Eph and *Ephrin* Variants in Malaysian Neural Tube Defect Families

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Abstract

Neural tube defects (NTDs) are among the most common of birth defects. Despite the many gene candidates identified in certain population; none have thus far been regarded as compelling enough as a candidate for different populations. As candidate genes for NTDs, this study focussed on *Ephs* and *ephrins* owing to growing evidence for the role of this gene family during neural tube closure in mouse models. *Eph* and *ephrin* genes were analysed in 31 Malaysian individuals comprising 7 individuals with sporadic spina bifida, 14 parents and siblings and 10 unrelated controls. Whole exome sequencing analysis was performed to identify variants in 22 known *EphAs, EphBs, ephrinAs* and *ephrinBs* genes. All candidate variants in *Eph-ephrin* genes were subjected to bioinformatics analysis to determine possible pathogenicity. We reported 3 out of 7 spina bifida probands and 3 out of 13 parents and 1 twin-sibling, carried a variant in either *EPHA2* (rs147977279), *EPHB6* (rs780569137) or *EFNB1* (rs772228172). Analysis of public databases like 1000 Genome phase 3, GnomAD, ExAC, ESP, TOPMED and SGVP shows that these variants are rare, and they were not present in any of the unrelated controls. In exome datasets of the probands and parent of the probands with *Eph* and *ephrin* variants, the genotypes of spina bifida-related genes were compared to investigate the probability of the gene-gene interaction in relation to environmental risk factors. In summary, we report the presence of *Eph* and *ephrin* gene variants that are prevalent in a small cohort of spina bifida patients in Malaysian families.

Introduction

Neural Tube Defects (NTDs) are a group of congenital malformations in which the formation of the brain and/or spinal cord are compromised as a result of failed closure of the embryonic neural tube, during the fourth week of post-fertilisation development (Nikolopoulou et al., 2017). NTDs are among of the commonest congenital malformations and occur in 0.5 to 10 per 1000 pregnancies globally (Greene & Copp, 2014). The clinical severity of NTDs varies greatly (Copp et al., 2013; Mohd-Zin et al., 2017), with open lesions affecting the brain (anencephaly), and craniorachischisis being the most severe phenotypes. Spina bifida describes lesions affecting the spinal region and affected individuals often exhibit motor and sensory neurological deficit below the level of the lesion (Copp et al., 2015; Oakeshott et al., 2012). In view of life-changing health and economic consequences of NTDs, considerable effort should be invested in exploring the pathophysiological mechanisms that governs the aetiology of NTDs in lieu of the ultimate goal of primary prevention (Mohd-Zin et al., 2017).

There is strong evidence that genetics play a role in the aetiology of NTDs. Similarly, genetic alterations have been found to cause NTDs in mice, with more than 300 genes having been shown to cause NTDs when mutated (Harris & Juriloff, 2010; Wilde et al., 2014). So far, our knowledge of the causative genes in humans is less complete and only around 59 genes have been reported to be potentially associated with spina bifida in humans (Wilde et al., 2014; Pangilinan et al., 2012; Greene et al., 2009) (Supplementary Table 1). Each gene has been involved in only a small proportion of NTD patients or specific populations (Lemay et al., 2015; Lemay et al., 2017; Chen et al., 2018; Wang et al., 2019), suggesting that there is considerable heterogeneity underlying the genetic basis of NTDs.

In particular, candidate human NTD genes include folate-related genes for example; *MTHFR, MTRR*, and *MTHFD1* (Lemay et al., 2019; Zhang et al., 2013; Fang et al., 2018), are being investigated in several cohorts to understand the mechanism of pathophysiology of NTDs as well as prevention of NTDs (Greene et al., 2009; Wolujewicz & Ross, 2019). For instance, folate metabolism and methionine metabolism has been associated to neural tube closure (Leung et al., 2017). Also, *MTHFR* C677T polymorphism has been reported affecting level of folate serum (folate metabolism), homocysteine and vitamin B12 (methionine metabolism) (Liew & Gupta, 2015). Thus, *MTHFR* 677TT is being evaluated as genetic risk for NTD and was reported susceptible among Caucasians and Asians in meta-analysis based on 1998-2018 case-control studies (Tabatabaei et al., 2020). However, the clinical significance of this mutation is not known for certain as it has not been well replicated across other groups.

Ephs and ephrins are particularly attractive candidates in view of the Eph-ephrin interactions and signalling shown involved during adhesion and fusion (Holmberg et al., 2000; Abdul-Aziz et al., 2009; Abdullah *et al*., 2007; Arvanitis et al., 2013; Wang et al., 2016; Ji et al., 2014; Laussu et al., 2017; Kemp et al., 2009). Evidence of Ephs and ephrins being involved in neural tube development exists in three reported mouse neural tube defect models (Holmberg et al., 2000; Abdullah et al, 2017; Arvanitis et al., 2013), and two *Xenopus* neural tube defect models (Wang et al., 2016; Ji et al., 2014) and one zebrafish embryo model (Kemp et al., 2009). The first murine Eph and ephrin knockouts exhibited an open neural tube defect (Holmberg et al., 2000), the second was a spina bifida occulta model (Abdullah et al., 2017) and the most recent one was undefined as the phenotype was not assessed during closure of primary neurulation (Arvanitis et al., 2013). In *Xenopus* embryo models, Eph and ephrin knockdown disrupt cranial neural tube closure (Wang et al 2016; Ji et al., 2014). Whilst, in mosaic Eph and ephrin morpholino (loss-of-function reagents) of Zebrafish embryo model, the Eph and ephrin are specifically and individually required to facilitate integration of progenitor cells during the cross-midline cell division that occurs at the neural keel (Kemp et al., 2009). Although teleost neural keel formation and mammalian neural tube closure are developmentally distinct events, defects in either process result in severe neural tube defects (Nikolopoulou et al., 2017; Ciruna et al., 2006; Tawk et al., 2007).

