm	0.26mm
PBS	21	1.83mm	0.34mm	0.37mm

6.5 Discussion

6.5.1 No likely candidate genes identified for Patient 1

The case reported involved a premature female infant born with syndromic spina bifida encompassing lumbosacral myelomeningocele, variant Turner syndrome and Arnold-Chiari malformation type-2. Myelomeningocele is the most common and severe form of spina bifida that is also known as open spina bifida or spina bifida aperta. It is a lifelong disability characterized by the extrusion of the spinal cord into a sac filled with cerebrospinal fluid (Adzick *et al.*, 2011). Most infants born with myelomeningocele are also associated with Arnold-Chiari malformation (Adzick *et al.*, 2011; Copp *et al.*, 2015) which is a type of abnormality present at birth comprising downward herniation of the cerebellum and medulla into the spinal cord (Khan *et al.*, 2010).

The cytogenetic analysis of Patient 1 revealed one normal X-chromosome and an isochromosome of the long arm of one X-chromosome with the karyotype 46,X,i(X)(q10) which was an isochromosome variant of Turner syndrome. An isochromosome is formed due to the abnormal separation of the chromosomes during meiosis and results in a single chromosome consisting entirely of the p or q arms, rather than a single p and q arm consisting of either two short or long arms, because of the abnormal transverse mis-division of the centromere (Margaret *et al.*, 2010). Normal clinical manifestations of isochromosome variant Turner syndrome include short stature, absent pubertal development, gonadal streak, drooping eyelids, edema of the hands and feet (Margaret *et al.*, 2010; Ramos & Lantion-Ang, 2010; Akbas *et al.*, 2012). Turner syndrome is the result of complete or partial loss of a critical region of the X-chromosome in a female (Bharath *et al.*, 2010). As the child is yet to reach puberty, the main Turner syndrome phenotypes are not obvious, and deeper phenotyping will be needed when she is older. Previous study concluded that the median age of female with i(Xq) to be diagnosed with Turner

syndrome is 14.2 years (Stochholm *et al.*, 2006; Ou *et al.*, 2010). Several cases of syndromic NTD due to Turner syndrome have been reported. The cases include a deletion of Xp in an anencephalic fetus, a lumbosacral spina bifida in a female foetus with *de novo* X/autosomal translocation, and mosaic Turner syndrome identified in five NTD patients (Plaja *et al.*, 1994; Fryns *et al.*, 1996; Lambert-Messerlian *et al.*, 2000).

Analysis of WES in Patient 1 found four rare and highly pathogenic candidate variants (*MTCH2*:p.Cys79Tyr, *MTCH2:*p.Cys79Arg, *RORA*: p.Leu539Phe and *HOXA10*:p.Ser372Arg). However, all four variants were ruled out as the candidate genes following the false-positive results. All variants were actually wild type in Patient 1. This could be due poor DNA quality of the sample which leads to high rate of false-positive results. Other issue might be the misinterpretation when analyzing the variant calling or error in WES data leading to the identification of artifact variants. WES errors can be caused by inaccuracies of reference sequence and misalignment of sequencing reads to a reference sequence (Fajardo *et al.*, 2012). In the future, as far as variant calling is concerned, several steps of filtration should be put into consideration. Apart from the selection of non-synonymous variant with MAF<0.1, variant with read depth<15, a quality score <20, alternate allele ratio below 85% for homozygous and greater than 70% or less than 30% for heterozygous variants should be excluded to get the most accurate candidate variants (Patel *et al.*, 2014).

Furthermore, as Patient 1 also presented with variant Turner syndrome, we hypothesized that this patient has higher copy number of genes located at the affected region of the X-chromosome compared to her mother and other female controls. As the genetic occurrence of spina bifida is complex and sporadic, we suggest that extra copy number of any reported spina bifida genes and/or combination of multiple sets of genes

on the X-chromosome could be the possible risk factor of the spina bifida phenotype in the patient. Therefore, future work including analysis of copy number variations using SYBR Green Real-time PCR assay is worth to be conducted. This should include the analysis in Zic family member 3 (*ZIC3)* gene, the known spina bifida risk gene located at the X-chromosome. This method will be helpful to quantify the copy number of the X-chromosome genes and the *GAPDH* as the internal control.

6.5.2 Identification of *SEC63* as the candidate variant in Patient 2

The case reported for non-syndromic spina bifida aperta involved an Indian female patient diagnosed with myelomeningocele without any associated syndrome. WES identified six *de novo* variants and from that list, the variant in *SEC63* (S. Cerevisiae 63) (rs17854547) was selected at the candidate variant for this patient due to the reports of mutations in the same gene family; the *Sec23a* and *Sec24b* in mouse models of NTD. In a *Sec23a* study, most of the mouse mutant embryos died during mid-embryogenesis and half of the surviving mice had exencephaly which is the open NTD of the midbrain and majority of the experimental embryos developed cranial neural tube openings (Zhu *et al.*, 2015). Another study reported severe craniorachischisis and a typical planar cell polarity (PCP) phenotype in *Sec24b* mouse mutant (Wansleeben *et al.*, 2010). The *Sec24b* is a gene involved in the cargo selection for the protein transport and works in concert with the *Scribble* (known PCP gene) and also the *Vangl1* that is the PCP core protein (Wansleeben *et al.*, 2010). The PCP is a pathway that controls the polarization of the epithelial cells in the event of convergent extension and disruption of this pathway leads to severe NTDs in mice (Wallingford, 2006; Copp *et al.*, 2013; De Marco *et al.*, 2014). The finding provides evidence that *Sec24b* deficiency affects the transport of the PCP protein and subsequently leads to the occurrence of NTDs (Wansleeben *et al.*, 2010).

The *SEC63* gene contains SEC63p protein that works as a protein translocation regulator which governs the protein translocation process at the endoplasmic reticulum (Fedeles *et al.*, 2011). Mutation of the *SEC63* gene mainly linked to autosomal dominant polycystic liver disease (ADPLD) (Davila *et al.*, 2004). To our knowledge, this is the first report suggesting potential variant of *SEC63* in the case of NTD. There was a study of *sec63* mutation in zebrafish which however is not related to NTD. In that study, mutation of *sec63* disrupts the myelination process in both central and peripheral nervous system (Monk *et al.*, 2013).

The identified non-synonymous *SEC63* variant creates a protein change from valine to isoleucine at position 556 of the amino acids and located at the large cytoplasmic SEC63 domain (Figure 6.6). Interestingly, this variant is not common (MAF ~ 0.02) in the whole world population and also not reported in the Singaporean population (as of October 2017) (Table 6.3). Preliminary screening of this variant in 50 unaffected Malaysian controls showed that none of the controls had the mutation. Although the *SEC63* variant is non-pathogenic, it is noteworthy that spina bifida could result from the haploinsuffiency (Mohd-Zin *et al.*, 2017). The *SEC63* variant acts in concert with other different underlying pathogenic variants and/or combination with environmental factors is more likely for the possible risk factor of the patient.

However, segregation analysis among the patient and other family members showed the *SEC63* variant did not exclusively segregate with the spina bifida phenotype and could be explained by a normal Mendelian pattern of inheritance (Figure 6.7). The variant was also identified in the mother and two other unaffected siblings. This variant could be affected by other factors including the *in utero* environment that contributes to spina bifida in the patient compared to unaffected members with the same genotype. Thus,

this finding suggest that the *SEC63* variant on its own is not able to cause spina bifida in human. The incidence of spina bifida may be due to environmental trigger or other genetic tribulation.

The potential role of *Sec63* in NTD was studied using whole embryo culture in mouse through antisense oligonucleotide technology. In this preliminary study, only 3 embryos treated with 5 pmol antisense oligonucleotide phosphorothioate, 5 pmol sense oligonucleotide phosphorothioate, and PBS were scored (Figure 6.12). In our raw data result, longer length of PNP was found in the antisense-treated embryos compared to both sense and PBS controls (Table 6.5). This suggests that antisense treatment was specific to the development of the spinal neural tube. However, in this study, it is unfortunate that the embryos obtained did not have duplicates or triplicates. Insufficient number of embryo was due to diminish quality of the rat serum. Further work is required to repeat these experiments to get a more accurate result. Hence, further experiment using high-quality rat serum is recommended as it is necessary to yield reliable result of embryo culture.

Apart from the *SEC63* variant, there were two other variants (*TAS2R19*: rs12424373 and *STARD9*: rs116745790) predicted to be deleterious according to SIFT (Table 6.3). Both genes and their respective gene families have never been implicated with the occurrence of NTD. The *TAS2R19* (Taste 2 Receptor Member 19) is a gene involved in the perception of salty and bitter tastes whereby mutations of this gene normally linked to the differences in bitterness sensation (Hayes *et al.*, 2013). Besides that, this gene also was found to be associated with Parkinson disease and respiratory syncytial virus disease (Salas *et al.*, 2017; Siitonen *et al.*, 2017). The *STARD9* (Start domain-containing protein 9) is a gene that encodes a mitotic kinesin which regulates the

assembly of spindle pole during cell mitosis (Senese *et al.*, 2015). Depletion of the *STARD9* have been implicated with dermatofibrosarcoma protuberans and language impairment, (Hong *et al.*, 2013; Chen *et al.*, 2017).

6.5.3 Identification of *ZIC2* as the candidate variant in Patient 3

The third patient in this study was diagnosed with lipomyelomeningocele that is the spina bifida occulta type with the presence of intermeshed lipid globules and spinal cord (Copp *et al.*, 2015). It is the only defect found in the patient without the presence of any associated syndrome. Among the 14 *de novo* variants identified in this patient, six variants (*HSPA6*:c.C890A; p.Thr297Lys, *USP49*: c.G37C; p.Ala13Pro, *AKNA*: c.A3287C; p.His1096Pro, *ZIC2*:c.C1357A; p.Leu453Met, *SYMPK*: c.C2123G; p.Ser708Cys, and *NLRP2*:c.C1151T; p.Thr384Ile) were rare, deleterious, and the most likely contributors of pathogenicity in Patient 3. From the list, *ZIC2* (Zic Family Member 2) variant has been selected as the variant of interest owing the fact that the *Zic2* is a well-known spina bifida gene in mouse (Nagai *et al.*, 2000). Knockout of *Zic2* gene causes neurulation delay which subsequently leads to the occurrence of spina bifida (Nagai *et al.*, 2000). The gene is uniquely expressed during somitogenesis where it promotes the formation of newly generated somites from the presomitic mesoderm and plays a crucial role during neural crest formation and hindbrain patterning of developing mouse embryos (Elms *et al.*, 2003; Inoue *et al.*, 2007).

The role of *Zic2* during neurulation may support the evidence that mutation of this gene causes spina bifida in mice. Interestingly, *Zic2* is located at the critical region of chromosome 13 near the *Zic5 gene* which is the other Zic family member established as a genetic risk factor in murine NTD (Inoue *et al.*, 2004). The *Zic2-Zic5* gene pair are the important element regulating midbrain growth and neural tube development in zebrafish

(Toyama *et al.*, 2004; Nyholm *et al.*, 2007). In humans, several mutations in the *ZIC2* gene have been reported in spina bifida. One study identified a 70bp insertion within the first *ZIC2* intron in a spina bifida patient but the same variant was also identified in the unaffected mother and sister (Brown *et al.*, 2002). In another study, a deletion of an alanine codon (c.94-96delGCG) in the exon 1 of the *ZIC2* gene was found in a male spina bifida aperta patient. The deletion was not identified in 364 controls alluding to a possible role of this variant in the etiology of human spina bifida (Klootwijk *et al.*, 2004). Notably, those two reported mutations of *ZIC2* gene were not identified in Patient 3.

The identified non-synonymous *ZIC2* variant in Patient 3 creates a protein change from leucine to methionine at position 453 of the amino acids. This is a rare variant as it is not reported in any genome databases (as of October 2017) and was deleterious based on the Polyphen 2 analysis (Table 6.4). Unfortunately, we were unable to verify the *ZIC2* genotype in the patient, parents and unaffected controls due to the high GC-content of the *ZIC2* sequence (Figure 6.11). In human, mutations in *ZIC2* were previously associated with holoprosencephaly (HPE), a structural malformation of the brain resulting non-separation of the prosencephalon (forebrain) (Brown *et al.*, 2001; Chabchoub *et al.*, 2012; Murillo *et al.*, 2015).

Other than *ZIC2*, five other novel variants were found namely c.C890A in *HSPA6*, c.G37C in *USP49*, c.A3287C in *AKNA*, c.C2123G in *SYMPK* , and*:* c.C1151T in *NLRP2* by which the variant in *HSPA6* was highly pathogenic as predicted by all the available bioinformatics analysis while the other four variants were probably damaging based on PolyPhen 2 (Table 6.4). None of these variants have been associated with spina bifida.

