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Pathogenic mutations in *ARX*, *CDKL5* and *STXBP1* genes are not associated with the early-onset epileptic encephalopathy in Malaysian pediatric patients: A pilot study

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Abstract: Gene mutation is one of the etiologies of early-onset epileptic encephalopathy (EOEE), an age-dependent seizure in infants, which leads to brain defects. Previous studies have shown that several genes namely, aristaless related homeobox (*ARX*), cyclin dependent kinase like 5 (*CDKL5*) and syntaxin binding protein 1 (*STXBP1*) are responsible for the pathophysiology of the syndrome. The study involved 20 EOEE patients and 60 control subjects, which aimed to investigate the clinical association of Malaysian EOEE subjects with 13 known pathogenic mutations in the genes of interest. In addition, the entire ARX exonic region was also sequenced for known and novel mutations. PCR specificity and efficiency were optimized using conventional PCR and High Resolution Melting Analysis (HRMA). All cases and approximately 10% of control amplicon samples were purified and subjected to DNA sequencing. All known mutations reported previously were not found in control subjects and Malaysian EOEE patients with 100% confirmation by sequencing results. Sequencing of ARX exonic regions of patient samples did not find any mutation in all exons. The preliminary study indicates that selected known pathogenic mutations of *ARX*, *CDKL5* and *STXBP1* are not associated with EOEE in Malaysian paediatric patients.

Keywords: Early-onset epileptic encephalopathy; *ARX* gene; *CDKL5* gene; *STXBP1* gene; mutation screening; pediatric epilepsy; high resolution melting analysis;

1. INTRODUCTION

Early-onset epileptic encephalopathy (EOEE) is an age-related, rare group of specific conditions characterized by an impaired cognitive, sensory and motor function caused by the abnormal inter-ictal (seizure-like) and electrical (electrographic) activity during brain maturation [1]. It may occur as syndromic cases observed in the first 3 months (neonates) with isolated tonic seizure accompanied by suppression-burst (Ohtahara syndrome) and sometimes, accompanied with myoclonia (early myoclonic epilepsy). At 6 to 12 months (infants), migrating focal discharge and mild seizure arise frequently with hypsarrhythmia (West syndrome) [2,3]. The incidence of Ohtahara syndrome was reported at ~0.3/10,000 live births, whereas 3-5/10,000 live births for West syndrome, and ~2.8/10,000 live births for Lennox Gastaut syndrome [1]. The epidemiologic information and research into the etiology of EOEE in Malaysia, however, is lacking due to the rarity of the disease. Therefore, a patient registry should be established to document EOEE cases in the country.

The genetic predisposition in EOEE could be monogenic (involving one gene) or polygenic (multiple genes). Many genes have been associated with EOEE patients. There are 3 commonest genes frequently found to be linked to EOEE; (1) aristaless related homeobox *(ARX),* (2) cyclin dependent kinase like 5 *(CDKL5)* and (3) syntaxin binding protein *(STXBP1)*. *ARX* and *CDKL5* are X-linked dominant and recessive genes, respectively, while *STXBP1* is an autosomal dominant gene located at chromosome 9. The X-linked *ARX* and *CDKL5* genes located at Xp21.3 and Xp22 loci have 5 and 24 exons, respectively. *ARX* encodes a transcription factor that is involved in axonal guidance, neuronal proliferation and differentiation [4] while CDKL5 is a protein kinase that regulate the catalytic activity and nuclear placement of other proteins in the brain [5]. *STXBP1* gene on the other hand is located on chromosome 9q34.11 locus encompasses 19 (Isoform a) to 20 (Isoform b) exons that plays a role in synaptic vesicle docking and fusion in the brain [6].

Most of the EOEE cases reported were due to *de novo* mutation(s) and very few familial cases were reported to date [7]. Splice-site, nonsense, missense, deletion, insertion, frameshift, and duplication mutations have been reported in *ARX* (44), *CDKL5* (53) and *STXBP1* (28) [8-15]. Interestingly, recurrent causative mutations have been reported in patients exhibit similar clinical characteristics. We are interested in identifying these causative mutations (Table 1) and correlate with various clinical findings in Malaysian EOEE patients.

The type and the site of mutation implicated the severity and time of onset of EOEE. In *ARX* mutation, Suri (2004) [16] suggested that the premature termination at 'homeodomain' and missense mutation in 'aristaless domain' affected patients with mental retardation and other forms of brain malformation while the expansion of first and second polyalanine domain affect those with brain non-malformation. In *CDKL5* mutation, Bahi-buisson *et al.* (2008) [9] and Russo *et al*. (2009) [11] reported that patients affected with nonsense mutation have a milder phenotype than those with missense or splicing mutations and those in 'catalytic domain' is more severe than in 'C-terminal domain'. The effect of specific type and site of mutation is not yet elucidated for *STXBP1* since the studies are still expanding. Based on previous reports, no specific 'hotspot' has been proven to cause more severe effects in epileptic encephalopathy patients [17].

Our group is interested in SNPs within *ARX, CDKL5* and *STXBP1* gene that are clinically associated with EOEE located at the coding sequences region and causes amino acid change in the encoded protein. *ARX* gene, being one of the most reported causative genes for EOEE, were also fully sequenced to determine novel

polymorphisms within the exons that are potentially associated with Malaysian EOEE subjects. We aimed to identify selected known mutations (Table 1) and correlate with EOEE patients in Malaysian cohort.