Ephs are the largest group of receptor tyrosine kinases (RTKs) and are known to not only bind to their ligand ephrins but Eph-ephrin complexes also known to interact or couple with co-receptors like the TrkB neurotrophin receptor (Meier et al., 2011; Marler et al., 2008; Marler et al., 2010), p75 neurotrophin receptor (Lim et al., 2008), and Ret tyrosine kinase receptor (Bonanomi et al., 2012; Lisabeth *et al*., 2000). Also, Eph-ephrin complexes activate or inhibit signalling effectors like protein tyrosine phosphatase (PTPase) SHP2 protein (Miao et al., 2000; Miao et al., 2003; Saxton & Pawson *et al*., 1999), Ras/Rho family GTPases (Pasquale, 2008), ADP-ribosylation factor 6 (Arf6) (Arvanitis et al., 2013), and focal adhesion kinase (FAK) (Wang et al., 2016). Ephs are integral membrane receptors, whereas ephrinA ligands are linked to the plasma membrane via cell surface glycosyl phosphatidylinositol (GPI)-anchor, and ephrinBs are transmembrane ligands. GPI-anchored molecules, ephrinA5 is implicated in interaction with different splice forms of EphA7 can mediate cellular adhesion or repulsion during neural fold fusion in mouse model (Holmberg et al., 2000). Whilst, ephrinB1 is associated to the maintenance of the structural integrity in apical cell and extracellular matrix (ECM) adhesion for mouse neuroepithelial development (Arvanitis et al., 2013). Besides, a functional role during neural tube closure is suggested by the finding that closure was delayed in whole mouse embryos cultured with EphA1 and EphA3 fusion proteins used to specifically disrupt ephrinA-EphA receptor interactions (Abdul-Aziz et al., 2009).

Whether there is also a role for Ephs-ephrins down-stream signalling within the cells of the neural fold tips were also questioned. In mouse embryos, *EphA2* is shown expressed in a lamellipodium-like protrusion structure which extends towards the opposite neural fold (Abdul-Aziz et al., 2009). In EphA2-deficient endothelial cells, ephrinA1 stimulation fails to activate phosphoinositide-3-kinase (PI3K)-mediated of Rac1 GTPase (Brantley-Sieders et al., 2004). Also, in primary rat embryo fibroblasts (REF) culture, inhibition of Rac function suppresses lamellipodia protrusive activity and movement (Nobes & Hall, 1999; Li et al., 2013; Rothenberg *et al*., 2019). Most recent, in mouse knockout embryos with perturbation of both alleles of one gene and a single allele of the second gene simultaneously in EphA2 and EphA4 crosses (*Epha2*$^{tm1Jrui/+}$*Epha4*$^{rb-2J/rb-2J}$ and *Epha2*$^{tm1Jrui/tm1Jrui}$*Epha4*$^{rb-2J/+}$), a large number of rounded cells were seen in the open cranial and open spinal neuropores (Abdullah et al., 2017). Furthermore, the double heterozygous embryos carrying loss of function alleles of *EphA2* and *EphA4* (*Epha2*$^{tm1Jrui/+}$*Epha4*$^{rb-2J/+}$) exhibit spina bifida occulta and exencephaly at a penetrance of more than 50%. These findings suggest a dual compensatory role of *EphA2* and *EphA4* during murine spinal neural tube closure (Abdullah et al., 2017). Comparatively, to date there has been no systematic study to implicate *Ephs* and *ephrins* in a human spina bifida cohort although a multitude of Ephs and ephrins have been implicated in human cancers (Lee et al., 2021; Gao et al., 2017; Efazat *et al*, 206; Karidis et al., 2011; Barquilla & Pasquale, 2015).

It is important to assess the genetic basis of NTDs in diverse populations. Progress has been made in identifying 'risk' variants for NTDs in a number of genes and some of these studies have identified risk factors that may show differing genetic predisposition among ethnic groups (Greene & Copp *et al*., 2014; Agopian et al., 2013). Potential variation in genetic predisposition among ethnic groups is suggested by differences in the NTD prevalence between ethnic groups, which in some cases persists after migration to other geographical locations. In the current study we focussed on a cohort from Malaysia. The Malaysian population is multi-ethnic, and this study included individuals of Malay, Chinese and Indian origin.