HSPA6 is a protein coding gene that serves as molecular chaperones and plays a pivotal role in a wide variety of cellular processes. These include proteome protection from stress and helping in folding of newly-synthesized proteins (Regeling *et al.*, 2016). The gene is expressed in most cells under stressful conditions and is present exclusively in mammals but not conserved in rodents (Leung *et al.*, 1990; Noonan *et al.*, 2007). Previously, mutations of this gene was linked to several types of cancer and autism (Garbett *et al.*, 2008; Court *et al.*, 2017; Su *et al.*, 2017).

USP49 comprises of ubiquitin-specific protease domain and UBP-type zinc finger domain. This gene regulates co-transcriptional pre-mRNA splicing but the cellular function of it remain unknown (Zhang *et al.*, 2013; Luo *et al.*, 2017). *USP49* has been studied to play a role in AKT pathway that facilitates multiple cellular processes including cell proliferation and glucose metabolism (Luo et al., 2017).

AKNA is involved in mediating immune responses. AKNA protein normally binds to AT-rich promoters that promote normal development and inflammatory processes (Moliterno & Resar, 2011). Study using murine *AKNA* gene discovered small and frail knockout mice that die at early age with diffuse inflammatory lesions (Ma *et al.*, 2011). This gene was also associated with cervical cancer and inflammatory disorders including the primary Sjögren's syndrome and Vogt-Koyanagi-Harada syndrome (Perales *et al.*, 2010; Mao *et al.*, 2011; Hernandez-Molina *et al.*, 2017).

SYMPK is involved for the polyadenylation regulation and promotes gene expression (Cappell *et al.*, 2010). It acts as a scaffold protein for polyadenylation complex which is crucial for the maturation of most pre-mRNAs and mRNA processing (Mandel

et al., 2008). The role of this gene during neurulation process has never been identified but this gene was shown to affect the *HOXA9*, an important gene during embryogenesis, leukemogenesis and hametopoiesis (Abramovich & Humphries, 2005; Largeot *et al.*, 2013).

Members of the NLR Family have a key role in immune response system but the role of *NLRP2* itself was ill-defined (Proell *et al.*, 2008; Kuchmiy *et al.*, 2016). *NLRP2* that expressed ectopically was revealed to regulate inflammasome signaling (component of the innate immune system) and inhibit NF-κB activation which is essential for DNA transcription and cell survival (Bruey *et al.*, 2004; Fontalba *et al.*, 2007; Kuchmiy *et al.*, 2016). Importantly, deletion of this gene was known to cause embryonic arrest in the mouse (Peng *et al.*, 2017).

6.6 Conclusion

This chapter extends the screening of potential candidate variants using *de novo* mutation approach from the exome datasets of three spina bifida patients presented with different spina bifida clinical phenotype. Based on the exome analysis in Patient 1 who represents the syndromic spina bifida aperta, no likely candidate variant was identified. The exome analysis in Patient 2 who represents the non-syndromic spina bifida aperta identified an uncommon variant in *S. Cerevisiae 63* (*SEC63*). Meanwhile a rare and pathogenic variant in Zic family member 2 (*ZIC2*) gene was selected as the candidate variant in Patient 3 who represents non-syndromic spina bifida occulta. This study however warrants more research in the identified candidate variants and spina bifida in larger populations. Future work including gene expression study and analysis at the protein level will be required to investigate the genetic contribution of the candidate variants in the aetiology of spina bifida.

CHAPTER 7: CONCLUSION

This thesis had a few objectives. Firstly, was to investigate the prevalence rate of spina bifida and anencephaly using the record of patients retrieved from the Department of Patient Information, University of Malaya Medical Centre (UMMC) from 2003 to 2016. We found that the occurrence of spina bifida (1.33 to 6.4 per 1000 births) was found to be higher compared to anencephaly (0.38 per 1000 births). The most common phenotype that was observed among the spina bifida patients were myelomeningocele. The lesion of spina bifida mostly occurred at the lumbar region and non-syndromic spina bifida was more common compared to the syndromic spina bifida.

Secondly, as spina bifida has the higher rate of occurrences compared to the anencephaly in the prevalence study, the genetics factor of spina bifida were studied through screening of the previously reported spina bifida potential risk variants in our patient cohort. From 107 variants in 40 potential risk genes, we found 10 non-synonymous pathogenic variants representing 7 different genes. Those identified variants include *MTHFR* (c.C677T and c.A1298C), *ALDH1L1* (c. A2378G), *MTRR* (c.A66G), *SARDH* (c.G1841A), *XPD* (c.G934A), *CUBN* (c.G5518A, c.A8950G and c.C10656A) and *BRCA1* (c.A4837G). However, the identified variants might contribute towards the risk of spina bifida in the individual spina bifida patients, but they are not common in our patient cohort.

Thirdly, we also focused on screening of potential candidate variants using *de novo* mutation approach in three patients with varying degrees of spina bifida clinical phenotype. There was no candidate variants identified in Patient 1 which has syndromic spina bifida aperta. On the other hand, patient 2 who has non-syndromic spina bifid aperta

has variant *SEC63* which is uncommon but non-pathogenic. Patient 3 with non-syndromic spina bifida occulta has rare and pathogenic candidate variant in the *ZIC2*. Thus, candidate variants found in the non-syndromic spina bifida aperta and occulta patients suggest the possibility of these variants to be potential risk factor for these individuals.

Fourthly, in order to further understand the potential role of *Sec63* in NTD, we opted an antisense oligonucleotide technology using whole embryo culture in mouse embryo. In this preliminary study, it is unfortunate that the embryos obtained did not have duplicates or triplicates. However, our raw data suggested that longer length of PNP was found in the antisense-treated embryos compared to both sense and PBS controls. This suggests that antisense treatment was specific to the development of the spinal neural tube. Nevertheless, it is recommended to repeat the experiment using high-quality rat serum in order to yield reliable result of embryo culture.

7.1 Future work

The prevalence and distribution study of NTDs will require a larger number of NTD cohort. In order to get convincing results on prevalence of NTDs, it would be informative to collect data from all main hospitals in every districts in Malaysia. Data collection should also include the data of periconceptional intake of folic acid, parental drug intake, and maternal behavior during pregnancy (drinking, prenatal care, maternal smoking, and maternal weight gain) to determine the specific causes of NTDs in Malaysian populations.

For the genetic analysis of spina bifida, segregation analysis in the triads should be conducted using larger number of spina bifida risk genes. In addition, screening of

these variants in large scale genome-wide association study (GWAS) is needed to confirm the variants as genetic risk factors for spina bifida. Besides that, more studies on the association between the identified candidate variants and spina bifida in larger populations is needed. Additionally, further validation experiments at the transcript level (RNA and protein) would help to assess the pathogenic or disease aetiology of the candidate variants.

7.2 Implication and application in research and medical fields

Due to lack of publications, the prevalence and distribution study was made significant at garnering current relevant data on the state of NTDs in Malaysia. The high estimated prevalence rate provide the overview of NTDs as the significant preventable public health problem. This subsequently helps to improve the prevention, treatment and management of NTDs. On the other hand, as far as the genetic causal of NTD remain largely unknown, the genetic study provide efforts to better understand the genetic contribution to the pathogenesis of NTDs. Further study and collection of genetics information can be done for the counselling and care of families of patients with NTDs. The application to the counselling will be more accurate prediction of recurrent rates and evidence-based prevention strategies.

REFERENCES

Abdul-Aziz, N. M., Turmaine, M., Greene, N. D., Copp, A. J. (2009). EphrinA-EphA receptor interactions in mouse spinal neurulation: implications for neural fold fusion. Int J Dev Biol, 53(4), 559-568. doi:10.1387/ijdb.082777na

Abramovich, C., Humphries, R. K. (2005). Hox regulation of normal and leukemic hematopoietic stem cells. Curr Opin Hematol, 12(3), 210-216.

Acuna-Hidalgo, R., Veltman, J. A., Hoischen, A. (2016). New insights into the generation and role of de novo mutations in health and disease. Genome Biology, 17(1), 241. doi:10.1186/s13059-016-1110-1

Adzhubei, I. A., Schmidt, S., Peshkin, L., Ramensky, V. E., Gerasimova, A., Bork, P., . . . Sunyaev, S. R. (2010). A method and server for predicting damaging missense mutations. Nat Methods, 7(4), 248-249. doi:10.1038/nmeth0410-248

Adzick, N. S. (2013). Fetal surgery for spina bifida: past, present, future. Semin Pediatr Surg, 22(1), 10-17. doi:10.1053/j.sempedsurg.2012.10.003

Adzick, N. S., Thom, E. A., Spong, C. Y., Brock, J. W., 3rd, Burrows, P. K., Johnson, M. P., . . . Farmer, D. L. (2011). A randomized trial of prenatal versus postnatal repair of myelomeningocele. N Engl J Med, 364(11), 993-1004. doi:10.1056/NEJMoa1014379

Afgan, E., Baker, D., van den Beek, M., Blankenberg, D., Bouvier, D., Cech, M., . . . Goecks, J. (2016). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. Nucleic Acids Res, 44(W1), W3w10. doi:10.1093/nar/gkw343

Ahern, M. M., Hendryx, M., Conley, J., Fedorko, E., Ducatman, A., Zullig, K. J. (2011). The association between mountaintop mining and birth defects among live births in central Appalachia, 1996–2003. Environmental Research, 111(6), 838-846.

Ailes, E. C., Gilboa, S. M., Gill, S. K., Broussard, C. S., Crider, K. S., Berry, R. J., . . . Reefhuis, J. (2016). Association between antibiotic use among pregnant women with urinary tract infections in the first trimester and birth defects, National Birth Defects Prevention Study 1997 to 2011. Birth Defects Res A Clin Mol Teratol, 106(11), 940-949. doi:10.1002/bdra.23570

Akbas, E., Altintas, Z. M., Celik, S. K., Dilek, U. K., Ali Delibas, Ozen, S., . . . Uyaniker, G. A. (2012). Rare Types of Turner Syndrome: Clinical Presentation and Cytogenetics in Five Cases. Labmedicine, 43(5).

Al Hosani, H., Salah, M., Abu-Zeid, H., Farag, H. M., Saade, D. (2005). The National Congenital Anomalies Register in the United Arab Emirates. East Mediterr Health J, 11(4), 690-699.

Alfirevic, Z. (2005). Failure to diagnose a fetal anomaly on a routine ultrasound scan at 20 weeks. Ultrasound Obstet Gynecol, 26(7), 797-798. doi:10.1002/uog.2631

Amorim, M. R., Lima, M. A., Castilla, E. E., Orioli, I. M. (2007). Non-Latin European descent could be a requirement for association of NTDs and MTHFR variant 677C > T: a meta-analysis. Am J Med Genet A, 143a(15), 1726-1732. doi:10.1002/ajmg.a.31812

Au, K.-S., Northrup, H., Kirkpatrick, T., Volcik, K., Fletcher, J., Townsend, I., … King, T. (2005). Promotor Genotype of the Platelet-derived Growth Factor Receptor-α gene Shows Population Stratification but Not Association with Spina Bifida Meningomyelocele. American Journal of Medical Genetics. Part A, 139(3), 194–198. http://doi.org/10.1002/ajmg.a.31002

Bassuk, A. G., Kibar, Z. (2009). Genetic basis of neural tube defects. Semin Pediatr Neurol, 16(3), 101-110. doi:10.1016/j.spen.2009.06.001

Bharath, R., Unnikrishnan, A. G., Thampy, M. V., Anilkumar, A., Nisha, B., Praveen, V. P., . . . Kumar, H. (2010). Turner syndrome and its variants. Indian J Pediatr, 77(2), 193-195. doi:10.1007/s12098-009-0226-7

Blom, H. J., Shaw, G. M., den Heijer, M., Finnell, R. H. (2006). Neural tube defects and folate: case far from closed. Nat Rev Neurosci, 7(9), 724-731. doi:10.1038/nrn1986

Boduroglu, K., Alikasifoglu, M., Anar, B., Tuncbilek, E. (1999). Association of the 677C->T mutation on the methylenetetrahydrofolate reductase gene in Turkish patients with neural tube defects. J Child Neurol, 14(3), 159-161. doi:10.1177/088307389901400305

Boo, N. Y., Cheah, I. G., Thong, M. K. (2013). Neural tube defects in Malaysia: data from the Malaysian National Neonatal Registry. J Trop Pediatr, 59(5), 338-342. doi:10.1093/tropej/fmt026

Botto, L. D., Moore, C. A., Khoury, M. J., Erickson, J. D. (1999). Neural-tube defects. N Engl J Med, 341(20), 1509-1519. doi:10.1056/nejm199911113412006

Boulet, S. L., Yang, Q., Mai, C., Kirby, R. S., Collins, J. S., Robbins, J. M., . . . Mulinare, J. (2008). Trends in the postfortification prevalence of spina bifida and anencephaly in the United States. Birth Defects Res A Clin Mol Teratol, 82(7), 527-532. doi:10.1002/bdra.20468

Boyles, A. L., Hammock, P., Speer, M. C. (2005). Candidate gene analysis in human neural tube defects. Am J Med Genet C Semin Med Genet, 135c(1), 9-23. doi:10.1002/ajmg.c.30048

Brender, J. D., Weyer, P. J. (2016). Agricultural Compounds in Water and Birth Defects. Curr Environ Health Rep, 3(2), 144-152. doi:10.1007/s40572-016-0085-0

Brook, F. A., Estibeiro, J. P., Copp, A. J. (1994). Female predisposition to cranial neural tube defects is not because of a difference between the sexes in the rate of embryonic growth or development during neurulation. J Med Genet, 31(5), 383387.