2. MATERIALS AND METHODS

2.1. Ethics approval, sampling method and recruitment of subjects

The study was approved by National Medical Research Register (NMRR), Ministry of Health (MOH) (NMRR-12-1159-13653) and Universiti Putra Malaysia (UPM) Ethics Committee (RUGS 04-04-11-1483RU). The study was carried out in accordance to Helsinki declaration. Information sheet were given to the patients before an informed consent was obtained from either one of the parents for cases. Control subjects (adults) could sign the consent form as all of them were 21 years old and above.

A total of 20 cases diagnosed with sporadic EOEE were recruited from Hospital Serdang, Hospital Kuala Lumpur, Hospital Penang, Hospital Raja Perempuan Zainab II, Kota Bharu, University Malaya Medical Centre while the controls were recruited from Universiti Putra Malaysia. The samples were analyzed in Medical Genetic Laboratory, Faculty of Medicine and Health Science, UPM. Diagnoses were based on clinical assessment by pediatric neurologists. Data collected includes demographic data, physical examination, EEG status, family history and prescribed medications. Key clinical inclusion criteria were: Age less than 7 years old, onset of first seizure at less than 12 months old, electrophysiological and clinical features of infantile spasm with hypsarrhythmia or Ohtahara syndrome/early myoclonic epileptic encephalopathy with burst suppression or malignant migrating partial epilepsy of infancy or Lennox-Gastaut syndrome with prior history of the above. On the other hand, control subjects must be older than 18 years with no medical complications and history of epilepsy. See Figure 1 for the flow of subjects' recruitment and list of inclusion and exclusion criteria.

2.2. Blood collection

Approximately 3 ml of peripheral blood was collected via venipuncture and transferred into ethylene-diaminetetraacetic acid (EDTA) tube. The blood sample was mixed soon after collection to avoid coagulation. The blood sample was packed and delivered on ice-packs to Medical Genetics Laboratory, UPM, within 24 hours for processing.

2.3. High-resolution melting analysis

Genomic DNA (gDNA) was extracted from the buffy coat using the QiaAmp DNA Blood Mini kit (Qiagen, USA) according to manufacturer's protocol. Eleven primer sets were designed using Primer3 program (http://www.bioinformatics.nl/) to flank selected SNPs (Table 1).

Pre-analysis of polymerase chain reaction (PCR) and real-time PCR were performed prior to High-Resolution Melting (HRM) analysis. The HRM analysis was performed in 10 µl reaction volumes containing 30 ng/µl of genomic DNA, 0.7 µl of each 10µM primers, 5 µl of Type-It HRM-PCR kit (HotStarTaq Plus DNA Polymerase; EvaGreen Dye; optimized concentration of Q-solution; dNTPs; $MgCl_2$; Qiagen, USA). Non-template controls were included in all run. The reactions were run on 5-plex HRM real-time PCR machine on Rotor Disc 72 mounted on Rotor-Gene™ 6000 (Qiagen, USA). PCR conditions involved a single pre-denaturation step of 95°C for 5 minutes followed by 45 cycles of 95°C for 10 seconds, 53-60°C (60°C for the SNP in *ARX*, 55°C for *CDKL5* SNPs and 53°C *STXBP1* SNPs) for 30 seconds and green channel of fluorescence acquisition (460 nm excitation; 510 nm detection) was carried out during the extension step. After cooling, HRM analysis was run by 0.1°C increment with 2 seconds hold and ramping from 65-95°C with a pre-melt condition of 90 seconds at the first step.

The total reactions ended approximately in 2.5 h. The data obtained were analyzed using the Rotor-Gene 6000 Series Software v1.7 (Qiagen, USA).