Evidence from published genetic research, suggests that the extensive genetic involvement in cranial neurulation is reflected to additional complexity and greater sensitivity to disruption compared with spinal neurulation (Greene & Copp *et al*., 2014; Harris & Juriloff, 2010). However, the human and mouse genetic basis delineating open (aperta) and close (occulta, most commonly referred to spinal dysraphism) have never before been studied nor compared (Mohd-Zin et al., 2017; Detrait et al., 2005; Harris & Juriloff, 2007; Harris & Juriloff, 2010) and we feel that there is a need to as the Malaysian population seems biased towards the close type of neural tube defect seen in preliminary datasets (Sahmat et al., 2017, unpublished data from Invertebrate & Vertebrate Neurobiology Lab Universiti Malaya). This is further supported in genetic models of mouse knockouts which exhibit both open and close neural tube defects in the same family of molecules (Holmberg et al., 2000; Abdul-Aziz et al., 2009; Abdullah et al., 2017). Furthermore, there exists a number of mouse models which display spinal dysraphism which aetiology remains unresolved as papers abound which do not capture the phenotype at E10.5 (primary neurulation commencing final closure) (Tian et al., 2021; Rocha et al., 2010; Pallerla et al., 2007). Therefore, in this study, both types of human spina bifida; 4 occulta and 3 aperta were recruited. The objective for this study is to screen pathogenic variants in *Eph* and *ephrin* genes through whole exome sequencing in a spina bifida cohort.

Methods

Proband selection for whole exome sequencing and candidate gene validation

Seven unrelated individuals with sporadic spina bifida (5 Malays, 1 Chinese, and 1 Indian) and their healthy family members, where available, were recruited for this study. The 7 patients were presented with spina bifida with neurological deficits, encompassing 4 spina bifida occulta and 3 spina bifida aperta. In total, 31 individuals participated in this study. The 7 patients (5 Malays, 1 Chinese and 1 Indian) with sporadic spina bifida were grouped into distinct families, comprising 5 complete trios (mother-father-proband), 1 triad-families (mother-father-twin sibling-proband), and 1 single-parent family (mother-proband). The remaining 10 subjects (6 Malays, 2 Chinese, and 2 Indians) were healthy individuals unrelated to the probands and were included as comparable controls.

There were no descriptions of NTD cases in other family members and none of the patients were the result of consanguineous parents. An assumption was made based on the possibility that NTD phenotypes may be due to mutations in shared genes, thus NTDs datasets were grouped based on type of spina bifida and similarity of neurological deficits (Supplementary Table 2).

Informed consent was obtained from all study participants and photo publication of probands were authorised by their family. Collection of samples was done in accordance with the ethical approval given by the Medical Ethics Committee (MEC) in University of Malaya Medical Centre (UMMC) (MEC reference number: 914.5).

Whole exome sequencing

DNA was isolated from peripheral blood, saliva samples (Oragene® OG500) or buccal mouthwash of spina bifida individuals. Samples were prepared in Illumina TruSeq kit and Agilent SureSelect Target Enrichment kit for whole exome sequencing (WES). The samples were sequenced using the Illumina HiSeq 2000 and HiSeq 4000 platforms. The platforms were able to generate an average read length of 100 bp and had a median depth of 50x coverage per sample. Data alignment and variant analysis were performed (Li *et al.,* 2013; Nielsen *et al.,* 2011).

Exome datasets analysis

The variants from the 7 probands of spina bifida were filtered for non-synonymous (missense), frameshift mutations, and non-frameshift mutations in the exonic region. The variants within splicing region located adjacent to exons were also included. Variants with minor allele frequency (MAF<0.01) based on 1000 Genome Phase 1 (annotated based on database build dbSNP135 with November 2010 and October 2011 allele frequency data) were first short-listed. The variant location and nucleotide changes were compared in probands and parents using the platform Galaxy biomedical data tool. Further, the variants were filtered based on mapping quality of above 50 and read depth of higher than 15 to ensure that the variants were mapped to the reference genome with a high degree of confidence and reduced error probabilities (Li & Durbin, 2008; Sims *et al.,* 2014). The homozygous variants with alternate allele to total read depth ratio (AD/TD) above 85% and heterozygous variants range between 30% - 70% will be included to get the most accurate candidate variants (Patel *et al.,* 2014).

Subsequently, the MAF of candidate variants were checked against 1000 Genome Phase 3 (annotated based on dbSNP 142 April 2020), GnomAD version 2.1.1, ExAC, ESP and TOPMED. Candidate variants that are rare (MAF < 0.01), low frequency (MAF 0.01-0.05), and not reported in the 5 global databases were short-listed (Mani, 2017; Chen *et al.,* 2011; Kryukov *et al.,* 2007). The MAF of variants in the specific population database which were Asian sub-database (GnomeAD and ExAC), East Asian (EAS) sub-database (1000 Genome Phase 3 and GnomeAD), South Asian (SAS) sub-database (1000 Genome Phase 3) and Singaporean (Singapore Genome Variation Project (SGVP)) variant calling files (VCF) (genotype data build on human genome 18 project assembly, Hg18, March 2006) (Teo *et al.,* 2009) were included for the inclusion of ancestry-matched controls (Manrai *et al.,* 2016).

All candidate variants in *EPHA1- EPHA8, EPHA10, EPHB1- EPHB4, EPHB6, EFNA1 – EFNA5,* and *EFNB1- EFNB3* genes were subjected to bioinformatics analysis to determine possible pathogenicity. The loss-of-function (LOF) effect were checked against *in silico* protein function and conservation tools: Polyphen-2 (Polymorphism Phenotype-2) (Adzhubei *et al.,* 2010), SIFT (Sorting Tolerance from Intolerance) (Kumar *et al.,* 2009), Provean (Protein Variant Effect Analyser) (Choi *et al.,* 2012), CADD (Rentzsch *et al.,* 2019), FATHMM-MKL (Shihab *et al.,* 2015), MutationTaster (Schwarz *et al.,* 2014), GERP (Davydov *et al.,* 2010), and PhyloP (Pollard *et al.,* 2010; Krupp *et al.,* 2014). The position of the mutations and the predicted structure of the protein were analysed using SMART (Simple Modular Architecture Research Tool).