Brown, K. S., Cook, M., Hoess, K., Whitehead, A. S., Mitchell, L. E. (2004). Evidence that the risk of spina bifida is influenced by genetic variation at the NOS3 locus. Birth Defects Res A Clin Mol Teratol, 70(3), 101-106. doi:10.1002/bdra.20002

Brown, L. Y., Hodge, S. E., Johnson, W. G., Guy, S. G., Nye, J. S., Brown, S. (2002). Possible association of NTDs with a polyhistidine tract polymorphism in the ZIC2 gene. Am J Med Genet, 108(2), 128-131.

Brown, L. Y., Odent, S., David, V., Blayau, M., Dubourg, C., Apacik, C., . . . Muenke, M. (2001). Holoprosencephaly due to mutations in ZIC2: alanine tract expansion mutations may be caused by parental somatic recombination. Hum Mol Genet, 10(8), 791-796.

Bruey, J. M., Bruey-Sedano, N., Newman, R., Chandler, S., Stehlik, C., Reed, J. C. (2004). PAN1/NALP2/PYPAF2, an inducible inflammatory mediator that regulates NF-kappaB and caspase-1 activation in macrophages. J Biol Chem, 279(50), 51897-51907. doi:10.1074/jbc.M406741200

Calhoun, C. L., Schottler, J., Vogel, L. C. (2013). Recommendations for Mobility in Children with Spinal Cord Injury. Topics in Spinal Cord Injury Rehabilitation, 19(2), 142-151. doi:10.1310/sci1902-142

Campbell, J. B., Moore, K. N., Voaklander, D. C., Mix, L. W. (2004). Complications associated with clean intermittent catheterization in children with spina bifida. J Urol, 171(6 Pt 1), 2420-2422.

Canfield, M. A., Mai, C. T., Wang, Y., O'Halloran, A., Marengo, L. K., Olney, R. S., . . . Kirby, R. S. (2014). The association between race/ethnicity and major birth defects in the United States, 1999-2007. Am J Public Health, 104(9), e14-23. doi:10.2105/ajph.2014.302098

Cappell, K. M., Larson, B., Sciaky, N., Whitehurst, A. W. (2010). Symplekin specifies mitotic fidelity by supporting microtubule dynamics. Mol Cell Biol, 30(21), 51355144. doi:10.1128/mcb.00758-10

Carroll, N., Pangilinan, F., Molloy, A. M., Troendle, J., Mills, J. L., Kirke, P. N., . . . Parle-McDermott, A. (2009). Analysis of the MTHFD1 promoter and risk of neural tube defects. Hum Genet, 125(3), 247-256. doi:10.1007/s00439-008-0616-

Cengiz, B., Soylemez, F., Ozturk, E., Cavdar, A. O. (2004). Serum zinc, selenium, copper, and lead levels in women with second-trimester induced abortion resulting from neural tube defects: a preliminary study. Biol Trace Elem Res, 97(3), 225-235. doi:10.1385/bter:97:3:225

Centers-for-Disease-Control-and-Prevention (2009). What Is PedNSS/PNSS? PedNSS Health Indicators. Retrieved from http://www.cdc.gov/pednss/whatis/pednsshealth indicators.htm

Chabchoub, E., Willekens, D., Vermeesch, J. R., Fryns, J. P. (2012). Holoprosencephaly and ZIC2 microdeletions: novel clinical and epidemiological specificities delineated. Clin Genet, 81(6), 584-589. doi:10.1111/j.1399-0004.2011.01684.x

Chen, G., Pei, L. J., Huang, J., Song, X. M., Lin, L. M., Gu, X., . . . Zheng, X. Y. (2009). Unusual patterns of neural tube defects in a high risk region of northern China. Biomed Environ Sci, 22(4), 340-344. doi:10.1016/s0895-3988(09)60065-9

Chen, S., Zhang, Q., Bai, B., Ouyang, S., Bao, Y., Li, H., Zhang, T. (2016). MARK2/Par1b Insufficiency Attenuates DVL Gene Transcription via Histone Deacetylation in Lumbosacral Spina Bifida. Mol Neurobiol. doi:10.1007/s12035016-0164-0

Chen, X. S., Reader, R. H., Hoischen, A., Veltman, J. A., Simpson, N. H., Francks, C., . . . Fisher, S. E. (2017). Next-generation DNA sequencing identifies novel gene variants and pathways involved in specific language impairment. Scientific Reports, 7, 46105. doi:10.1038/srep46105

Choi, M., Scholl, U. I., Ji, W., Liu, T., Tikhonova, I. R., Zumbo, P., . . . Lifton, R. P. (2009). Genetic diagnosis by whole exome capture and massively parallel DNA sequencing. Proc Natl Acad Sci U S A, 106(45), 19096-19101. doi:10.1073/pnas.0910672106

Choi, Y., Chan, A. P. (2015). PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. Bioinformatics, 31(16), 2745-2747. doi:10.1093/bioinformatics/btv195

Copp, A. J. (2005). Neurulation in the cranial region--normal and abnormal. J Anat, 207(5), 623-635. doi:10.1111/j.1469-7580.2005.00476.x

Copp, A. J., Adzick, N. S., Chitty, L. S., Fletcher, J. M., Holmbeck, G. N., Shaw, G. M. (2015). Spina bifida. Nat Rev Dis Primers, 1, 15007. doi:10.1038/nrdp.2015.7

Copp, A. J., Brook, F. A., Estibeiro, J. P., Shum, A. S., Cockroft, D. L. (1990). The embryonic development of mammalian neural tube defects. Prog Neurobiol, 35(5), 363-403.

Copp, A. J., Greene, N. D. (2010). Genetics and development of neural tube defects. J Pathol, 220(2), 217-230. doi:10.1002/path.2643

Copp, A. J., Greene, N. D., Murdoch, J. N. (2003). The genetic basis of mammalian neurulation. Nat Rev Genet, 4(10), 784-793. doi:10.1038/nrg1181

Copp, A. J., Greene, N. D. E. (2013). Neural tube defects – disorders of neurulation and related embryonic processes. Wiley interdisciplinary reviews. Developmental biology, 2(2), 213-227. doi:10.1002/wdev.71

Copp, A. J., Stanier, P., Greene, N. D. (2013). Neural tube defects: recent advances, unsolved questions, and controversies. Lancet Neurol, 12(8), 799-810. doi:10.1016/s1474-4422(13)70110-8

Cordier, S., Bergeret, A., Goujard, J., Ha, M. C., Ayme, S., Bianchi, F., . . . Mandereau, L. (1997). Congenital malformation and maternal occupational exposure to glycol ethers. Occupational Exposure and Congenital Malformations Working Group. Epidemiology, 8(4), 355-363.

Court, K. A., Hatakeyama, H., Wu, S. Y., Lingegowda, M. S., Rodriguez-Aguayo, C., Lopez-Berestein, G., . . . Torres-Lugo, M. (2017). HSP70 Inhibition Synergistically Enhances the Effects of Magnetic Fluid Hyperthermia in Ovarian Cancer. Mol Cancer Ther, 16(5), 966-976. doi:10.1158/1535-7163.mct-16-0519

Dane, B., Dane, C., Aksoy, F., Cetin, A., Yayla, M. (2007). Jarcho-Levin syndrome presenting as neural tube defect: report of four cases and pitfalls of diagnosis. Fetal Diagn Ther, 22(6), 416-419. doi:10.1159/000106345

Davidson, C. M., Northrup, H., King, T. M., Fletcher, J. M., Townsend, I., Tyerman, G. H., Au, K. S. (2008). Genes in glucose metabolism and association with spina bifida. Reprod Sci, 15(1), 51-58. doi:10.1177/1933719107309590

Davila, S., Furu, L., Gharavi, A. G., Tian, X., Onoe, T., Qian, Q., . . . Somlo, S. (2004). Mutations in SEC63 cause autosomal dominant polycystic liver disease. Nat Genet, 36(6), 575-577. doi:10.1038/ng1357

de Franchis, R., Botto, L. D., Sebastio, G., Ricci, R., Iolascon, A., Capra, V., . . . Mastroiacovo, P. (2002). Spina bifida and folate-related genes: a study of genegene interactions. Genet Med, 4(3), 126-130. doi:10.1097/00125817-20020500000005

de Franchis, R., Buoninconti, A., Mandato, C., Pepe, A., Sperandeo, M. P., Del Gado, R., . . . Mastroiacovo, P. (1998). The C677T mutation of the 5,10methylenetetrahydrofolate reductase gene is a moderate risk factor for spina bifida in Italy. J Med Genet, 35(12), 1009-1013.

De Marco, P., Merello, E., Calevo, M. G., Mascelli, S., Raso, A., Cama, A., & Capra, V. (2006). Evaluation of a methylenetetrahydrofolate-dehydrogenase 1958G>A polymorphism for neural tube defect risk. J Hum Genet, 51(2), 98-103. doi:10.1007/s10038-005-0329-6

De Marco, P., Merello, E., Piatelli, G., Cama, A., Kibar, Z., Capra, V. (2014). Planar cell polarity gene mutations contribute to the etiology of human neural tube defects in our population. Birth Defects Res A Clin Mol Teratol, 100(8), 633-641. doi:10.1002/bdra.23255

Deak, K. L., Boyles, A. L., Etchevers, H. C., Melvin, E. C., Siegel, D. G., Graham, F. L., . . . Speer, M. C. (2005a). SNPs in the neural cell adhesion molecule 1 gene (NCAM1) may be associated with human neural tube defects. Hum Genet, 117(23), 133-142. doi:10.1007/s00439-005-1299-7

Deak, K. L., Dickerson, M. E., Linney, E., Enterline, D. S., George, T. M., Melvin, E. C., . . . Speer, M. C. (2005b). Analysis of ALDH1A2, CYP26A1, CYP26B1, CRABP1, and CRABP2 in human neural tube defects suggests a possible association with alleles in ALDH1A2. Birth Defects Res A Clin Mol Teratol, 73(11), 868-875. doi:10.1002/bdra.20183

Detrait, E. R., George, T. M., Etchevers, H. C., Gilbert, J. R., Vekemans, M., Speer, M. C. (2005). Human neural tube defects: developmental biology, epidemiology, and genetics. Neurotoxicol Teratol, 27(3), 515-524. doi:10.1016/j.ntt.2004.12.007

Division-of-Family-Health-Development. (2013). Perinatal Care Manual 3rd Edition. Retrieved from http://fh.moh.gov.my/v3/index.php/component/jdownloads/send/18-sektor- kesihatan-ibu/224-perinatal-care-manual-3rd edition2013?option=com_ jdownloads

Dolk, H., Loane, M., Garne, E. (2010). The prevalence of congenital anomalies in Europe. Adv Exp Med Biol, 686, 349-364. doi:10.1007/978-90-481-9485-8_20

Doudney, K., Grinham, J., Whittaker, J., Lynch, S. A., Thompson, D., Moore, G. E., . . . Stanier, P. (2009). Evaluation of folate metabolism gene polymorphisms as risk factors for open and closed neural tube defects. Am J Med Genet A, 149a(7), 15851589. doi:10.1002/ajmg.a.32937

Doudney, K., Stanier, P. (2005). Epithelial cell polarity genes are required for neural tube closure. Am J Med Genet C Semin Med Genet, 135c(1), 42-47. doi:10.1002/ajmg.c.30052

Ekanem, T. B., Okon, D. E., Akpantah, A. O., Mesembe, O. E., Eluwa, M. A., Ekong, M. B. (2008). Prevalence of congenital malformations in Cross River and Akwa Ibom states of Nigeria from 1980-2003. Congenit Anom (Kyoto), 48(4), 167-170. doi:10.1111/j.1741-4520.2008.00204.x

Elms, P., Siggers, P., Napper, D., Greenfield, A., Arkell, R. (2003). Zic2 is required for neural crest formation and hindbrain patterning during mouse development. Dev Biol, 264(2), 391-406.

