CHAPTER IV

RESULTS

The goal of this study was to identify the underlying genetic defect in patients with MR and epilepsy. Mutation analysis from the syndromic patients were performed, from the non syndromic patients then do further screening to confirm the mutation to the parents of the suspected patient.

IV.1 Obtaining MR with Epilepsy patients

First step of this study was obtaining MR with Epilepsy patients list. Starting total 527 MR patients that already collected from previous study [16], screening of the clinical data from all patients performed. Patients that positively diagnosed with epilepsy by medical doctor from Bandung and Semarang Indonesia are all included in this study.

The patients clinical features were collected, possible dysmorphology data from the pictures, results of measurements, and cytogenetic analysis. Unfortunately those patients have had no previous additional brain imaging examination, or blood metabolite analysis yet. History and pedigree was already taken from the parents and or the teacher in the special school and was written in the medical record, although not completely captured or reliable.

The clinical features with pictures already presented to the clinical geneticist in charge, Dr. Tjitske Kleefstra, MD, PhD and Bregje van Bon, MD, PhD in power point format. Differential diagnosis gained based on LMD (London Medical Database) software and discussion with clinical geneticists RUNMC.

Table 3. Differential Diagnosis

No	Name	Sex	Epilepsy onset	Seizures type	Differential Diagnosis for suspect Syndromic cases

1	MR	M	7y	Absence	
2	DY	M	8m	FS,GE	
3	JT	F	4y	GPE	Angelman syndrome
4	RA	M	1y	GPE	Lujan syndrome
5	RGP	M	5y	GPE	Suspect Mozaicism 22
6	DM	M	6m	GE	
7	NTW	M	6m	GPE	
8	DS	F	3y	?	
9	AAI	M	4y	?	
10	JO	F	6m	FS,GE	
11	RA	M	1,5y	?	
12	AK	M	9m	FS	
13	DAT	M	?	GPE	
14	PH	M	4m	?	
15	RA	F	?	Absence	Possible 46,XX, der(9)
16	D	M	9m	GPE	
17	LA	F	1,5y	?	
18	AA	M	1,5m	FS,GE	
19	R	F	7m	FS,GE	
20	FAE	M	?	?	
21	DB	F	?	?	
22	SB	M	?	?	
23	DBP	M	?	?	
24	EA	F	?	?	Cowden syndrome
25	FRHP	M	?	?	
26	D	F	?	?	Pitt Hopkins Syndrome
27	J	F	?	?	
28	Yosepin	F	?	?	
29	Aris Masari	M	?	?	
30	Yuda	M	?	?	
31	Evi	F	?	?	
32	SA	M	11y	GPE	FISH: Marker 15: exclude

DOB: Date of Birth; FS: Febrile Seizures; GE: General Epilepsy; GPE: General Partial Epilepsy.

Those patients had no history of environmental disturbance that may cause MR and epilepsy such as toxins and brain infections. They had normal developmental history during prenatal, natal, and post natal period. Several genetic factors that could contribute to the MR and

epilepsy were excluded.One patient with marker at chromosome 15 was excluded from patients list, so in total there were 31 subjects left.

IV.2 Mutation Screening from The Syndromic Patients List

For the syndromic patients list, there are four patients that were suspected have dysmorphology that fit to some features in some syndromes. For patient with the possible mosaic 22 (named RGP, male), cytogenetic analysis for mosaicism were recheck, but did not find anything. Need more specific cytogenetic tools to help the diagnosis for this patient (example: whole genome array). Explanationof the details can be seen below.

1. *PTEN* gene: Cowden syndrome patient

Dysmorphology and physical examination of this patient found macrocephaly, hamartomas on chest and inside mouth and severe MR.

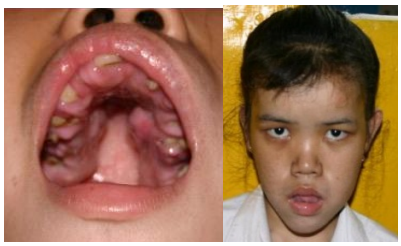

Figure 3. Picture of suspected Cowden syndrome patient.

The pictures of the girl showed narrow palate, and hamarthomas inside the mouth. She also has hamarthomas on her chest.

2. *UPF3B* and *MED12* gene: Lujan syndrome

Dysmorphology and physical examination of this patient characterized male, had severe MR, marfanoid habitus positive, and microcephaly.

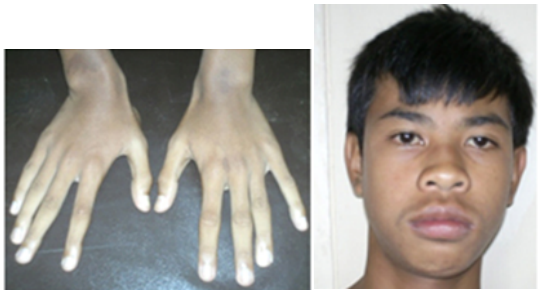

Figure 4. Picture of suspected Lujan syndrome patient.

The pictures of the boy showed arachnoid fingers, typically for marfanoid phenotype.