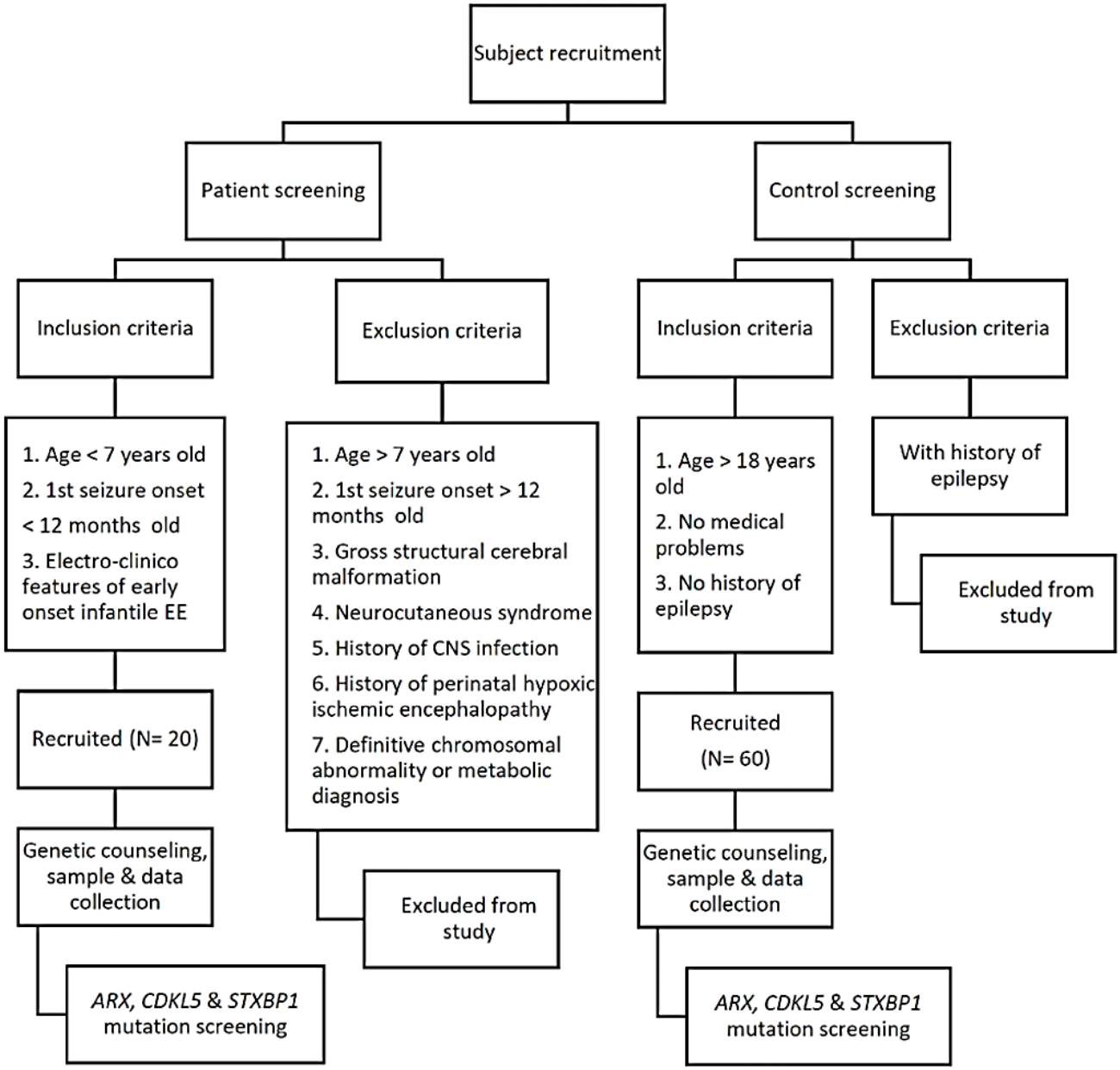

Figure 1. Flow of subjects' recruitment based on approved inclusion/exclusion criteria.

The analysis was performed in three steps which includes normalization, curve overlaid, and variant clustering. Amplification plot was analyzed using comparative quantitation by assessing Ct value (threshold cycle), end-point fluorescence and amplification rate. Data outside the standard criteria were omitted due to late amplification that may produce variable results. Derivative melt curve of optimized assays was kept as single bimodal peak for all samples prior to genotyping.

In HRM analysis, the comparative quantitation, derivative melt-curve, melting graph and difference graph enables the discrimination of SNP variations in different samples. Based on the Qiagen Type-it HRM-PCR handbook (2009), comparative quantitation analysis is used to check on Ct value of "less than 30" and amplification rate of "more than 1.4" for a successful analysis.

Table 1: Targeted known single nucleotide mutation in *ARX, CDKL5* and *STXBP1* and their associated primer set used for high resolution melting analysis

Gene	dbSNP ID	Exon	Mutation	Protein change	Allele status on dbSNP	References	Primers (5'→3')	Amplicon size (bp)
ARX	N/A	1	c.81C>G	p.(Val27X)	Untested allele	[18]	F: CAGCAACCGCATTTTGCAC R: CCAGCCATGAGCAATCAGT	138
CDKL5	rs 62653623	5	c.175C>T	p.(Arg59X)	Untested allele	[19,20]	F: TGGGATGTTTTCAGTGTTCT R: ATGCTTCCTTCAACTCCACAAT	176
	rs 62643608	5	c.183delT	p.(Met63fs)	Pathogenic	[21]		
	rs 62641235	5	c.215T>A/T>C	p.(Ile72Asn)/p.(Ile72Ile)/ p.(Ile72Thr)	Pathogenic	[11,22,23]	F: AGCTTAAAATGCTTCGGACTCTC R: TGCACATTGGCAATTAATGACT	129
	rs 122460157	7	c.455G>T	p.(Cys152Phe)	Pathogenic	[19,24]	F: TGACACTCCAGATATAAAACCAGA R: CATGTGACTCAAAAGAATGTTCC	207
	rs 61749700	8	c.525A>T	p.(Arg175Ser)	Pathogenic	[11,25]	F: GCTATCTTTCAGGTTTTGCTCGT R: ATCAGCAGATGTGGAAATGTCA	149
	rs 61749704	8	c.539C>T	p.(Pro180Leu)	Pathogenic	[23]		
	rs 61750250	11	c.838_847del10	p.(Leu280Ala)	Pathogenic	[26]	F: TCTGCAATGACTGTGTATTTCTTT R: AGAAGTCTCTGGGTTTGAAATGT	190
STXBP1	rs 121918320	5	c.251T>A	p.(Val84Asp)	Pathogenic	[27]	F: ACAGGTCCCATTTGGCTCTA R: AATGCTAACCTGCCTGATGG	188
	rs 121918318	7	c.539G>A	p.(Cys180Tyr)	Pathogenic		F: CCTTGGACTCTGCTGACTCTTT R: CAGACTGGTGCACTGCCTTAC	163
	rs 121918321	14	c.1162C>T	p.(Arg388Ter)	Pathogenic		F: GGAGCCAATGAGGTGTGTTT R: CCACAGCCCTACCATTCTTC	178
	rs 121918319	15	c.328T>G	p.(Met443Arg)	Pathogenic		F: TCACGGAGGAAAACCTGAAC R: CAGTCAGAGCAGAAGCAGACA	179
	rs 121918317	18	c.1631G>A	p.(Gly544Asp)	Pathogenic		F: CTATGGGCACTGGCATAAGAAC R: ACCTATCAGCACCTCCCACTT	151