Enaw, J. O., Zhu, H., Yang, W., Lu, W., Shaw, G. M., Lammer, E. J., Finnell, R. H. (2006). CHKA and PCYT1A gene polymorphisms, choline intake and spina bifida risk in a California population. BMC Med, 4, 36. doi:10.1186/1741-70154-36

EUROCAT. (2012). European Surveillance of Congenital Anomalies. Prevalence Tables Retrieved from http://www.eurocat-network.eu/accessprevalencedata/ prevalencetables

Faden, D. L., Asthana, S., Tihan, T., DeRisi, J., Kliot, M. (2017). Correction: Whole Exome Sequencing of Growing and Non-Growing Cutaneous Neurofibromas from a Single Patient with Neurofibromatosis Type 1. PLoS One, 12(2), e0172620. doi:10.1371/journal.pone.0172620

Fajardo, K. V. F., Adams, D., Program, N. C. S., Mason, C. E., Sincan, M., Tifft, C., . . . Markello, T. (2012). Detecting false positive signals in exome sequencing. Human Mutation, 33(4), 609-613. doi:10.1002/humu.22033

Farlow, J. L., Robak, L. A., Hetrick, K., Bowling, K., Boerwinkle, E., Coban-Akdemir, Z. H., … Foroud, T. (2016). Whole-Exome Sequencing in Familial Parkinson Disease. JAMA Neurology, 73(1), 68–75.

Fedeles, S. V., Tian, X., Gallagher, A. R., Mitobe, M., Nishio, S., Lee, S. H., . . . Somlo, S. (2011). A genetic interaction network of five genes for human polycystic kidney and liver diseases defines polycystin-1 as the central determinant of cyst formation. Nat Genet, 43(7), 639-647. doi:10.1038/ng.860

Fontalba, A., Gutierrez, O., Fernandez-Luna, J. L. (2007). NLRP2, an inhibitor of the NFkappaB pathway, is transcriptionally activated by NF-kappaB and exhibits a nonfunctional allelic variant. J Immunol, 179(12), 8519-8524.

Franke, B., Vermeulen, S. H., Steegers-Theunissen, R. P., Coenen, M. J., Schijvenaars, M. M., Scheffer, H., . . . Blom, H. J. (2009). An association study of 45 folaterelated genes in spina bifida: Involvement of cubilin (CUBN) and tRNA aspartic acid methyltransferase 1 (TRDMT1). Birth Defects Res A Clin Mol Teratol, 85(3), 216-226. doi:10.1002/bdra.20556

Fryns, J. P., Devriendt, K., Moerman, P. (1996). Lumbosacral spina bifida and myeloschizis in a female foetus with de novo X/autosomal translocation (t(X;22)(q27;q121)). Genet Couns, 7(2), 159-160.

Garbett, K., Ebert, P. J., Mitchell, A., Lintas, C., Manzi, B., Mirnics, K., Persico, A. M. (2008). Immune transcriptome alterations in the temporal cortex of subjects with autism. Neurobiol Dis, 30(3), 303-311. doi:10.1016/j.nbd.2008.01.012

Gebbia, M., Ferrero, G. B., Pilia, G., Bassi, M. T., Aylsworth, A., Penman-Splitt, M., . . . Casey, B. (1997). X-linked situs abnormalities result from mutations in ZIC3. Nat Genet, 17(3), 305-308. doi:10.1038/ng1197-305

Gonzalez-Herrera, L., Garcia-Escalante, G., Castillo-Zapata, I., Canto-Herrera, J., Ceballos-Quintal, J., Pinto-Escalante, D., . . . Orozco-Orozco, L. (2002). Frequency of the thermolabile variant C677T in the MTHFR gene and lack of association with neural tube defects in the State of Yucatan, Mexico. Clin Genet, 62(5), 394-398.

Gonzalez-Herrera, L., Castillo-Zapata, I., Garcia-Escalante, G., Pinto-Escalante, D. (2007). A1298C polymorphism of the MTHFR gene and neural tube defects in the state of Yucatan, Mexico. Birth Defects Res A Clin Mol Teratol, 79(8), 622626. doi:10.1002/bdra.20381

Gosalia, N., Economides, A. N., Dewey, F. E., Balasubramanian, S. (2017). MAPPIN: a method for annotating, predicting pathogenicity and mode of inheritance for nonsynonymous variants. Nucleic Acids Research, 45(18), 10393-10402. doi:10.1093/nar/gkx730

Gowen, L. C., Johnson, B. L., Latour, A. M., Sulik, K. K., Koller, B. H. (1996). Brca1 deficiency results in early embryonic lethality characterized by neuroepithelial abnormalities. Nat Genet, 12(2), 191-194. doi:10.1038/ng0296-191

Grandone, E., Corrao, A. M., Colaizzo, D., Vecchione, G., Di Girgenti, C., Paladini, D., . . . Margaglione, M. (2006). Homocysteine metabolism in families from southern Italy with neural tube defects: role of genetic and nutritional determinants. Prenat Diagn, 26(1), 1-5. doi:10.1002/pd.1359

Greene, N. D., Copp, A. J. (2009). Development of the vertebrate central nervous system: formation of the neural tube. Prenat Diagn, 29(4), 303-311. doi:10.1002/pd.2206

Greene, N. D., Copp, A. J. (2014). Neural tube defects. Annu Rev Neurosci, 37, 221-242. doi:10.1146/annurev-neuro-062012-170354

Greene, N. D., Stanier, P., Copp, A. J. (2009). Genetics of human neural tube defects. Hum Mol Genet, 18(R2), R113-129. doi:10.1093/hmg/ddp347

Grewal, J., Carmichael, S. L., Ma, C., Lammer, E. J., Shaw, G. M. (2008). Maternal periconceptional smoking and alcohol consumption and risk for select congenital anomalies. Birth Defects Res A Clin Mol Teratol, 82(7), 519-526. doi:10.1002/bdra.20461

Gueant-Rodriguez, R. M., Rendeli, C., Namour, B., Venuti, L., Romano, A., Anello, G., . . . Gueant, J. L. (2003). Transcobalamin and methionine synthase reductase mutated polymorphisms aggravate the risk of neural tube defects in humans. Neurosci Lett, 344(3), 189-192.

Gutierrez-Angulo, M., Lazalde, B., Vasquez, A. I., Leal, C., Corral, E., Rivera, H. (2002). del(X)(p22.1)/r(X)(p22.1q28) Dynamic mosaicism in a Turner syndrome patient. Ann Genet, 45(1), 17-20. doi: 10.1016/S0003-3995(02)01109-7

Hackshaw, A., Rodeck, C., Boniface, S. (2011). Maternal smoking in pregnancy and birth defects: a systematic review based on 173 687 malformed cases and 11.7 million controls. Hum Reprod Update, 17(5), 589-604. doi:10.1093/humupd/dmr022

Hall, J. G., Friedman, J. M., Kenna, B. A., Popkin, J., Jawanda, M., Arnold, W. (1988). Clinical, genetic, and epidemiological factors in neural tube defects. Am J Hum Genet, 43(6), 827-837.

Hansen, D. K., Barbee, S. A., Grafton, T. F., Gu, Y., Streck, R. D. (2001). Antisense modulation of 5,10-methylenetetrahydrofolate reductase expression produces neural tube defects in mouse embryos. Reprod Toxicol, 15(1), 21-29.

Harris, M. J., Juriloff, D. M. (2007). Mouse mutants with neural tube closure defects and their role in understanding human neural tube defects. Birth Defects Res A Clin Mol Teratol, 79(3), 187-210. doi:10.1002/bdra.20333

Harris, M. J., Juriloff, D. M. (2010). An update to the list of mouse mutants with neural tube closure defects and advances toward a complete genetic perspective of neural tube closure. Birth Defects Res A Clin Mol Teratol, 88(8), 653-669. doi:10.1002/bdra.20676

Hayes, J. E., Feeney, E. L., Allen, A. L. (2013). Do polymorphisms in chemosensory genes matter for human ingestive behavior? Food Qual Prefer, 30(2), 202-216. doi:10.1016/j.foodqual.2013.05.013

Hernández-Díaz, S., Werler, M. M., Walker, A. M., Mitchell, A. A. (2001). Neural Tube Defects in Relation to Use of Folic Acid Antagonists during Pregnancy. American Journal of Epidemiology, 153(10), 961-968. doi:10.1093/aje/153.10.961

Hernandez-Molina, G., Rodriguez-Perez, J. M., Fernandez-Torres, J., Lima, G., PerezHernandez, N., Lopez-Reyes, A., Martinez-Nava, G. A. (2017). HIF1A (rs11549465) and AKNA (rs10817595) Gene Polymorphisms Are Associated with Primary Sjogren's Syndrome. Biomed Res Int, 2017, 5845849. doi:10.1155/2017/5845849

Hill, D. S., Wlodarczyk, B. J., Palacios, A. M., Finnell, R. H. (2010). Teratogenic effects of antiepileptic drugs. Expert review of neurotherapeutics, 10(6), 943-959. doi:10.1586/ern.10.57

Hindin, R., Brugge, D., Panikkar, B. (2005). Teratogenicity of depleted uranium aerosols: A review from an epidemiological perspective. Environmental Health, 4, 17-17. doi:10.1186/1476-069X-4-17

Ho, M. K. (2016). Current Population Estimates, Malaysia, 2014-2016. Retrieved from The Office of Chief Statistician Malaysia:

Hoang, T., Nguyen, D. T., Nguyen, P. V., Tran, D. A., Gillerot, Y., Reding, R., Robert, A. (2013). External birth defects in Southern Vietnam: a population-based study at the grassroots level of health care in Binh Thuan Province. BMC Pediatr, 13, 67. doi:10.1186/1471-2431-13-67

Hong, J. Y., Liu, X., Mao, M., Li, M., Choi, D. I., Kang, S. W., . . . La Choi, Y. (2013). Genetic Aberrations in Imatinib-Resistant Dermatofibrosarcoma Protuberans Revealed by Whole Genome Sequencing. PLoS ONE, 8(7), e69752. doi:10.1371/journal.pone.0069752

Houcher B, Akar N, Begag S, Y., E. (2012). Neural tube defects in Algeria. In D. K. L. Narasimhan (Ed.), Neural Tube Defects - Role of Folate, Prevention Strategies and Genetics: InTech.