PCR and Sanger sequencing for validation

PCR amplification and Sanger sequencing were used to verify candidate variants from the WES data and to analyse the variants in the family members (where available) and 10 controls (without NTD). A total of 3 sets of primers were designed (Supplementary Table 3).

Results

Whole exome sequencing analysis of Eph and ephrin identifies 3 mutations

The possible contribution of the *Eph-ephrins* were investigated. There were no shared exonic variants in all known mammalian *Eph* and *ephrin* genes (*EPHA1- EPHA8, EPHA10, EPHB1- EPHB4, EPHB6, EFNA1 – EFNA5*, and *EFNB1- EFNB3*) that fulfilled the MAF<0.01 criteria in all 7 spina bifida probands. Each exome dataset was screened individually in all 7 probands to look for potential variants. We compared whole exome analysis on 5 complete trios (SB1A, SB2A, SB5A, SB7A, and SB13A), 1 triad-families (SB27A) and 1 single-parent family (SB3A) and evaluated all possible means of transmission for *Eph* and *ephrin* variant: *de novo* mutations and homozygous and/or compound heterozygous variants for a recessive transmission. From 13 *Eph* and *ephrin* variants, there were no variants that followed *de novo* mutations or emulated recessive transmission and appear to segregate exclusively with the phenotype (Supplementary Table 4).

Nevertheless, from the 13 candidate variants, 3 variants in EPHA2 *(*rs147977279), EPHB6 (rs780569137) and EFNB1 (rs772228172) were unreported or MAF<0.01 and predicted as pathogenic and conserved in at least 7 *in silico* prediction tools. The EPHA2 variant (rs147977279) was found heterozygous in exome of proband SB2A and father of SB2A. The EPHB6 variant (rs780569137) was found heterozygous in exome of proband SB5A and mother of SB5A. The EFNB1 (rs772228172) was found heterozygous in exome of proband SB1A and genotyped as hemizygous in father of SB1A (Table 1). Each of the candidate variants found in one of SB2A (rs147977279 in *EPHA2*), SB5A (rs780569137 in *EPHB6*), and SB1A (rs772228172 in *EFNB1*) were detected in single families, but, not in other spina bifida families and control group.

Table 1
Genotypes of spina bifida probands from exome datasets

Probands	Genotypes						
	EPHA2 (rs147977279)		EPHB6 (rs780569137)		EFNB1 (rs772228172)		
	G/G	G/C (Heterozygous mutation)	A/A	A/G (Heterozygous mutation)	C/C	C/T (Heterozygous mutation)	T (Hemizygous mutation)
Proband	-	-	1 (SB5A)	-	1 (SB1A)	-	
Parent of probands	1 (Mother of SB2A)	1 (Father of SB2A)	1 (Father of SB5A)	1 (Mother of SB5A)	1 (Mother of SB1A)	-	1 (Father of SB1A)
Other NTD Probands	6	-	6	-	6	-	-
Parents and unaffected twin-sibling of other NTD probands	12	-	12	-	12	-	-
Controls	10	-	10	-	10	-	-
Total	29	29	2	29	1	1	

The rs147977279 variant in EphA-type receptor, EPHA2 is a G to C base change at 16,477,423 on chromosome 1, in an individual with spina bifida occulta, SB2A (Table 2). This alters the coding sequence resulting in a leucine to valine substitution in the ligand binding domain of **EPHA2** (Fig. 1). The rs780569137 variant in EphB-type receptor, EPHB6 is a A to G base change at 142,562,247 on chromosome 7, in an individual with spina bifida aperta, SB5A (Table 2). This alters the coding sequence resulting in a tyrosine to cysteine substitution in the ligand binding domain of **EPHB6** (Fig. 1). The rs772228172 variant in ephrinB-type transmembrane ligand, EFNB1 is a C to T base change at 68,049,626 on chromosome X, in an individual with spina bifida occulta, SB1A (Table 2). This alters the coding sequence resulting in an arginine to trypsin substitution in the signal peptide domain of **EFNB1** (Fig. 1).

Table 2
Position and changes induced by the 3 gene variants

Gene	Location	Transcript	cDNA change	Amino acid change	Nucleotide change
EPHA2 rs147977279	Chr1: 16477423	NM_004431 (exon 2)	c.G121C	p.L41V	G to C
EPHB6 rs780569137	Chr7: 142562247	NM_004445 (exon 7)	c.A689G	p.Y230C	A to G
EFNB1 rs772228172	ChrX: 68049626	NM_004429 (exon 1)	c.C7T	p.R3W	C to T

Unreported and rare Eph **and** ephrin **variants**

Among the three variants, MAF of rs780569137 (EPHB6) were not listed in the 5 global population databases with total alleles (2N) between 5,008 to 282,912 alleles, suggesting that the variant is novel (Table 2). Nonetheless, the rs147977279 (EPHA2) was considered rare

(MAF<0.01) in the 5 global databases in total alleles between 5,008 to 251,420 alleles. Similarly, the MAF of rs772228172 (EFNB1*)* was considered very rare (MAF<0.01) in GnomAD (MAF= 0.000014; 2n= 4), and TOPMED (MAF= 0.000008; 2n=1) in total alleles between 124,568 to 282,912 alleles. Furthermore, the MAF of rs772228172 (EFNB1*)* was not reported in the ExAC and ESP global population databases with total alleles between 13,006 to 121,250 alleles, suggesting that the variant is novel in the population covered by the ExAC and ESP databases (Table 3).