Genotypes of the unknown samples were auto-called by the software using standard option and displayed using two curves; normalized curve and difference curve. The normalized curve was generated based on the dissociation of the fluorescence dye from double-stranded DNA to form single-stranded DNA as the temperature increases. The difference curve measured the difference between selected samples with samples of known genotypes as baseline. Using a standard normalized region and confidence threshold set to a minimum of 90%, the normalize curve distinguished homozygote based on changes in shape and heterozygotes in temperature shifting on x-axis while the difference curve enhances the difference between selected samples as compared to the wild-type profile.

For validation, suspected positive mutant samples were purified (Intron Biotechnology, South Korea) and sent for DNA sequencing services. All cases and 10% of control samples in each HRM cluster of normalized curves were purified and sequenced. Trace files were analyzed, and sequence alignment was conducted using DNA Baser v3.5.4 software. Phred score value of 20 or more was used to define the quality of the sequencing result.

2.4. Sequencing of ARX exons

A total of 50 µl PCR reaction was prepared in 1.5 µl microcentrifuge tube containing 2.5 U Expand long template PCR mix (Roche), 1X Failsafe Premix J (Epicentre Technology), 50-100 ng/ul of DNA, 150 nM forward and reverse primer, and RNase-free water. The primers used to amplify all the five *ARX* exons were based on Strømme *et al*., 2002 (Table 2). Touchdown PCR were performed on all samples using the Mastercycler™ Gradient S (Eppendorf, Germany) with the following steps; 94°C for 5 minutes followed by 35 cycles of 94°C for 30 s, 65-60°C for 30 s and 68 °C for 2 min. A final step was carried out at 68°C for 7 min before the samples were held at 4°C until further analysis. PCR products were electrophoresed on 2.0% (w/v) agarose gel to examine the size of amplicons. Samples were purified and sequenced using the Sanger's method. Trace files were analyzed, and sequence alignment was conducted using DNA Baser v3.5.4 software. Phred score value of 20 or more was used to define the quality of the sequence.

Table 2: Primers used for the amplification of *ARX* exons

Exon	Primer (5'→3')	Amplicon length (bp)	Amplicon T_m (°C)
1	F: GCTCACTACACTTGTTACCGC R: AATTGACAATT CCAGGCCACTG	520	63
2	F: ACGCCTGGGCCTAGGCACTG R: CTCGGTGCCGGTGCCACCAC	584	62
2	F: GCAAGTCGTACCGCGAGAACG R: TGCGCTCTCTGCCGCTGCGA	602	62
3	F: CTGCCATAGAGGAGGAAATAG R: GGTTTTGTGAAGGGGATCTCAC	239	60
4	F: GACGCGTCCGAAAACAACCTGAG R: CCCCAGCCTCTGTGTGTATG	551	60
5	F: ACAGCTCCCGAGGCCATGAC R: GAGTGGTGCTGAGTGAGGTGA	347	60

3. RESULTS

The screening involves 20 cases and 60 control samples. Patient age group ranged from 25-day to two years old. Their samples were provided by Hospital Serdang (n=7), Hospital Raja Perempuan Zainab II (n=6), Hospital Kuala Lumpur (n=1), Hospital Pulau Pinang (n=2), Women and Children Hospital Sabah (n=3) and University Malaya Medical Centre (n=1). Approximately 45% of the patients were above 6 months to 2 years old, 30% below 2 months old and 25% between 3 to 6 months old.

Among 20 cases, 55% of the patients were male while 45% female (Table 3). They were presented with spasm (50%), generalized tonic clonic seizure (GTCS) (10%) and some were accompanied with myoclonic (5%). Collectively, 15% had spasm accompanied with focal and myoclonic seizures while 5% had focal accompanied with myoclonic seizures. Based on the outcome of syndrome, 55% of cases is West syndrome, 20% Ohtahara syndrome, 10% migrating partial epilepsy of

Table 3: Clinical diagnosis and EOEE patients

Gender	Age[a]	Seizure type[b]	Epilepsy syndrome[c]	Development	Family history[d]	Physical examination	EEG features	MRI findings	Metabolic profiles[e]	Medication (current and past)
F	8m	Spasm	West	Normal	Negative	Normal	Hypsarrhythmia	Normal	Normal	Epilim; Vigabatrin
F	1m	GTCS and spasm	MPEI	Normal	Negative	Normal	Hypsarrhythmia	Normal	Normal	Clonazepam
F	4m	Spasm	West	Delayed	Negative	Normal	Multifocal epileptiform discharge	Non-specific white matter changes	Normal	Valproate; Keppra; Clobazam; Ketogenic diet
F	1w	Spasm, focal and myoclonic	Ohtahara	Delayed	Negative	Abnormal (autistic)	Burst suppression	Not available	Not available	Prednisolone; Nitrazepam
F	1w	GTCS	West	Delayed	Negative	Normal	Hypsarrhythmia	Subarachnoid space & hypertrophic of arachnoid membrane, Left-frontal region, mild cerebral atrophy	Not available	Clobazam
F	2m	Spasm	West	Normal	Negative	Normal	Burst-suppression	Not available	Not available	Phenobarbitone; Clonazepam
F	2m	Focal and spasm	EME	Delayed	Positive	Normal	Multifocal epileptiform discharge	Normal	Normal	Levetiracetam; Clobazam; Topiramate
F	4m	Spasm, focal and myoclonic	Ohtahara	Delayed	Negative	Abnormal (hypotonia)	Multifocal epileptiform discharge	Prominent	Not available	Prednisolone; Pyridoxine; Nitrazepam
F	1m	Spasm	West	Delayed	Negative	Normal	Hypsarrhythmia	Mild thinning of corpus collosum	Normal	Vigabatrin
M	1m	Focal	West	Normal	Negative	Normal	Hypsarrhythmia	Normal	Normal	Prednisolone
M	1m	Spasm	West	Normal	Negative	Normal	Hypsarrhythmia	Normal	Normal	Prednisolone
M	2m	Spasm	EME	Delayed	Negative	Abnormal (autistic)	Hypsarrhythmia	Normal	Normal	Sodium valproate
M	1m	Spasm	EME	Delayed	Negative	Abnormal (generalised hypotonia)	Hypsarrhythmia	Delayed myelination	Normal	Pyridoxine; Sodium Valproate; Nitrazepam