Howards, P. P., Johnson, C. Y., Honein, M. A., Flanders, W. D. (2015). Adjusting for bias due to incomplete case ascertainment in case-control studies of birth defects. Am J Epidemiol, 181(8), 595-607. doi:10.1093/aje/kwu323

Imbard, A., Benoist, J.-F., Blom, H. J. (2013). Neural Tube Defects, Folic Acid and Methylation. International Journal of Environmental Research and Public Health, 10(9), 4352-4389. doi:10.3390/ijerph10094352

Inoue, T., Hatayama, M., Tohmonda, T., Itohara, S., Aruga, J., Mikoshiba, K. (2004). Mouse Zic5 deficiency results in neural tube defects and hypoplasia of cephalic neural crest derivatives. Dev Biol, 270(1), 146-162. doi:10.1016/j.ydbio.2004.02.017

Inoue, T., Ota, M., Mikoshiba, K., Aruga, J. (2007). Zic2 and Zic3 synergistically control neurulation and segmentation of paraxial mesoderm in mouse embryo. Dev Biol, 306(2), 669-684. doi:10.1016/j.ydbio.2007.04.003

Isakov, O., Perrone, M., Shomron, N. (2013). Exome sequencing analysis: a guide to disease variant detection. Methods Mol Biol, 1038, 137-158. doi:10.1007/978-162703-514-9_8

Jaruratanasirikul, S., Kor-anantakul, O., Limpitikul, W., Dissaneevate, P., Khunnarakpong, N., Sattapanyo, A. (2014). Prevalence of neural tube defect in southern Thailand: a population-based survey during 2009-2012. Childs Nerv Syst, 30(7), 1269-1275. doi:10.1007/s00381-014-2410-y

Jaquier, M., Klein, A., Boltshauser, E. (2006). Spontaneous pregnancy outcome after prenatal diagnosis of anencephaly. Bjog, 113(8), 951-953. doi:10.1111/j.14710528.2006.01014.x

Jensen, L. E., Barbaux, S., Hoess, K., Fraterman, S., Whitehead, A. S., Mitchell, L. E. (2004). The human T locus and spina bifida risk. Hum Genet, 115(6), 475-482. doi:10.1007/s00439-004-1185-8

Jin, L., Zhang, L., Li, Z., Liu, J. M., Ye, R., Ren, A. (2013). Placental concentrations of mercury, lead, cadmium, and arsenic and the risk of neural tube defects in a Chinese population. Reprod Toxicol, 35, 25-31. doi:10.1016/j.reprotox.2012.10.015

Johnson, W. G., Stenroos, E. S., Spychala, J. R., Chatkupt, S., Ming, S. X., & Buyske, S. (2004). New 19 bp deletion polymorphism in intron-1 of dihydrofolate reductase (DHFR): a risk factor for spina bifida acting in mothers during pregnancy? Am J Med Genet A, 124A(4), 339-345. doi:10.1002/ajmg.a.20505

Jong, T. d., Chrzan, R., Klijn, A. J., Dik, P. (2008). Treatment of the neurogenic bladder in spina bifida. Pediatric Nephrology (Berlin, Germany), 23(6), 889-896. doi:10.1007/s00467-008-0780-7

Kallen, B., Robert, E., Harris, J. (1998). Associated malformations in infants and fetuses with upper or lower neural tube defects. Teratology, 57(2), 56-63. doi:10.1002/(sici)1096-9926(199802)57:2<56::aid-tera3>3.0.co;2-4

Kang, S. S., Wong, P. W. (1996). Genetic and nongenetic factors for moderate hyperhomocyst(e)inemia. Atherosclerosis, 119(2), 135-138.

Karczewski, K. J., Weisburd, B., Thomas, B., Solomonson, M., Ruderfer, D. M., Kavanagh, D., . . . MacArthur, D. G. (2016). The ExAC browser: displaying reference data information from over 60 000 exomes. Nucleic Acids Res. doi:10.1093/nar/gkw971

Kase, B. A., Northrup, H., Morrison, A. C., Davidson, C. M., Goiffon, A. M., Fletcher, J. M., . . . Au, K. S. (2012). Association of copper-zinc superoxide dismutase (SOD1) and manganese superoxide dismutase (SOD2) genes with nonsyndromic myelomeningocele. Birth Defects Res A Clin Mol Teratol, 94(10), 762-769. doi:10.1002/bdra.23065

Kaur, J., Singh, H. (2011). Maternal Health in Malaysia: A Review. WebmedCentral PUBLIC HEALTH, 2(12):WMC002599. doi:10.9754/journal.wmc.2011.002599

Khan, A. A., Bhatti, S. N., Khan, G., Ahmed, E., Aurangzeb, A., Ali, A., . . . Afzal, S. (2010). Clinical and Radiological Findings in Arnold Chiari Malformation. J Ayub Med Coll Abbottabad.

Khattak, S. T., Khan, M., Naheed, T., Khattak, I., Ismail, M. (2010). Prevalence and management of anencephaly at Saidu Teaching Hospital, Swat. J Ayub Med Coll Abbottabad, 22(4), 61-63.

Khoo, T. B., Kassim, A. B., Omar, M. A., Hasnan, N., Amin, R. M., Omar, Z., Yusoff, A. F. (2009). Prevalence and impact of physical disability on Malaysian schoolaged children: a population-based survey. Disabil Rehabil, 31(21), 1753-1761. doi:10.1080/09638280902751964

Kibar, Z., Bosoi, C. M., Kooistra, M., Salem, S., Finnell, R. H., De Marco, P., . . . Gros, P. (2009). Novel mutations in VANGL1 in neural tube defects. Hum Mutat, 30(7), E706-715. doi:10.1002/humu.21026

Kibar, Z., Torban, E., McDearmid, J. R., Reynolds, A., Berghout, J., Mathieu, M., . . . Gros, P. (2007). Mutations in VANGL1 associated with neural-tube defects. N Engl J Med, 356(14), 1432-1437. doi:10.1056/NEJMoa060651

Kibar, Z., Vogan, K. J., Groulx, N., Justice, M. J., Underhill, D. A., Gros, P. (2001). Ltap, a mammalian homolog of Drosophila Strabismus/Van Gogh, is altered in the mouse neural tube mutant Loop-tail. Nat Genet, 28(3), 251-255. doi:10.1038/90081

Kim, Y. C., Soliman, A. S., Cui, J., Ramadan, M., Hablas, A., Abouelhoda, M., . . . Wang, S. M. (2017). Unique Features of Germline Variation in Five Egyptian Familial Breast Cancer Families Revealed by Exome Sequencing. PLoS One, 12(1), e0167581. doi:10.1371/journal.pone.0167581

King, T. M., Au, K. S., Kirkpatrick, T. J., Davidson, C., Fletcher, J. M., Townsend, I., . . . Northrup, H. (2007). The impact of BRCA1 on spina bifida meningomyelocele lesions. Ann Hum Genet, 71(Pt 6), 719-728. doi:10.1111/j.14691809.2007.00377.x

Kirke, P. N., Mills, J. L., Molloy, A. M., Brody, L. C., O'Leary, V. B., Daly, L., . . . Scott, J. M. (2004). Impact of the MTHFR C677T polymorphism on risk of neural tube defects: case-control study. BMJ : British Medical Journal, 328(7455), 15351536. doi:10.1136/bmj.38036.646030.EE

Klootwijk, R., Groenen, P., Schijvenaars, M., Hol, F., Hamel, B., Straatman, H., . . . Franke, B. (2004). Genetic variants in ZIC1, ZIC2, and ZIC3 are not major risk factors for neural tube defects in humans. Am J Med Genet A, 124a(1), 40-47. doi:10.1002/ajmg.a.20402

Koch, M. C., Stegmann, K., Ziegler, A., Schroter, B., Ermert, A. (1998). Evaluation of the MTHFR C677T allele and the MTHFR gene locus in a German spina bifida population. Eur J Pediatr, 157(6), 487-492.

Kole, R., Krainer, A. R., Altman, S. (2012). RNA therapeutics: Beyond RNA interference and antisense oligonucleotides. Nature reviews. Drug discovery, 11(2), 125-140. doi:10.1038/nrd3625

Kozyraki, R., Fyfe, J., Kristiansen, M., Gerdes, C., Jacobsen, C., Cui, S., . . . Moestrup, S. K. (1999). The intrinsic factor–vitamin B12 receptor, cubilin, is a high-affinity apolipoprotein A-I receptor facilitating endocytosis of high-density lipoprotein. Nature Medicine, 5, 656. doi:10.1038/9504

Krupenko, N. I., Dubard, M. E., Strickland, K. C., Moxley, K. M., Oleinik, N. V., Krupenko, S. A. (2010). ALDH1L2 Is the Mitochondrial Homolog of 10Formyltetrahydrofolate Dehydrogenase. The Journal of Biological Chemistry, 285(30), 23056-23063. doi:10.1074/jbc.M110.128843

Krupp, D. R., Soldano, K. L., Garrett, M. E., Cope, H., Ashley-Koch, A. E., Gregory, S. G. (2014). Missing genetic risk in neural tube defects: can exome sequencing yield an insight? Birth Defects Res A Clin Mol Teratol, 100(8), 642-646. doi:10.1002/bdra.23276

Kuchmiy, A. A., D'Hont, J., Hochepied, T., Lamkanfi, M. (2016). NLRP2 controls ageassociated maternal fertility. The Journal of Experimental Medicine, 213(13), 2851-2860. doi:10.1084/jem.20160900

Kumar, P., Henikoff, S., Ng, P. C. (2009). Predicting the effects of coding nonsynonymous variants on protein function using the SIFT algorithm. Nat Protoc, 4(7), 1073-1081. doi:10.1038/nprot.2009.86

Kuper, J., Braun, C., Elias, A., Michels, G., Sauer, F., Schmitt, D. R., . . . Kisker, C. (2014). In TFIIH, XPD helicase is exclusively devoted to DNA repair. PLoS Biol, 12(9), e1001954. doi:10.1371/journal.pbio.1001954

Kyrklund-Blomberg, N. B., Granath, F., Cnattingius, S. (2005). Maternal smoking and causes of very preterm birth. Acta Obstet Gynecol Scand, 84(6), 572-577. doi:10.1111/j.0001-6349.2005.00848.x

Lambert-Messerlian, G. M., Palomaki, G. E., Canick, J. A. (2000). Second trimester levels of maternal serum inhibin A in pregnancies affected by fetal neural tube defects. Prenat Diagn, 20(8), 680-682. doi:10.1002/1097-0223

Largeot, A., Paggetti, J., Broseus, J., Aucagne, R., Lagrange, B., Martin, R. Z., . . . Delva, L. (2013). Symplekin, a polyadenylation factor, prevents MOZ and MLL activity on HOXA9 in hematopoietic cells. Biochim Biophys Acta, 1833(12), 3054-3063. doi:10.1016/j.bbamcr.2013.08.013

Lawrenson, R., Wyndaele, J. J., Vlachonikolis, I., Farmer, C., Glickman, S. (2000). A UK general practice database study of prevalence and mortality of people with neural tube defects. Clin Rehabil, 14(6), 627-630. doi:10.1191/0269215500

Lei, Y., Zhu, H., Duhon, C., Yang, W., Ross, M. E., Shaw, G. M., Finnell, R. H. (2013). Mutations in planar cell polarity gene SCRIB are associated with spina bifida. PLoS One, 8(7), e69262. doi:10.1371/journal.pone.0069262

Lei, Y., Zhu, H., Yang, W., Ross, M. E., Shaw, G. M., Finnell, R. H. (2014). Identification of novel CELSR1 mutations in spina bifida. PLoS One, 9(3), e92207. doi:10.1371/journal.pone.0092207

Lemay, P., Guyot, M. C., Tremblay, E., Dionne-Laporte, A., Spiegelman, D., Henrion, E., . . . Kibar, Z. (2015). Loss-of-function de novo mutations play an important role in severe human neural tube defects. J Med Genet, 52(7), 493-497. doi:10.1136/jmedgenet-2015-103027

Leung, T. K., Rajendran, M. Y., Monfries, C., Hall, C., Lim, L. (1990). The human heatshock protein family. Expression of a novel heat-inducible HSP70 (HSP70B') and isolation of its cDNA and genomic DNA. Biochem J, 267(1), 125-132.

Liao, Y., Wang, J., Wu, J., Driskell, L., Wang, W., Zhang, T., . . . Zheng, X. (2010). Spatial analysis of neural tube defects in a rural coal mining area. Int J Environ Health Res, 20(6), 439-450. doi:10.1080/09603123.2010.491854

Lin, Z., Liu, Z., Li, X., Li, F., Hu, Y., Chen, B., . . . Liu, Y. (2017). Whole-exome sequencing identifies a novel de novo mutation in DYNC1H1 in epileptic encephalopathies. Scientific Reports, 7, 258. doi:10.1038/s41598-017-00208-6

Lloyd, K. A. (2013). A scientific review: mechanisms of valproate-mediated teratogenesis. Bioscience Horizons: The International Journal of Student Research, 6, hzt003-hzt003. doi:10.1093/biohorizons/hzt003

Lu, W., Zhu, H., Wen, S., Laurent, C., Shaw, G. M., Lammer, E. J., & Finnell, R. H. (2007). Screening for novel PAX3 polymorphisms and risks of spina bifida. Birth Defects Res A Clin Mol Teratol, 79(1), 45-49. doi:10.1002/bdra.20322

Luo, K., Li, Y., Yin, Y., Li, L., Wu, C., Chen, Y., . . . Yuan, J. (2017). USP49 negatively regulates tumorigenesis and chemoresistance through FKBP51-AKT signaling. Embo j, 36(10), 1434-1446. doi:10.15252/embj.201695669

Ma, W., Ortiz-Quintero, B., Rangel, R., McKeller, M. R., Herrera-Rodriguez, S., Castillo, E. F., . . . Martinez-Valdez, H. (2011). Coordinate activation of inflammatory gene networks, alveolar destruction and neonatal death in AKNA deficient mice. Cell Res, 21(11), 1564-1577. doi:10.1038/cr.2011.84

Mahadevan, B., Bhat, B. V. (2005). Neural tube defects in Pondicherry. Indian J Pediatr, 72(7), 557-559.