Table 3
Minor Allele Frequency (MAF) databases information specific for EPHA2 (rs147977279), EPHB6 (rs780569137), and *EFNB1* (rs772228172)

MAF databases	***EPHA2*** **(rs147977279)**	***EPHB6*** **(rs780569137)**	***EFNB1*** **(rs772228172)**
1000 Genome Phase 3 (2N= 5,008 alleles)	Global: 0.0022 (2n=11) EAS= Not reported (2N= 1,008) SAS= 0.011 (2N= 978)	Not listed	Not listed
GnomeAD v2.1.1 (2N= 282,912 alleles)	GnomAD_exomes Global= 0.002013 (2n= 506/ 251,420) Asian= 0.00853 (2N= 49,008) GnomAD Global= 0.000019 (2n= 6/31,384) EAS= Not reported (2n= 0/1,556)	Not listed	GnomeAD_exomes Global= 0.000014 (2n= 2/ 142,298) Asian= 0.00004 (2N= 26,746)
ExAC (2N= 121,250 alleles)	Global= 0.0024 (2n=291) Asian= 0.00927 (2N= 25,142)	Not listed	Not reported
ESP (2N= 13,006 alleles)	Global= 0.0000308 (2n= 4/13,006)	Not listed	Not reported
TOPMED (2N= 124,568 alleles)	Global= 0.000334 (2n= 42/125,568)	Not listed	Global= 0.000008 (2n= 1/125,568)
Singapore Genome Variation Project (SGVP)	Not listed	Not listed	Not listed

2n is allele count of variants in 1 individual (n); 2N is total number of alleles counts in 1 individual (N); EAS= East Asian population; SAS= South Asian population; Not listed= the reported SNP (rs) were not called in variant call database (VCF); Not reported= The reported SNP (rs) is not found (2n=0) in the database

From the databases surveyed, rs147977279 (EPHA2) were not reported in the Asian population in GnomAD exome samples (2N= 49,008 alleles) and ExAC (2N= 25,142 alleles). In particular, rs147977279 (*EPHA2*) was not reported in East Asian population in 1000 Genome Phase 3 (2N= 1,008), and GnomAD (2N=1,556 alleles). However, rs147977279 (EPHA2) was found as low frequency at 0.011 of total 978 alleles in South Asian sub-population (SAS) (Table 3). Likewise, rs772228172 (EFNB1*)* were not reported in Asian population in GnomAD exome samples (2N= 26,746 alleles). Interestingly, in the SGVP database whose population group (98 Malays, 99 Chinese, and 95 Indians) is a closer representation of the ethnic origins of Malaysians, all three reported SNP were not listed in the genotype data built on human genome 18 project assembly (Hg18; March 2006), suggesting the variants were principally absent in 292 Singapore individuals (Table 3).

In silico **prediction of the effect of the mutations on the protein**

The deleterious impact scores onto protein function of each variant were predicted as probably damaging in Polyphen2 HumDiv with scores between 0.998 to 1.00 where 1.00 is predicted most damaging. In SIFT, the variants were predicted as damaging with scores between 0.000 to 0.001 with 0.05 is the cut-off value for variants with deleterious impact. In CADD prediction analysis, the variants were ranked as top 1 % most deleterious in the genome with scores above 20 as a cut-off value (Table 4). Whereas, in Polyphen2 HumVar and FATHMM-MKL, rs147977279 (EPHA2) and rs780569137 (EPHB6) were predicted damaging with high deleterious impact scores (between 0.94- 0.997) compared to rs772228172 (EFNB1*)*'s scores (between 0.61563- 0.709), whereby 1.00 is predicted most damaging or pathogenic.

Table 4
Predictions of pathogenicity potential and sequence conservation analysis of *Eph* and *ephrin* candidate variants

Function	Tool	Score Cut-off / Range	*EPHA2* (rs147977279)		*EPHB6* (rs780569137)		*EFNB1* (rs772228172)	
			Score	Prediction	Score	Prediction	Score	Prediction
Pathogenicity	Polyphen2 HumDiv	0 - 1	0.998	Probably Damaging	1.00	Probably Damaging	0.999	Probably Damaging
	Polyphen2 HumVar	0 – 1	0.997	Probably Damaging	0.962	Probably Damaging	0.709	Possibly Damaging
	SIFT	Cut-off = 0.05	0.001	Damaging	0.000	Damaging	0.001	Damaging
	Provean	Cut-off = -2.5	-2.27	Neutral	-1.92	Neutral	-0.51	Neutral
	CADD	0 - 10 = Bottom 90% 10 - 20 = Top 10% > 20 = Top 1%	25.2	Top 1% most deleterious in the genome	24.0	Top 1% most deleterious in the genome	32	Top 1% most deleterious in the genome
	FATHMM-MKL	0 - 1	0.94351	Pathogenic	0.94876	Pathogenic	0.61563	Pathogenic
	MutationTaster	-	Disease Causing	Probably deleterious	Polymorphism	Probably harmless	Polymorphism	Probably harmless
Sequence conservation	GERP	-12.36 to +6.16	4.78	Evolutionary constrained	5.21	Evolutionary constrained	4.17	Evolutionary constrained
	PhyloP	-14 to +6	3.25157	Conserved	3.28894	Conserved	1.538	Conserved