M	2m	Tonic	Ohtahara	Delayed	Negative	Abnormal (quadriplegia)	Burst-suppression	Normal	Normal	Vigabatrin; Phenobarbitone
M	8m	GTCS	West	Delayed	Negative	Normal	Multifocal epileptiform discharge	Normal	Normal	Clonazepam
M	5m	Spasm	West	Delayed	Negative	Normal	Hypsarrhythmia	Normal	Normal	Vigabatrin; Ketogenic diet
M	2m	Focal	MPEI	Delayed	Negative	Abnormal (hypotonia)	Multifocal epileptiform discharge	Not available	Not available	Levetiracetam; Vigabatrin; Ketogenic diet; Clonazepam; Prednisolone
M	2m	Spasm, focal and myoclonic	Ohtahara	Delayed	Positive	Abnormal (microcephaly)	Burst-suppression	Absent corpus collosum, Cerebellar hypoplasia, Atrophy	Normal	Keppra; Topiramate; Carbamazepine
M	6m	Spasm	West	Normal	Negative	Normal	Hypsarrhythmia	Normal	Normal	Prednisolone
M	5m	Spasm	West	Delayed	Negative	Normal	Multifocal epileptiform discharge	Normal	Normal	Prednisolone; Vigabatrin

[a] refers to age at onset. "w" denotes week(s), "m" denotes month(s).

[b] "GTCS" denotes generalized tonic clonic seizure.

[c] "MPEI" denotes migrating partial epilepsy of infancy, "EME" denotes early myoclonic encephalopathy.

[d] Family history was traced up to 3rd degree relatives.

[e] Metabolic profiles interpretation was based on blood and urine tests for biochemical profiles.

infancy (MPEI), and 15% early myoclonic epilepsy (EME). The results were in concordance with electroencephalogram (EEG) reports showing 50% of cases had hypsarrhythmia which was a typical EEG profile of patients with West syndrome, 20% burst-suppression of Ohtahara syndrome patients and 30% had epileptiform discharge. Other than age, gender, seizure type and EEG type, family history was also recorded. Among the cases, 15% subjects were positive while 85% negative of epileptic family background. Other findings on magnetic resonance imaging, metabolic profile and prescribed treatment are listed in Table 3. Gradient PCR optimization (Figure 2), HRM analysis (Figure 3) and DNA sequencing of all cases and selected control samples (Figure 4) did not find any of the selected causative mutations in *ARX*, *CDKL5* and *STXBP1* genes in all samples. In addition, direct sequencing of all *ARX* exons (data not shown) did not reveal any polymorphisms or mutations in both cases and control samples. Our preliminary results show that the reported causative mutations do not exist in the studied pilot group of Malaysian EOEE patients.