Mandel, C. R., Bai, Y., Tong, L. (2008). Protein factors in pre-mRNA 3'-end processing. Cell Mol Life Sci, 65(7-8), 1099-1122. doi:10.1007/s00018-007-7474-3

Mao, L., Yang, P., Hou, S., Li, F., Kijlstra, A. (2011). Label-free proteomics reveals decreased expression of CD18 and AKNA in peripheral CD4+ T cells from patients with Vogt-Koyanagi-Harada syndrome. PLoS One, 6(1), e14616. doi:10.1371/journal.pone.0014616

Marco, P. D. (2012). Advances in Genetics of Non Syndromic Neural Tube Defects, Neural Tube Defects In D. K. L. Narasimhan (Ed.), Role of Folate, Prevention Strategies and Genetics (pp. 142-154). Rijeka, Croatia InTech.

Margaret, M., Tilak, P., Rajangam, S. (2010). 45,X/47,X,i(X)(q10),i(X)(q10)/ 46,X,i(X)(q10) Isochromosome Xq in Mosaic Turner syndrome. Int J Hum Genet, 10(1-3), 77-80.

Martin, I., Gibert, M. J., Pintos, C., Noguera, A., Besalduch, A., Obrador, A. (2004). Oxidative stress in mothers who have conceived fetus with neural tube defects: the role of aminothiols and selenium. Clin Nutr, 23(4), 507-514. doi:10.1016/j.clnu.2003.09.010

Matlow, J., Koren, G. (2012). Is carbamazepine safe to take during pregnancy? Canadian Family Physician, 58(2), 163-164.

Mazumdar, M., Ibne Hasan, M. O., Hamid, R., Valeri, L., Paul, L., Selhub, J., . . . Christiani, D. C. (2015). Arsenic is associated with reduced effect of folic acid in myelomeningocele prevention: a case control study in Bangladesh. Environ Health, 14, 34. doi:10.1186/s12940-015-0020-0

Missmer, S. A., Suarez, L., Felkner, M., Wang, E., Merrill, A. H., Rothman, K. J., Hendricks, K. A. (2006). Exposure to Fumonisins and the Occurrence of Neural Tube Defects along the Texas–Mexico Border. Environmental Health Perspectives, 114(2), 237-241. doi:10.1289/ehp.8221

Mitchell, L. E. (2008). Spina Bifida Research Resource: study design and participant characteristics. Birth Defects Res A Clin Mol Teratol, 82(10), 684-691. doi:10.1002/bdra.20465

Mitchell, L. E., Adzick, N. S., Melchionne, J., Pasquariello, P. S., Sutton, L. N., Whitehead, A. S. (2004). Spina bifida. Lancet, 364(9448), 1885-1895. doi:10.1016/s0140-6736(04)17445-x

Mlodzik, M. (2002). Planar cell polarization: do the same mechanisms regulate Drosophila tissue polarity and vertebrate gastrulation? Trends Genet, 18(11), 564571.

Mohd-Zin, S. W., Marwan, A. I., Abou Chaar, M. K., Ahmad-Annuar, A., Abdul-Aziz, N. M. (2017). Spina Bifida: Pathogenesis, Mechanisms, and Genes in Mice and Humans. Scientifica, 2017, 29. doi:10.1155/2017/5364827

Moliterno, A. R., Resar, L. M. S. (2011). AKNA: Another AT-hook transcription factor "hooking-up" with inflammation. Cell Research, 21(11), 1528-1530. doi:10.1038/cr.2011.96

Molloy, A. M., Mills, J. L., Kirke, P. N., Ramsbottom, D., McPartlin, J. M., Burke, H., . . . Scott, J. M. (1998). Low blood folates in NTD pregnancies are only partly explained by thermolabile 5,10-methylenetetrahydrofolate reductase: low folate status alone may be the critical factor. Am J Med Genet, 78(2), 155-159.

Monk, K. R., Voas, M. G., Franzini-Armstrong, C., Hakkinen, I. S., Talbot, W. S. (2013). Mutation of sec63 in zebrafish causes defects in myelinated axons and liver pathology. Disease Models & Mechanisms, 6(1), 135-145. doi:10.1242/dmm.009217

Moretti, M. E., Bar-Oz, B., Fried, S., Koren, G. (2005). Maternal hyperthermia and the risk for neural tube defects in offspring: systematic review and meta-analysis. Epidemiology, 16(2), 216-219.

Mornet, E., Muller, F., Lenvoise-Furet, A., Delezoide, A. L., Col, J. Y., Simon-Bouy, B., Serre, J. L. (1997). Screening of the C677T mutation on the methylenetetrahydrofolate reductase gene in French patients with neural tube defects. Hum Genet, 100(5-6), 512-514.

MRC-Vitamin-Study-Research-Group. (1991). Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. Lancet, 338(8760), 131-137.

Mund, M., Louwen, F., Klingelhoefer, D., Gerber, A. (2013). Smoking and Pregnancy — A Review on the First Major Environmental Risk Factor of the Unborn. Int J Environ Res Public Health, 10(12), 6485-6499. doi:10.3390/ijerph10126485

Murillo, B., Ruiz-Reig, N., Herrera, M., Fairen, A., Herrera, E. (2015). Zic2 Controls the Migration of Specific Neuronal Populations in the Developing Forebrain. J Neurosci, 35(32), 11266-11280. doi:10.1523/jneurosci.0779-15.2015

Nagai, T., Aruga, J., Minowa, O., Sugimoto, T., Ohno, Y., Noda, T., Mikoshiba, K. (2000). Zic2 regulates the kinetics of neurulation. Proc Natl Acad Sci U S A, 97(4), 1618-1623.

Nayarisseri, A., Yadav, M., Bhatia, M., Pandey, A., Elkunchwar, A., Paul, N., . . . Kumar, G. (2013). Impact of Next-Generation Whole-Exome sequencing in molecular diagnostics. Drug Invention Today, 5(4), 327-334. doi:10.1016/j.dit.2013.07.005

Ngo, A. D., Taylor, R., Roberts, C. L. (2010). Paternal exposure to Agent Orange and spina bifida: a meta-analysis. Eur J Epidemiol, 25(1), 37-44. doi:10.1007/s10654009-9401-4

Nielsen, L. A., Maroun, L. L., Broholm, H., Laursen, H., Graem, N. (2006). Neural tube defects and associated anomalies in a fetal and perinatal autopsy series. Apmis, 114(4), 239-246. doi:10.1111/j.1600-0463.2006.apm_325.x

Noel, J. K., Namazi, S., Haddock, R. L. (2016). Response to Commentary on Disparities in Infant Mortality Due to Congenital Anomalies on Guam. Hawai'i Journal of Medicine & Public Health, 75(9), 260-261.

Noonan, E. J., Place, R. F., Giardina, C., Hightower, L. E. (2007). Hsp70B′ regulation and function. Cell Stress & Chaperones, 12(4), 393-402. doi:10.1379/CSC-278e.1

Norman, S. M., Odibo, A. O., Longman, R. E., Roehl, K. A., Macones, G. A., Cahill, A. G. (2012). Neural tube defects and associated low birth weight. Am J Perinatol, 29(6), 473-476. doi:10.1055/s-0032-1304830

Nyholm, M. K., Wu, S.-F., Dorsky, R. I., Grinblat, Y. (2007). The zebrafish zic2a-zic5 gene pair acts downstream of canonical Wnt signaling to control cell proliferation in the developing tectum. Development, 134(4), 735.

O'Leary, V. B., Mills, J. L., Parle-McDermott, A., Pangilinan, F., Molloy, A. M., Cox, C., . . . Birth Defects Research, G. (2005). Screening for new MTHFR polymorphisms and NTD risk. Am J Med Genet A, 138A(2), 99-106. doi:10.1002/ajmg.a.30846

Olshan, A. F., Shaw, G. M., Millikan, R. C., Laurent, C., Finnell, R. H. (2005). Polymorphisms in DNA repair genes as risk factors for spina bifida and orofacial clefts. Am J Med Genet A, 135(3), 268-273. doi:10.1002/ajmg.a.30713

Olteanu, H., Banerjee, R. (2001). Human methionine synthase reductase, a soluble P-450 reductase-like dual flavoprotein, is sufficient for NADPH-dependent methionine synthase activation. J Biol Chem, 276(38), 35558-35563. doi:10.1074/jbc.M103707200

Ong, L. C., Lim, Y. N., Sofiah, A. (2002). Malaysian children with spina bifida: relationship between functional outcome and level of lesion. Singapore Med J, 43(1), 12-17.

Ou, Z., Li, S., Li, Q., Chen, X., Liu, W., Sun, X. (2010). Duchenne muscular dystrophy in a female patient with a karyotype of 46,X,i(X)(q10). Tohoku J Exp Med, 222(2), 149-153.

Padmanabhan, R. (2006). Etiology, pathogenesis and prevention of neural tube defects. Congenital Anomalies, 46(2), 55-67. doi:10.1111/j.1741-4520.2006.00104.x

Padula, A. M., Tager, I. B., Carmichael, S. L., Hammond, S. K., Lurmann, F., Shaw, G. M. (2013). The association of ambient air pollution and traffic exposures with selected congenital anomalies in the San Joaquin Valley of California. Am J Epidemiol, 177(10), 1074-1085. doi:10.1093/aje/kws367

Palacios, J., Gamallo, C., Garcia, M., Rodriguez, J. I. (1993). Decrease in thyrocalcitonincontaining cells and analysis of other congenital anomalies in 11 patients with DiGeorge anomaly. Am J Med Genet, 46(6), 641-646. doi:10.1002/ajmg.1320460608

Pangilinan, F., Molloy, A. M., Mills, J. L., Troendle, J. F., Parle-McDermott, A., Signore, C., . . . Brody, L. C. (2012). Evaluation of common genetic variants in 82 candidate genes as risk factors for neural tube defects. BMC Medical Genetics, 13, 62-62. doi:10.1186/1471-2350-13-62

Parker, S. E., Mai, C. T., Canfield, M. A., Rickard, R., Wang, Y., Meyer, R. E., . . . Correa, A. (2010). Updated National Birth Prevalence estimates for selected birth defects in the United States, 2004-2006. Birth Defects Res A Clin Mol Teratol, 88(12), 1008-1016. doi:10.1002/bdra.20735

Parle-McDermott, A., Kirke, P. N., Mills, J. L., Molloy, A. M., Cox, C., O'Leary, V. B., . . . Scott, J. M. (2006). Confirmation of the R653Q polymorphism of the trifunctional C1-synthase enzyme as a maternal risk for neural tube defects in the Irish population. Eur J Hum Genet, 14(6), 768-772. doi:10.1038/sj.ejhg.5201603

Parle-McDermott, A., Pangilinan, F., Mills, J. L., Kirke, P. N., Gibney, E. R., Troendle, J., . . . Brody, L. C. (2007). The 19-bp deletion polymorphism in intron-1 of dihydrofolate reductase (DHFR) may decrease rather than increase risk for spina bifida in the Irish population. Am J Med Genet A, 143A(11), 1174-1180. doi:10.1002/ajmg.a.31725

Patel, Z. H., Kottyan, L. C., Lazaro, S., Williams, M. S., Ledbetter, D. H., Tromp, h., . . . Kaufman, K. M. (2014). The struggle to find reliable results in exome sequencing data: filtering out Mendelian errors. Frontiers in Genetics, 5, 16. doi:10.3389/fgene.2014.00016

Paulussen, A. D., Schrander-Stumpel, C. T., Tserpelis, D. C., Spee, M. K., Stegmann, A. P., Mancini, G. M., . . . Herbergs, J. (2010). The unfolding clinical spectrum of holoprosencephaly due to mutations in SHH, ZIC2, SIX3 and TGIF genes. Eur J Hum Genet, 18(9), 999-1005. doi:10.1038/ejhg.2010.70

Peng, H., Liu, H., Liu, F., Gao, Y., Chen, J., Huo, J., . . . Zhang, W. (2017). NLRP2 and FAF1 deficiency blocks early embryogenesis in the mouse. Reproduction, 154(3), 145-151. doi:10.1530/rep-16-0629

Perales, G., Burguete-Garcia, A. I., Dimas, J., Bahena-Roman, M., Bermudez-Morales, V. H., Moreno, J., Madrid-Marina, V. (2010). A polymorphism in the AT-hook motif of the transcriptional regulator AKNA is a risk factor for cervical cancer. Biomarkers, 15(5), 470-474. doi:10.3109/1354750x.2010.485332

Piao, W., Guo, J., Bao, Y., Wang, F., Zhang, T., Huo, J., Zhang, K. (2016). Analysis of polymorphisms of genes associated with folate-mediated one-carbon metabolism and neural tube defects in Chinese Han Population. Birth Defects Res A Clin Mol Teratol, 106(4), 232-239. doi:10.1002/bdra.23478

Pitkin, R. M. (2007). Folate and neural tube defects. Am J Clin Nutr, 85(1), 285s-288s. Retrieved from http://ajcn.nutrition.org/content/85/1/285S.full.pdf

Plaja, A., Vendrell, T., Sarret, E., Toran, N., Mediano, C. (1994). Terminal deletion of Xp in a dysmorphic anencephalic fetus. Prenat Diagn, 14(5), 410-412. doi:10.1002/pd.1970140512

Proell, M., Riedl, S. J., Fritz, J. H., Rojas, A. M., Schwarzenbacher, R. (2008). The NodLike Receptor (NLR) Family: A Tale of Similarities and Differences. PLoS ONE, 3(4), e2119. doi:10.1371/journal.pone.0002119

Ramensky, V., Bork, P., Sunyaev, S. (2002). Human non-synonymous SNPs: server and survey. Nucleic Acids Research, 30(17), 3894-3900.