Moreover, in MutationTaster, rs147977279 (EPHA2) was predicted as probably deleterious (disease causing) compared to rs780569137 (EPHB6) and rs772228172 (EFNB1*)* with prediction analysis as probably harmless (polymorphism). In Provean, all variants were predicted as neutral with scores ranging between -0.51 to -2.27 with a cut-off value of -2.5. In sequence evolutionary conservation analysis, the wild-type nucleotide at c.G121 for rs147977279 (EPHA2), c.A689 for rs780569137 (EPHB6) and c.C7 for rs772228172 (EFNB1*)* are highly constrained with a GERP score of 4.17 to 5.21 (cut-off value is -12.36 to 6.16), and PhyloP score of 1.538 to 3.28894 (cut-off value is -14 to 6) (Table 4).

Spina bifida-related genes in probands with Eph **and** ephrin **variants**

This study extends the investigation into the possible involvement of spina bifida-related genes (Supplementary Table 1) (Mohd-Zin et al., 2017; Greene et al., 2009) amongst the datasets with *Eph* and *ephrin* variants which we found in SB1A, SB1C, SB2A, SB2C, SB5A and SB5B (Table 1). Amongst the 6 exome datasets, proband SB5A have 6 variants with different genotype to mother of proband SB5A (SB5B) in genes involved in folate one-carbon metabolism (*MTHFR*, and *MTRR*), planar cell polarity (*VANGL1)* and methylation reaction (*APEX1, XPD* and *PCMT1*) (Table 5). *MTHFR* (rs1801133), *VANGL1* (rs4839469), *APEX1* (rs1130409), *XPD* (rs1799793) and *PCMT1* (rs4816) variants were annotated as heterozygous in exome dataset of SB5A, but not annotated (wildtype) in SB5B. Interestingly, another variant in *MTRR* (rs1801394) was annotated as homozygous in proband SB5A, and by contrast, heterozygous in SB5B (Table 5).

Table 5
Variants in spina bifida-related genes with different genotypes in probands and parent of probands with *Eph* and *ephrin* variants

	SB1A	SB1C (Father of proband SB1A)	SB2A	SB2C (Father of proband SB2A)	SB5A	SB5B (Mother of proband SB5A)
Ephs **and** ephrins	*EFNB1* (het)	*EFNB1* (hemi)	*EPHA2* (het)	*EPHA2* (het)	*EPHB6* (het)	*EPHB6* (het)
Reported spina bifida genes	*BRCA1* (het) CELSR1 (hom) COMT (hom) *CUBN* (hom) *LEPR*.2 (het) *MTRR*.1 (het) *MTRR.4* (het) *MTRR.5* (het) *PCMT1* (hom) *PTCH1* (hom)	Exome dataset not available	*BHMT* (het) *CUBN* (hom) *GRHL3* (het) *MTHFD1* (het) *MTRR*.2 (het) *MTRR*.3 (het) *PARD3* (het) *TNIP1* (het) *VANGL1* (het)	*BHMT* (wt) *CUBN* (het) *GRHL3* (wt) *MTHFD1* (wt) *MTRR*.2 (wt) *MTRR*.3 (wt) *PARD3* (wt) *TNIP1* (wt) *VANGL1* (wt)	*APEX1* (het) *MTHFR* (het) *MTRR*.1 (hom) *PCMT1* (het) *VANGL1* (het) *XPD* (het)	*APEX1* (wt) *MTHFR* (wt) *MTRR*.1 (het) *PCMT1* (wt) *VANGL1* (wt) *XPD* (wt)

The rs number for Variants found in *Ephs* and *ephrins* were represented as *EFNB1*= rs772228172, *EPHA2*= rs147977279, and *EPHB6*= rs780569137. Whereas, the rs number for variants found in spina bifida-related genes were represented in the following alphabetical order as *APEX1*= rs1130409, *BHMT*= rs3733890, *BRCA1*= rs1799966, *CELSR1*= rs4823561, *COMT*= rs4680, *CUBN*= rs1801231, *GRHL3*= rs2486668, *LEPR*.2= rs1137101, *MTHFD1*= rs2236225, *MTHFR*= rs1801133, *MTRR*.1= rs1801394, *MTRR*.2= rs162036, *MTRR*.3= rs10380, *MTRR.4*= rs2287780, *MTRR.5*= rs16879334, *PARD3*= rs118153230, *PCMT1*= rs4816, *PTCH1*= rs357564, *TNIP1*= rs2233311, *VANGL1*= rs4839469, and *XPD*= rs1799793 (wt= wildtype/ reference genotype; hom= homozygous genotype; het= heterozygous genotype)

Likewise, in exome dataset of SB2A, there were 8 out of 9 variants were annotated as heterozygous in genes involved in one-carbon metabolism including folate metabolism (*MTRR*, and *MTHFD1*) and homocysteine remethylation (*BHMT)*, planar cell polarity (*VANGL1*) and other spina bifida-related genes (*GRHL3, PARD3*, and *TNIP1*) (Table 5). In comparison to the exome dataset of father of proband SB2A (SB2C) with variant in *EPHA2* (rs147977279), the 8 variants were not annotated or have low alternate read depth (wildtype). Also, in proband SB2A, variant in *CUBN* gene (rs1801231) was annotated as homozygous in proband SB2A, and by contrast, heterozygous in SB2C (Table 5).