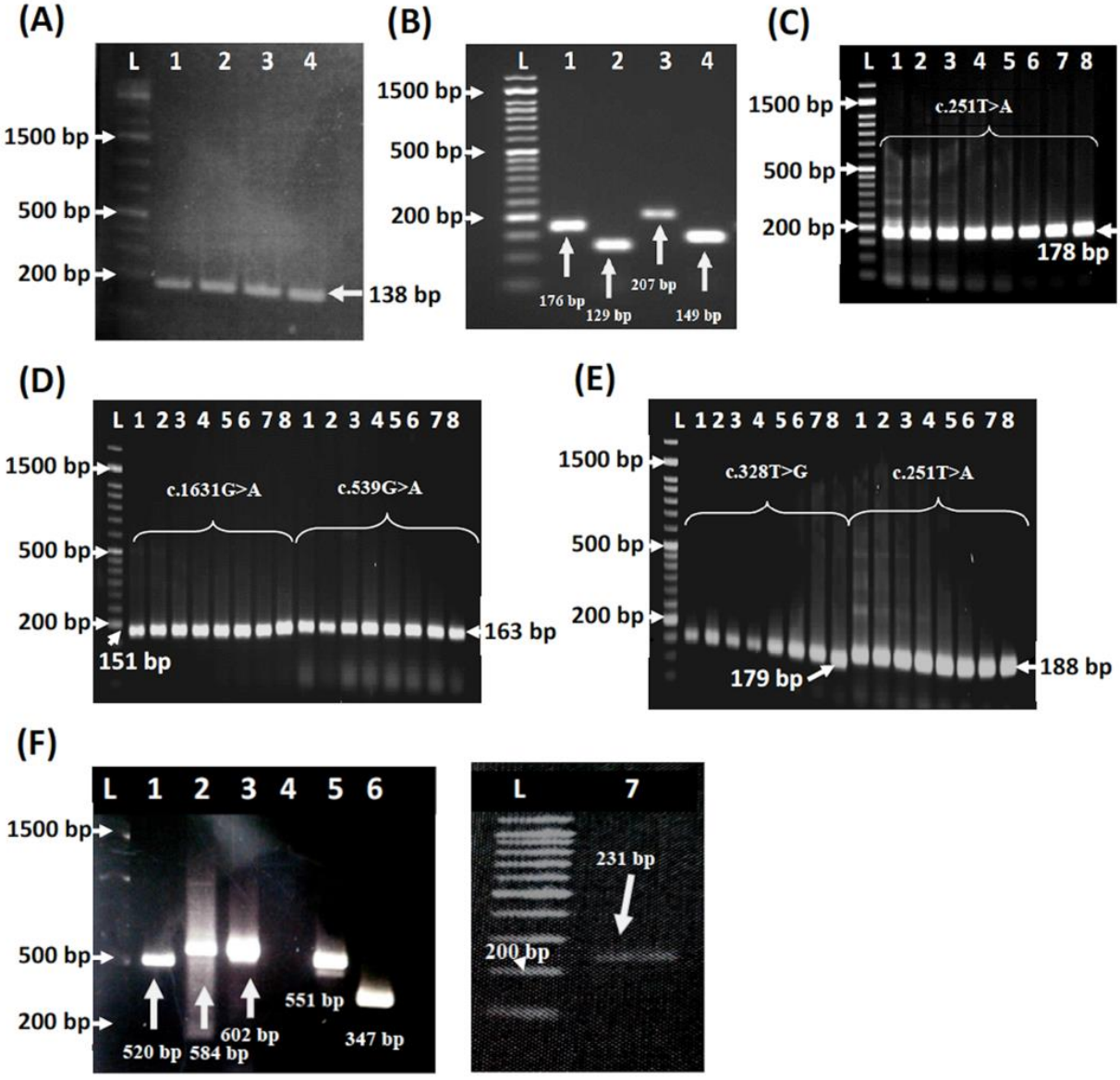

Figure 2. Gradient PCR gel electrophoresis for **(A)** *ARX* c.81C>G (138 bp). PCR was performed at different temperature ranging from 59.0°C to 62.3°C (L=1 kb ladder, 1=59.0°C, 2=60.1°C, 3=61.2°C, 4=62.3°C), **(B)** *CDKL5* (1) c.175C>T, c.183delT, (2) c.215T>A/C, (3) c.455G>T (4) c.525A>T, c.539C>T, **(C)** *STXBP1* c.1162C>T, **(D)** c.1631G>A, c.539G>A, **(E)** c.328T>G and c.251T>A (L=1 kb ladder, 1=44.1°C, 2=46.2°C, 3=47.8°C, 4=49.6°C, 5=51.5°C, 6=53.4°C, 7=55.2°C, 8=57.9°C). **(F)** Touch-down PCR amplification of *ARX* exons ranging from 65°C to 60°C (L=1 kb ladder, 1=exon 1, 2=exon 2P1, 3=exon 2P2, 4=failed amplification for exon 3, 5=exon 4, 6=exon 5 and 7=exon 3).