Ramos, H. C., Lantion-Ang, F. L. (2010). A Variant of Turner's Syndrome Presenting with Secondary Amenorrhea. Philippine Journal of Internal Medicine, 48(2).

Rashed, H., Awaluddin SM, Ahmad NA, Supar NHM , Lani ZM, Aziz F, . . . T, A. (2016). Advanced Maternal Age and Adverse Pregnancy Outcomes in Muar, Johor, Malaysia Sains Malaysiana, 45(10), 1537–1542.

Rasmussen, S. A., Chu, S. Y., Kim, S. Y., Schmid, C. H., Lau, J. (2008). Maternal obesity and risk of neural tube defects: a metaanalysis. Am J Obstet Gynecol, 198(6), 611619. doi:10.1016/j.ajog.2008.04.021

Regeling, A., Imhann, F., Volders, H. H., Blokzijl, T., Bloks, V. W., Weersma, R. K., . . . Faber, K. N. (2016). HSPA6 is an ulcerative colitis susceptibility factor that is induced by cigarette smoke and protects intestinal epithelial cells by stabilizing anti-apoptotic Bcl-XL. Biochim Biophys Acta, 1862(4), 788-796. doi:10.1016/j.bbadis.2016.01.020

Relton, C. L., Wilding, C. S., Laffling, A. J., Jonas, P. A., Burgess, T., Binks, K., . . . Burn, J. (2004). Low erythrocyte folate status and polymorphic variation in folaterelated genes are associated with risk of neural tube defect pregnancy. Mol Genet Metab, 81(4), 273-281. doi:10.1016/j.ymgme.2003.12.010

Robinson, A., Escuin, S., Doudney, K., Vekemans, M., Stevenson, R. E., Greene, N. D. E., . . . Stanier, P. (2012). Mutations in the planar cell polarity genes CELSR1 and SCRIB are associated with the severe neural tube defect, craniorachischisis. Human mutation, 33(2), 440-447. doi:10.1002/humu.21662

Robinson, A., Partridge, D., Malhas, A., De Castro, S. C., Gustavsson, P., Thompson, D. N., . . . Greene, N. D. (2013). Is LMNB1 a susceptibility gene for neural tube defects in humans? Birth Defects Res A Clin Mol Teratol, 97(6), 398-402. doi:10.1002/bdra.23141

Rochtus, A., Winand, R., Laenen, G., Vangeel, E., Izzi, B., Wittevrongel, C., . . . Freson, K. (2016). Methylome analysis for spina bifida shows SOX18 hypomethylation as a risk factor with evidence for a complex (epi)genetic interplay to affect neural tube development. Clin Epigenetics, 8, 108. doi:10.1186/s13148-016-0272-8

Roessler, E., Lacbawan, F., Dubourg, C., Paulussen, A., Herbergs, J., Hehr, U., . . . Muenke, M. (2009). The Full Spectrum of Holoprosencephaly-Associated Mutations within the ZIC2 Gene in Humans Predicts Loss-of-Function as the Predominant Disease Mechanism. Human mutation, 30(4), E541-E554. doi:10.1002/humu.20982

Ruiz-Martínez, J., Azcona, L. J., Bergareche, A., Martí-Massó, J. F., Paisán-Ruiz, C. (2017). Whole-exome sequencing associates novel CSMD1 gene mutations with familial Parkinson disease. Neurology Genetics, 3(5). doi:10.1212/nxg.0000000000000177

Sadler, T. W., Langman, J. (2012). Langman's Medical Embryology (12th ed. ed.). United States: Philadelphia : Wolters Kluwer Health/Lippincott Williams & Wilkins, c2012.

Salas, A., Pardo-Seco, J., Cebey-Lopez, M., Gomez-Carballa, A., Obando-Pacheco, P., Rivero-Calle, I., . . . Martinon-Torres, F. (2017). Whole Exome Sequencing reveals new candidate genes in host genomic susceptibility to Respiratory Syncytial Virus Disease. Sci Rep, 7(1), 15888. doi:10.1038/s41598-017-15752-4

Salbaum, J. M., Kappen, C. (2010). Neural tube defect genes and maternal diabetes during pregnancy. Birth Defects Res A Clin Mol Teratol, 88(8), 601-611. doi:10.1002/bdra.20680

Sarris, C. E., Tomei, K. L., Carmel, P. W., Gandhi, C. D. (2012). Lipomyelomeningocele: pathology, treatment, and outcomes. Neurosurg Focus, 33(4), E3. doi:10.3171/2012.7.focus12224

Schmidt, R. J. (2007). Maternal caffeine intake, select metabolic gene variants, and neural tube defects (NTDs). University of Lowa, Graduate Collage.

Seaver, L. H., Stevenson, R. E. (2006). Syndromes with neural tube defects. In D. F. Wyszynski (Ed.), In. Neural tube defects: from origin to treatment. (pp. 76-83): Oxford: Oxford U niversity Press.

Seller, M. J. (1987). Neural-Tube Defects and Sex-Ratios. American Journal of Medical Genetics, 26(3), 699-707. doi:DOI 10.1002/ajmg.1320260325

Seller, M. J. (1995). Sex, neural tube defects, and multisite closure of the human neural tube. Am J Med Genet, 58(4), 332-336. doi:10.1002/ajmg.1320580406

Senese, S., Cheung, K., Lo, Y.-C., Gholkar, A. A., Xia, X., Wohlschlegel, J. A., Torres, J. Z. (2015). A unique insertion in STARD9's motor domain regulates its stability. Molecular Biology of the Cell, 26(3), 440-452. doi:10.1091/mbc.E14-03-0829

Sepulveda, W., Corral, E., Ayala, C., Be, C., Gutierrez, J., Vasquez, P. (2004). Chromosomal abnormalities in fetuses with open neural tube defects: prenatal identification with ultrasound. Ultrasound Obstet Gynecol, 23(4), 352-356. doi:10.1002/uog.964

Shaik, A. P., Alsaeed, A. H., Kiranmayee, S., Bammidi, V. K., Sultana, A. (2013). Phylogenetic analysis of cubilin (CUBN) gene. Bioinformation, 9(1), 29-36. doi:10.6026/97320630009029

Shaw, G. M., Carmichael, S. L., Yang, W., Selvin, S., Schaffer, D. M. (2004). Periconceptional Dietary Intake of Choline and Betaine and Neural Tube Defects in Offspring. American Journal of Epidemiology, 160(2), 102-109. doi:10.1093/aje/kwh187

Shaw, G. M., Lu, W., Zhu, H., Yang, W., Briggs, F. B., Carmichael, S. L., . . . Finnell, R. H. (2009). 118 SNPs of folate-related genes and risks of spina bifida and conotruncal heart defects. BMC Med Genet, 10, 49. doi:10.1186/1471-2350-1049

Shaw, G. M., Quach, T., Nelson, V., Carmichael, S. L., Schaffer, D. M., Selvin, S., Yang, W. (2003). Neural tube defects associated with maternal periconceptional dietary intake of simple sugars and glycemic index. Am J Clin Nutr, 78(5), 972-978.

Shaw, G. M., Todoroff, K., Velie, E. M., Lammer, E. J. (1998). Maternal illness, including fever and medication use as risk factors for neural tube defects. Teratology,57(1),1-7.doi:10.1002/(sici)1096-9926(199801)57:1<1

Shaw, G. M., Velie, E. M., Schaffer, D. (1996). Risk of neural tube defect—affected pregnancies among obese women. JAMA, 275(14), 1093-1096. doi:10.1001/jama.1996.03530380035028

Shi, L. M., Chia, S. E., Chan, O. Y., Chew, S. K., Foong, B. H. (2002). Prevalence of birth defects and parental work in Singapore live births from 1994 to 1998: a population-based study. Occup Med (Lond), 52(6), 325-331.

Shields, D. C., Kirke, P. N., Mills, J. L., Ramsbottom, D., Molloy, A. M., Burke, H., . . . Whitehead, A. S. (1999). The "thermolabile" variant of methylenetetrahydrofolate reductase and neural tube defects: An evaluation of genetic risk and the relative importance of the genotypes of the embryo and the mother. Am J Hum Genet, 64(4), 1045-1055.

Shih, J., Keller, R. (1992). Cell motility driving mediolateral intercalation in explants of Xenopus laevis. Development, 116(4), 901-914.

Shoob, H. D., Sargent, R. G., Thompson, S. J., Best, R. G., Drane, J. W., Tocharoen, A. (2001). Dietary methionine is involved in the etiology of neural tube defectaffected pregnancies in humans. J Nutr, 131(10), 2653-2658.

Shum, A. S., Copp, A. J. (1996). Regional differences in morphogenesis of the neuroepithelium suggest multiple mechanisms of spinal neurulation in the mouse. Anat Embryol (Berl), 194(1), 65-73.

Siitonen, A., Nalls, M. A., Hernandez, D., Gibbs, J. R., Ding, J., Ylikotila, P., . . . Majamaa, K. (2017). Genetics of early-onset Parkinson's disease in Finland: exome sequencing and genome-wide association study. Neurobiol Aging, 53, 195.e197-195.e110. doi:10.1016/j.neurobiolaging.2017.01.019

Silver, D. P., Livingston, D. M. (2012). Mechanisms of BRCA1 Tumor Suppression. Cancer discovery, 2(8), 679-684. doi:10.1158/2159-8290.CD-12-0221

Smithells, R. W., Sheppard, S., Schorah, C. J. (1976). Vitamin deficiencies and neural tube defects. Arch Dis Child, 51(12), 944-950.

Soldano, K. L., Garrett, M. E., Cope, H. L., Rusnak, J. M., Ellis, N. J., Dunlap, K. L., . . . Ashley-Koch, A. E. (2013). Genetic association analyses of nitric oxide synthase genes and neural tube defects vary by phenotype. Birth Defects Res B Dev Reprod Toxicol, 98(5), 365-373. doi:10.1002/bdrb.21079

Sokal, R., Tata, L. J., Fleming, K. M. (2014). Sex prevalence of major congenital anomalies in the United Kingdom: a national population-based study and international comparison meta-analysis. Birth Defects Res A Clin Mol Teratol, 100(2), 79-91. doi:10.1002/bdra.23218

Sood, M., Agarwal, N., Verma, S., Bhargava, S. K. (1991). Neural tubal defects in an east Delhi hospital. Indian J Pediatr, 58(3), 363-365.