As in exome dataset of SB1A, 5 variants were annotated as homozygous (*CUBN, PCMT1, CELSR1, COMT*, and *PTCH1)* and 5 as heterozygous (*MTRR, LEPR*, and *BRCA1*) in reported spina bifida genes (Table 5). However, there was no comparison could be made as the exome dataset for the father of proband SB1A (SB1C) was not available.

Discussion

In this study, nearly half of the Malaysian spina bifida families (3 out of 7 families) recruited were found to carry variants in *Eph* or *ephrin* encoding genes, based on whole exome sequencing and subsequent validation by PCR. In each of the families, other than the proband, at least one unaffected member was detected as a heterozygous or hemizygous carrier of the variant allele, suggesting an uncaptured additive gene-gene or gene-environmental interactions that potentially disrupted neural tube closure in the probands.

Based on 1000 Genome Phase 3, GnomAD, ExAC, ESP and TOPMED databases, these variants are novel or rare with an unreported allele (novel) or MAF of 0.0024 and below (Table 3), based on the accepted convention that any mutations recorded in less than 1% or 0.01 of the general population are considered to be rare (Chen et al., 2011). Furthermore, EPHB6 (rs780569137) and EFNB1 (rs772228172) variants were not reported, not listed (based on variant calling files, vcf), or rare (MAF<0.01) in the population specific databases (East Asian, South Asian

and Singaporean). The Singaporean database (SGVP) is especially relevant, as the population group genotyped consisting of 292 Singaporean Malay, Chinese and Indian individuals who are a closer representation in genetic ancestries to the Malaysian individuals in our study. Although, EPHA2 variant (rs147977279) is reported less common with low frequency (0.01<MAF<0.1) based on South Asian population (1000 Genome Phase 3 database) however, the variant was not listed in the Singaporean database (SGVP). Thus, the reported rs147977279 (EPHA2*)* allele number (2n= 978 alleles) in South Asian subpopulation (SAS) was less likely screened from Singaporean or Malaysian individuals (Table 2).

By considering the potential contributing functions of Ephs and ephrins in neural tube closure (Holmberg et al., 2000; Abdul-Aziz et al., 2009; Abdullah et al., 2017), and *in silico* prediction of the effect of the mutations on the protein (in 6 to 8 out of the 9 prediction tools), we suggest that these variants may be play a causative role in the phenotypes. Moreover, the variants scored high in deleterious impact of its protein function and are remarkably close to the cut-off values (scores 0.94351 to 1.00 for cut-off value 1.00; score 0.000 to 0.001 for cut-off value 0.05) indicating the high probability for pathogenicity by amino acid substitutions (Table 4). However, the prediction for the EPHB6 and EFNB1 variants were probably harmless in FATHMM-MKL, might be due to categorisation and algorithm's predefined threshold scores compared to other prediction tools (Raychaudhuri, 2011). In Provean, all three variants were predicted to have neutral effect on the protein function, which might be due to the Provean scoring algorithm being more generic in evaluating the amino acid sequence (multiple amino acids) compared to others like Polyphen2 and SIFT which is more specific at the position of interest (single amino acid) (Adzhubei *et al*., 2010; Kumar et al., 2009; Choi et al., 2012; Krupp et al., 2014).

Based on the exome datasets and genetic screening, probands in our cohort with rare mutations in *Eph* and *ephrin*, were also detected with a set of genes in relation to environmental factor like *MTRR, MTFHR, CUBN* and others (Table 5). Also, the rare mutations in *Eph* and *ephrin*, and strong genetic component like planar cell polarity genes (*VANGL1*, and *CESLR1)* and spina bifida-related gene (*GRHL3)* were also found in probands with both types of spina bifida occulta (SB1A, SB2A) and aperta (SB5A) (Table 5).

Among the three probands with *Ephs* and *ephrins* mutations, probands SB1A and SB5A both have variants in *Ephs* and *ephrins*, and *MTRR* (rs1801394) that would suggest the additive gene-gene interaction (Table 5). Whilst, in exome datasets of proband SB2A and father of proband SB2A (SB2C), with both heterozygous for *EPHA2* variant (rs147977279), 2 different variants in *MTRR* (rs162036, and rs10380) were also annotated as heterozygous in SB2A but not in SB2C (wildtype). Our previous study on the same cohort revealed that 57% of patients and 83% of parents carried rs1801394 mutation in their *MTRR* gene, based on either homozygous (G/G) or heterozygous (A/G) genotypes (Tan et al., 2020). In that study, we concluded that *MTRR* rs1801394 variant may be an NTD risk factor in the Malaysian population based on the prevalence of this variant in other studies and found that its association with NTDs differed across populations worldwide (Tan et al., 2020). Hereto, the polymorphisms in a *Eph* and *ephrin* genes, single one-carbon metabolism gene, or planar cell polarity genes could play a limited role in overall NTD risk determination (Greene et al., 2009; Relton et al., 2004; Kibar et al., 2011; Kibar et al., 2007).