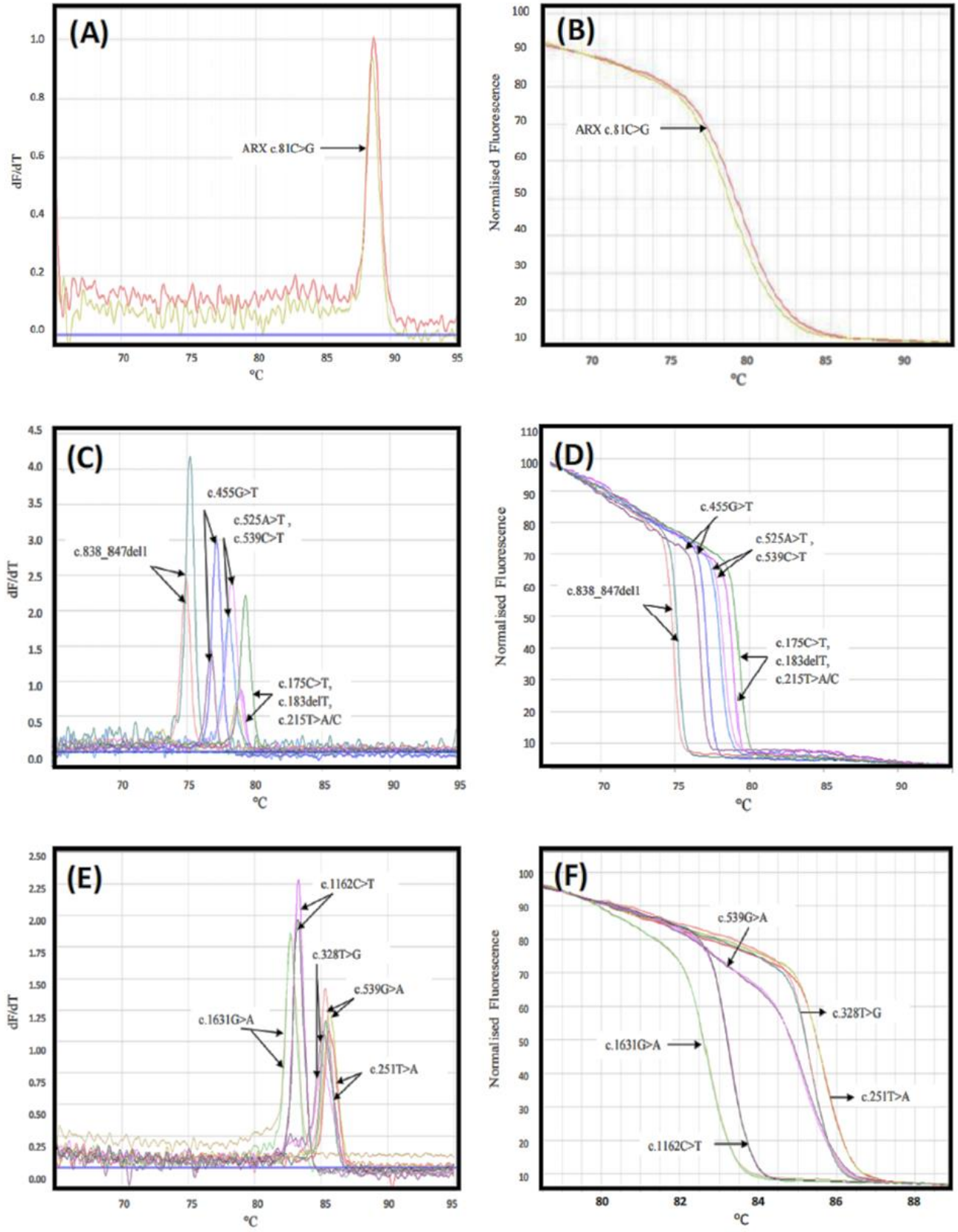

Figure 3. High-Resolution Melting analysis of *ARX* **(A & B)**, *CDKL5* **(C & D)** and *STXBP1* **(E & F)** selected causative mutations/SNPs based on the **(A, C & E)** derivative melting and **(B, D & F)** normalized curves. The derivative melting curves contain a pair of data points for temperature and fluorescence intensity that denote the dissociation-characteristics of double-stranded DNA. The normalized curve shows the intensity between 0-100% after uniformly normalized all the samples against their pre-*melt* (initial fluorescence) and post-*melt* (final fluorescence) signals.