Stenson, P. D., Mort, M., Ball, E. V., Shaw, K., Phillips, A. D., Cooper, D. N. (2014). The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. Human Genetics, 133(1), 1-9. doi:10.1007/s00439-013-13584

Stevenson, R. E., Seaver, L. H., Collins, J. S., Dean, J. H. (2004). Neural tube defects and associated anomalies in South Carolina. Birth Defects Res A Clin Mol Teratol, 70(9), 554-558. doi:10.1002/bdra.20062

Stochholm, K., Juul, S., Juel, K., Naeraa, R. W., Gravholt, C. H. (2006). Prevalence, incidence, diagnostic delay, and mortality in Turner syndrome. J Clin Endocrinol Metab, 91(10), 3897-3902. doi:10.1210/jc.2006-0558

Stoll, C., Dott, B., Alembik, Y., Roth, M. P. (2011). Associated malformations among infants with neural tube defects. Am J Med Genet A, 155a(3), 565-568. doi:10.1002/ajmg.a.33886

Su, P. H., Hsu, Y. W., Huang, R. L., Weng, Y. C., Wang, H. C., Chen, Y. C., . . . Lai, H. C. (2017). Methylomics of nitroxidative stress on precancerous cells reveals DNA methylation alteration at the transition from in situ to invasive cervical cancer. Oncotarget, 8(39), 65281-65291. doi:10.18632/oncotarget.18370

Taksande, A., Vilhekar, K., Chaturvedi, P., Jain, M. (2010). Congenital malformations at birth in Central India: A rural medical college hospital based data. Indian J Hum Genet, 16(3), 159-163. doi:10.4103/0971-6866.73412

Toepoel, M., Steegers-Theunissen, R. P., Ouborg, N. J., Franke, B., Gonzalez-Zuloeta Ladd, A. M., Joosten, P. H., & van Zoelen, E. J. (2009). Interaction of PDGFRA promoter haplotypes and maternal environmental exposures in the risk of spina bifida. Birth Defects Res A Clin Mol Teratol, 85(7), 629-636. doi:10.1002/bdra.20574

Teo, Y. Y., Sim, X., Ong, R. T., Tan, A. K., Chen, J., Tantoso, E., . . . Chia, K. S. (2009). Singapore Genome Variation Project: a haplotype map of three Southeast Asian populations. Genome Res, 19(11), 2154-2162. doi:10.1101/gr.095000.109

The-Ministry-of-Health-Malaysia. (2013). List of Government Hospitals. Retrieved from http://www.moh.gov.my/english.php/database_stores/store_view/3

Thong, M. K., Ho, J. J., Khatijah, N. N. (2005). A population-based study of birth defects in Malaysia. Ann Hum Biol, 32(2), 180-187. doi:10.1080/03014460500075332

Toru, H. S., Sanhal, C. Y., Uzun, O. C., Ocak, G. A., Mendilcioglu, I., Karaveli, F. S. (2016). Associated anomalies with neural tube defects in fetal autopsies. J Matern Fetal Neonatal Med, 29(5), 798-802. doi:10.3109/14767058.2015.1019456

Toyama, R., Gomez, D. M., Mana, M. D., Dawid, I. B. (2004). Sequence relationships and expression patterns of zebrafish zic2 and zic5 genes. Gene Expression Patterns, 4(3), 345-350. doi:10.1016/j.modgep.2003.09.011

Trudell, A. S., Odibo, A. O. (2014). Diagnosis of spina bifida on ultrasound: always termination? Best Pract Res Clin Obstet Gynaecol, 28(3), 367-377. doi:10.1016/j.bpobgyn.2013.10.006

Ulman, C., Taneli, F., Oksel, F., Hakerlerler, H. (2005). Zinc-deficient sprouting blight potatoes and their possible relation with neural tube defects. Cell Biochem Funct, 23(1), 69-72. doi:10.1002/cbf.1172

van der Linden, I. J., den Heijer, M., Afman, L. A., Gellekink, H., Vermeulen, S. H., Kluijtmans, L. A., & Blom, H. J. (2006). The methionine synthase reductase 66A>G polymorphism is a maternal risk factor for spina bifida. *J Mol Med (Berl), 84*(12), 1047-1054. doi:10.1007/s00109-006-0093-x

van der Linden, I. J., Heil, S. G., Kouwenberg, I. C., den Heijer, M., Blom, H. J. (2007). The methylenetetrahydrofolate dehydrogenase (MTHFD1) 1958G>A variant is not associated with spina bifida risk in the Dutch population. Clin Genet, 72(6), 599-600. doi:10.1111/j.1399-0004.2007.00904.x

van der Put, N. M., Steegers-Theunissen, R. P., Frosst, P., Trijbels, F. J., Eskes, T. K., van den Heuvel, L. P., . . . Blom, H. J. (1995). Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. Lancet, 346(8982), 1070-1071.

Van Dijk, E. L., Auger, H., Jaszczyszyn, Y., Thermes, C. (2014). Ten years of nextgeneration sequencing technology. Trends Genet, 30(9), 418-426. doi:10.1016/j.tig.2014.07.001

Vargesson, N. (2015). Thalidomide- induced teratogenesis: History and mechanisms. Birth Defects Research, 105(2), 140-156. doi:10.1002/bdrc.21096

Veltman, J. A., Brunner, H. G. (2012). De novo mutations in human genetic disease. Nature Reviews Genetics, 13, 565. doi:10.1038/nrg3241

Vogel LC, B. R., Mulcahey MJ. Lipomeningocele. In: Lin VW, Cardenas DD, Cutter NC,. (2003). Spinal Cord Medicine: Principles and Practice. (pp. 1176).

Volcik, K. A., Shaw, G. M., Zhu, H., Lammer, E. J., Laurent, C., & Finnell, R. H. (2003). Associations between polymorphisms within the thymidylate synthase gene and spina bifida. Birth Defects Res A Clin Mol Teratol, 67(11), 924-928. doi:10.1002/bdra.10029

Voyvodic, F., Scroop, R., Sanders, R. R. (1999). Anterior sacral meningocele as a pelvic complication of Marfan syndrome. Aust N Z J Obstet Gynaecol, 39(2), 262-265.

Wallingford, J. B. (2006). Planar cell polarity, ciliogenesis and neural tube defects. Hum Mol Genet, 15 Spec No 2, R227-234. doi:10.1093/hmg/ddl216

Wallingford, J. B., Fraser, S. E., Harland, R. M. (2002). Convergent extension: the molecular control of polarized cell movement during embryonic development. Dev Cell, 2(6), 695-706.

Wallingford, J. B., Harland, R. M. (2001). Xenopus Dishevelled signaling regulates both neural and mesodermal convergent extension: parallel forces elongating the body axis. Development, 128(13), 2581-2592.

Wang, X., Wang, R. H., Li, W., Xu, X., Hollander, M. C., Fornace, A. J., Jr., Deng, C. X. (2004). Genetic interactions between Brca1 and Gadd45a in centrosome duplication, genetic stability, and neural tube closure. J Biol Chem, 279(28), 29606-29614. doi:10.1074/jbc.M312279200

Wansleeben, C., Feitsma, H., Montcouquiol, M., Kroon, C., Cuppen, E., Meijlink, F. (2010). Planar cell polarity defects and defective Vangl2 trafficking in mutants for the COPII gene Sec24b. Development, 137(7), 1067-1073. doi:10.1242/dev.041434

Wasserman, C. R., Shaw, G. M., Selvin, S., Gould, J. B., Syme, S. L. (1998). Socioeconomic status, neighborhood social conditions, and neural tube defects. Am J Public Health, 88(11), 1674-1680.

Welcsh, P. L., King, M. C. (2001). BRCA1 and BRCA2 and the genetics of breast and ovarian cancer. Hum Mol Genet, 10(7), 705-713.

Wen, S., Lu, W., Zhu, H., Yang, W., Shaw, G. M., Lammer, E. J., . . . Finnell, R. H. (2009). Genetic polymorphisms in the thioredoxin 2 (TXN2) gene and risk for spina bifida. Am J Med Genet A, 149A(2), 155-160. doi:10.1002/ajmg.a.32589

Werler, M. M., Louik, C., Shapiro, S., Mitchell, A. A. (1996). Prepregnant weight in relation to risk of neural tube defects. JAMA, 275(14), 1089-1092. doi:10.1001/jama.1996.03530380031027

Wilson, A., Platt, R., Wu, Q., Leclerc, D., Christensen, B., Yang, H., . . . Rozen, R. (1999). A common variant in methionine synthase reductase combined with low cobalamin (vitamin B12) increases risk for spina bifida. Mol Genet Metab, 67(4), 317-323. doi:10.1006/mgme.1999.2879

Worthey, E. A., Mayer, A. N., Syverson, G. D., Helbling, D., Bonacci, B. B., Decker, B., . . . Dimmock, D. P. (2011). Making a definitive diagnosis: successful clinical application of whole exome sequencing in a child with intractable inflammatory bowel disease. Genet Med, 13(3), 255-262. doi:10.1097/GIM.0b013e3182088158

Wu, L., Lu, X., Guo, J., Zhang, T., Wang, F., Bao, Y. (2016). Association between ALDH1L1 gene polymorphism and neural tube defects in the Chinese Han population. Neurol Sci, 37(7), 1049-1054. doi:10.1007/s10072-016-2527-8

Yaliwal, L. V., Desai, R. M. (2012). Methylenetetrahydrofolate reductase mutations, a genetic cause for familial recurrent neural tube defects. Indian Journal of Human Genetics, 18(1), 122-124. doi:10.4103/0971-6866.96680

Yamaguchi, Y., Shinotsuka, N., Nonomura, K., Takemoto, K., Kuida, K., Yosida, H., Miura, M. (2011). Live imaging of apoptosis in a novel transgenic mouse highlights its role in neural tube closure. The Journal of Cell Biology, 195(6), 1047-1060. doi:10.1083/jcb.201104057

Yang, Y., Chen, J., Wang, B., Ding, C., Liu, H. (2015). Association between MTHFR C677T polymorphism and neural tube defect risks: A comprehensive evaluation in three groups of NTD patients, mothers, and fathers. Birth Defects Res A Clin Mol Teratol, 103(6), 488-500. doi:10.1002/bdra.23361

Yang, Y., Muzny, D. M., Xia, F., Niu, Z., Person, R., Ding, Y., . . . Eng, C. M. (2014). Molecular Findings Among Patients Referred for Clinical Whole-Exome Sequencing. JAMA, 312(18), 1870-1879. doi:10.1001/jama.2014.14601

Ybot-Gonzalez, P., Copp, A. J. (1999). Bending of the neural plate during mouse spinal neurulation is independent of actin microfilaments. Dev Dyn, 215(3), 273-283. doi:10.1002/(sici)1097-0177(199907)215:3<273::aid-aja9>3.0.co;2-h

Zaganjor, I., Sekkarie, A., Tsang, B. L., Williams, J., Razzaghi, H., Mulinare, J., . . . Rosenthal, J. (2016). Describing the Prevalence of Neural Tube Defects Worldwide: A Systematic Literature Review. PLoS One, 11(4). doi:10.1371/journal.pone.0151586

Zhang, Q., Bai, B., Liu, X., Miao, C., Li, H. (2014). Association of folate metabolism genes MTHFR and MTRR with multiple complex congenital malformation risk in Chinese population of Shanxi. Translational Pediatrics, 3(3), 259-267. doi:10.3978/j.issn.2224-4336.2014.07.10

Zhang, T., Lou, J., Zhong, R., Wu, J., Zou, L., Sun, Y., . . . Xiong, G. (2013). Genetic Variants in the Folate Pathway and the Risk of Neural Tube Defects: A MetaAnalysis of the Published Literature. PLoS ONE, 8(4), e59570. doi:10.1371/journal.pone.0059570

Zhang, Z., Jones, A., Joo, H. Y., Zhou, D., Cao, Y., Chen, S., . . . Wang, H. (2013). USP49 deubiquitinates histone H2B and regulates cotranscriptional pre-mRNA splicing. Genes Dev, 27(14), 1581-1595. doi:10.1101/gad.211037.112

Zhou, T., Souzeau, E., Sharma, S., Landers, J., Mills, R., Goldberg, I., . . . Craig, J. E. (2017). Whole exome sequencing implicates eye development, the unfolded protein response and plasma membrane homeostasis in primary open-angle glaucoma. PLoS One, 12(3), e0172427. doi:10.1371/journal.pone.0172427

Zhu, H., Curry, S., Wen, S., Wicker, N. J., Shaw, G. M., Lammer, E. J., . . . Finnell, R. H. (2005). Are the betaine-homocysteine methyltransferase (BHMT and BHMT2) genes risk factors for spina bifida and orofacial clefts? *Am J Med Genet A, 135*(3), 274-277. doi:10.1002/ajmg.a.30739

Zhu, H., Yang, W., Lu, W., Zhang, J., Shaw, G. M., Lammer, E. J., & Finnell, R. H. (2006). A known functional polymorphism (Ile120Val) of the human PCMT1 gene and risk of spina bifida. *Mol Genet Metab, 87*(1), 66-70. doi:10.1016/j.ymgme.2005.09.008

Zhu, H., Enaw, J. O., Ma, C., Shaw, G. M., Lammer, E. J., & Finnell, R. H. (2007). Association between CFL1 gene polymorphisms and spina bifida risk in a California population. *BMC Med Genet, 8*, 12. doi:10.1186/1471-2350-8-12

Zohn, I. E., Chesnutt, C. R., Niswander. (2003). Cell polarity pathways converge and extend to regulate neural tube closure. Trends Cell Biol 13(9): 451-454.

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