Conclusion

In summary, we report the existence of *Eph* and *ephrin* gene candidates in 3 out of 7 NTD families in Malaysia. Even though the sample size in this study is small, it provides initial evidence of the need to screen for these predicted pathogenic variants in a larger Malaysian cohort. In each of the families, other than the proband, at least one unaffected member was detected as a heterozygous or hemizygous carrier of the variant allele, suggesting *Eph* and *ephrin* variants as a potential screening tool for NTD development among Malaysians. The Malaysian population may benefit from the prenatal screening of *Eph* and *ephrin* mutants. Further validation experiments at the transcript level and gene perturbation study would help to assess the gene-gene interactions and gene function to support the pathophysiology of NTD aetiology amongst *Eph* and *ephrin* candidate genes.

Declarations

Competing interests: The authors declare no conflict of interest

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Data availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions: N.M.A-A., conceived and designed the study. M-K.T., A.B.A and S.W.M-Z. performed the experiment. N.M.A-A, N.D.E.G, M-K.T., W.M.A., A.C.W.T. and S.W.M-Z. analysed the data. N.M.A-A., N.D.E.G, M-K.T. and S.W.M-Z. drafted the manuscript. All authors contributed to manuscript revision, proofread, and approved the submitted version.

Ethics approval: Collection of samples were done in accordance with the ethical approval given by the Medical Ethics Committee (MEC) in University of Malaya Medical Centre (UMMC) (MEC reference number: 914.5)

Consent to participate: Informed consents were obtained from all patients and their families

Consent for publication: Data publication of probands were authorised by patients and their families

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

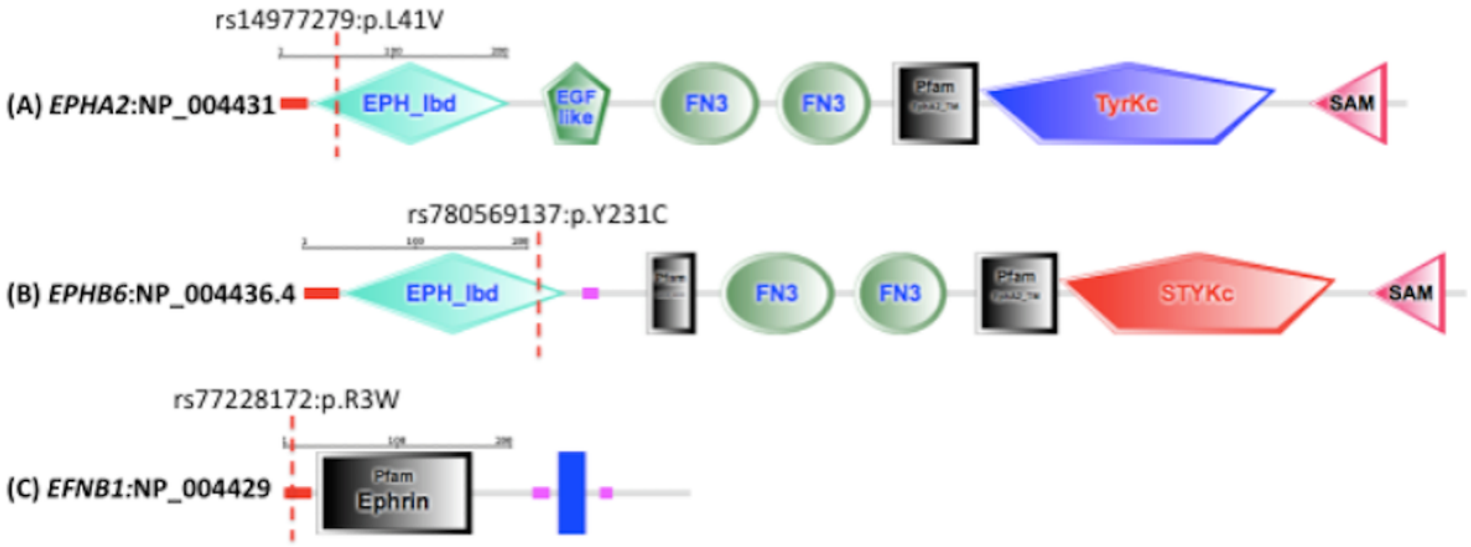

Figure 1

The EPHA2, EPHB6 and EFNB1 variants in their protein form The position of the non-synonymous variants (EPHA2:rs147977279, EPHB6:rs780569137 and EFNB1:rs772228172) were represented by red dashed bar (L=Leucine; V= Valine; Y= Tyrosine; C= Cysteine; R= Arginine; W= Trypsin; EPH_lbd= Eph ligand binding domain; EGF like= epidermal growth factor-like; FN3= fibronectin-3; Pfam EphA2_TM= protein family of EphA2 transmembrane; TyrKc= tyrosine kinase catalytic domain; SAM= sterile alpha domain; Pfam GCC2_GCC3= protein domain putative ephrin receptor like; STYKc= Protein Ser/Thr/Tyr kinase or phosphotransferases; Pfam ephrin= protein family ephrin; red box= signal peptide; blue box= transmembrane region; pink box= low complexity region)

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