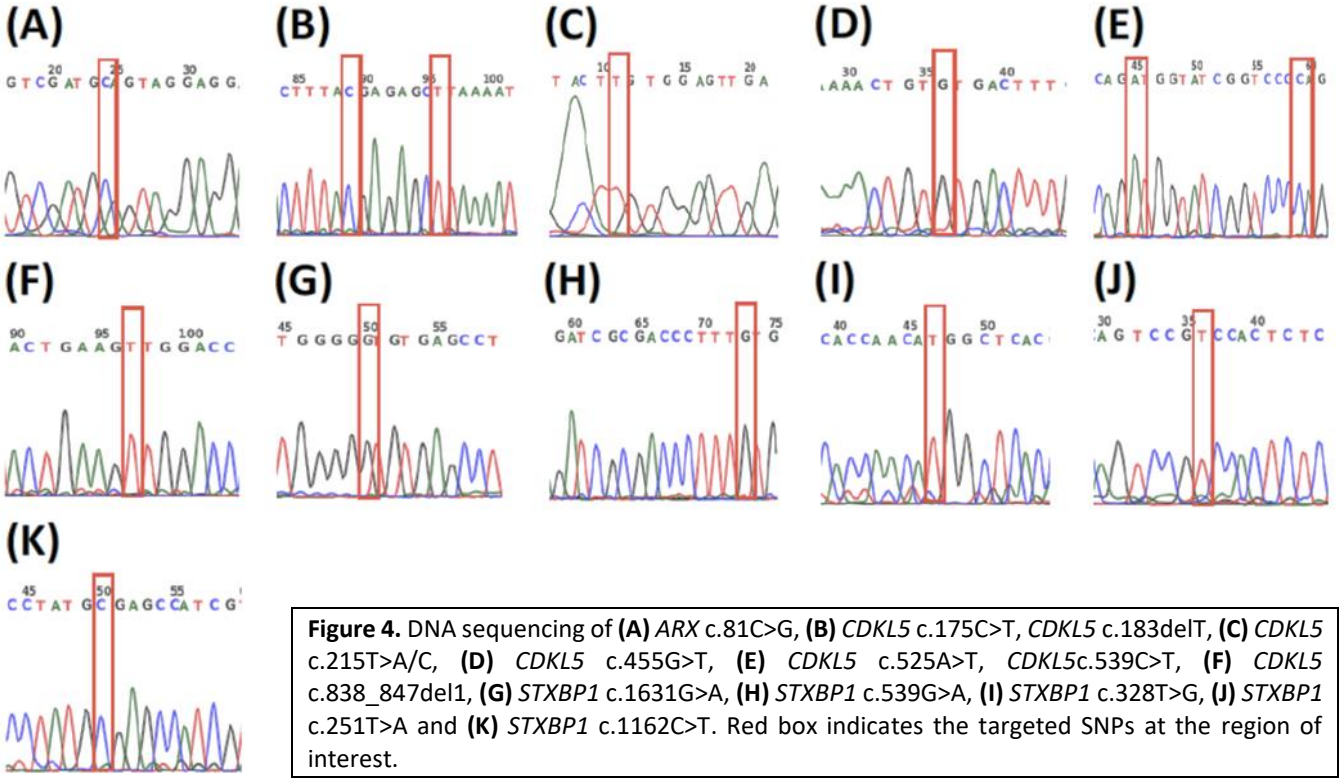

Figure 4. DNA sequencing of **(A)** *ARX* c.81C>G, **(B)** *CDKL5* c.175C>T, *CDKL5* c.183delT, **(C)** *CDKL5* c.215T>A/C, **(D)** *CDKL5* c.455G>T, **(E)** *CDKL5* c.525A>T, *CDKL5*c.539C>T, **(F)** *CDKL5* c.838_847del1, **(G)** *STXBP1* c.1631G>A, **(H)** *STXBP1* c.539G>A, **(I)** *STXBP1* c.328T>G, **(J)** *STXBP1* c.251T>A and **(K)** *STXBP1* c.1162C>T. Red box indicates the targeted SNPs at the region of interest.

4. DISCUSSION

The pilot study was limited by the small sample size due to the rareness of the disease in Malaysia [28]. Furthermore, a comprehensive screening is required to cover complete coding region whilst including mutation in the promoter and other regulatory sequences. The negative results did not preclude possible epigenetic contributions that may alter the expression profile of these genes leading to the clinical observations.

The absence of previously reported causative mutations in idiopathic Malaysian EOEE patients may possibly due to the heterogeneity of the disease manifested with a spectrum of clinical presentation. Fullston and colleagues (2010) [18] reported that *ARX c.81C>G* was found in two male cousins, one with West syndrome and another with Ohtahara. The first one was presented with focal seizure at four weeks old, infantile spasm at four months, delayed in all areas of brain development at 5-year-old, refractory to anticonvulsant therapy, myoclonic, and occasional tonic clonic. The cousin had tonic clonic, myoclonic on day five, Ohtahara syndrome on three weeks old, hypotonia at five months old. However, the body development was normal. The clinical presentation was different in Malaysian patients suggesting c.81C>G causes severe clinical manifestation that is specific in nature that was due to the formation of truncated protein [18]. Despite being one of the most reported causative genes for EOEE and additional exonic sequencing, no *ARX* mutations or polymorphisms were detected in our samples.

Most of the EOEE patients with *CDKL5* causative mutations were reported as having intellectual disability, which was absent in all Malaysian EOEE patients recruited in this study. The difference in clinical manifestation may be affected by different causative mutations found to be associated with the wide spectrum of clinical findings that presented at different time of onset [9]. For example, c.175C>T was described by Archer *et. al* (2006) [19] and Castrén *et al.* (2006) [13] in a 6-year old patient in two separate studies. The same mutation was again found by Bahi-Buisson *et al.* (2008) [9] in a 21-year old patient. Clinical presentation described on two patients who were unable to sit while another was able with aid. They had autistic features, hand stereotypies and deceleration of head growth. The seizure started at the age of four weeks and stop for the first reported patient while the second patient had severe epilepsy onset at twelve hours after born which develop into infantile spasm. When comparing with Malaysian EOEE patients, they are developmentally normal. Although two subjects were reported as autistic, no such mutation was found in both samples.

Saitsu *et al.* (2008) [2] described on five patients with c.1631G>A, c.539G>A, c.328T>G, c.251T>A and c.1162C>T have age of onset at less than three months old presented with Ohtahara syndrome with suppression-burst pattern on EEG. They had poor visual attention, no head control, non-verbal, weak eye pursuit and late walking ability and neurological examination displayed profound mental retardation with spastic quadriplegia, and diplegia. These causative mutations, however, were absent in four Malaysian Ohtahara patients whom we recruited in the study. Developmental and neurological examinations were normal in the recruited patients suggesting the causative mutations are likely to be associated with more severe clinical manifestations.

Sampaio *et al.* (2015) [29] suggested the need to screen *STXBP1* gene when both *ARX* in male and *CDKL5* in female were not at all associated with the disease. Subsequently, all samples in this study were subjected to *STXBP1* causative mutations screening. Moreover, these SNPs that have been found in Ohtahara syndrome patients were also screened in West syndrome, MPEI and EME patients to increase the targeted pool. However, the results were all negatives. One of the limitations of this study was the low sample size.

The cases are very rare and frequently presented with complex overlapping electro-clinical findings. Other than that, our screening only focusses on thirteen reported causative mutations in three commonest genes. Other novel genetic markers pertaining to Malaysian subjects may have been overlooked. Common allele in other populations may be rare in Malaysian population. Unlike Caucasian, EOEE is considered rare with very low prevalence in Malaysia [28].

5. CONCLUSIONS

In conclusion, thirteen *ARX, CDKL5* and *STXBP1* causative mutations that were reported to be associated with EOEE patients were absent in both cases and control Malaysian subjects. No novel mutation was found in *ARX* exons in all the EOEE cases. Nonetheless, HRMA was proven as effective and efficient genetic screening method to serve as a routine genetic screening in a clinical setting due to its robustness and accuracy during the screening process. This is the first targeted genetic screening of EOEE subjects in Malaysia. In future, it is recommended to increase the sample size and sub-classifying patients according to different clinical characteristics or EEG profiles for genetic comparison. Unbiased approaches such as whole genome sequencing and SNP array analysis should be adopted to identify novel genetic markers for the disease.

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