3. *UBE3A* gene: Angelman syndrome

Dysmorphology and physical examination of this patient foundsevere MR, speech impairment, seizures and attention disorder.

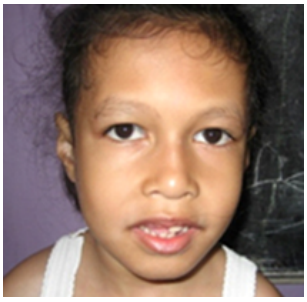

Figure5. Picture of suspected Angelman syndrome patient.

The picture of the girl does not show much dysmorphological appearances, but she has severe MR and atenntion disorder on her behaviour.

4. *TCF4* gene: Pitt-Hopkins syndrome

Dysmorphology and physical examination of this patient foundspecific facial features & wide mouth that closely depicted as PHS patients.

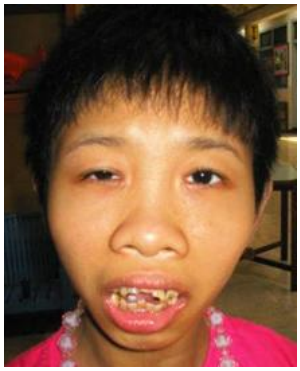

Figure 6. Picture of the suspect Pitt-Hopkins syndrome patient.

The picture of the girl showed crowded teeth on her open mouth, facial express of severed MR patients.

No mutation found in *UBE3A, UPF3B* and *MED12, PTEN,* and *TCF4* genes sequencing.

IV.3 Mutation Screening from the non syndromic patients list

From the LMD, differential diagnosis for suspected syndromic patients were made. But for the rest of them, which had no specific dysmorphology, screening with known genes that responsible for MR and epilepsy were the best option. Those gene are *SCN1A, ARX, STXBP1,* and *LGI1* genes. Only *ARX* testing is already performed as routine diagnostic gene in DNA Diagnostic Human Genetic Department RUN MC Nijmegen.

The rest of them are chosen based on similarity of some clinical features in the patients list. The tests need to be designed and tested first before applied to the patients list. *SCN1A, ARX* and *STXBP1* genes were chosen based on supported journals and characteristic of patients list that matched with characteristics of those genes.

Table 5. List of the patients with the screened genes.

No	Name	Sex	Tested genes for MR/epilepsy			
			SCN1A	*ARX*	*STXBP1*	*LGI1*
1	MR	M	tested	tested	tested	tested

2	DY	M	tested	tested	tested	tested
3	JT	F	tested	-	tested	tested
4	RA	M	tested	tested	tested	tested
5	RGP	M	tested	tested	tested	tested
6	DM	M	tested	tested	tested	tested
7	NTW	M	tested	tested	tested	tested
8	DS	F	tested	-	tested	tested
9	AAI	M	tested	tested	tested	tested
10	JO	F	tested	-	tested	tested
11	RA	M	tested	tested	tested	tested
12	AK	M	tested	tested	tested	tested
13	DAT	M	tested	tested	tested	tested
14	PH	M	tested	tested	tested	tested
15	RA	F	tested	-	tested	tested
16	D	M	tested	tested	tested	tested
17	LA	F	tested	-	tested	tested
18	AA	M	tested	tested	tested	tested
19	R	F	tested	-	tested	tested
20	FAE	M	tested	tested	tested	tested
21	DB	F	tested	-	tested	tested
22	SB	M	tested	tested	tested	tested
23	DBP	M	tested	tested	tested	tested
24	EA	F	tested	-	tested	tested
25	FRHP	M	tested	tested	tested	tested
26	D	F	tested	-	tested	tested
27	J	F	tested	-	tested	tested
28	Yosepin	F	tested	-	tested	tested
29	Aris Masari	M	tested	tested	tested	tested
30	Yuda	M	tested	tested	tested	tested
31	Evi	F	tested	-	tested	tested

IV.3.1 SCN1A

SCN1A were chosen because it is involved in channelopathies, one of the basic pathogenesis of epilepsy. It is tested for all patients (males and females), it was chosen to be first tested due to its high prevalence that in the society. It is also a well known gene for FS, GEFS+, and SMEI[13].

First step was designed the primers of *SCN1A*. PCR product were run on the gel and got the results. If there are product on the gel, then the primer worked. Here is the example of the PCR product of the tested *SCN1A* primers. After purified the DNA then sequencing were performed.

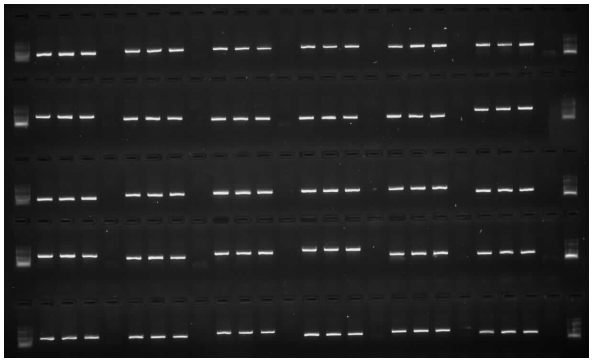

Figure 7. Results of *SCN1A* primers with three controls and 26 exons tested.

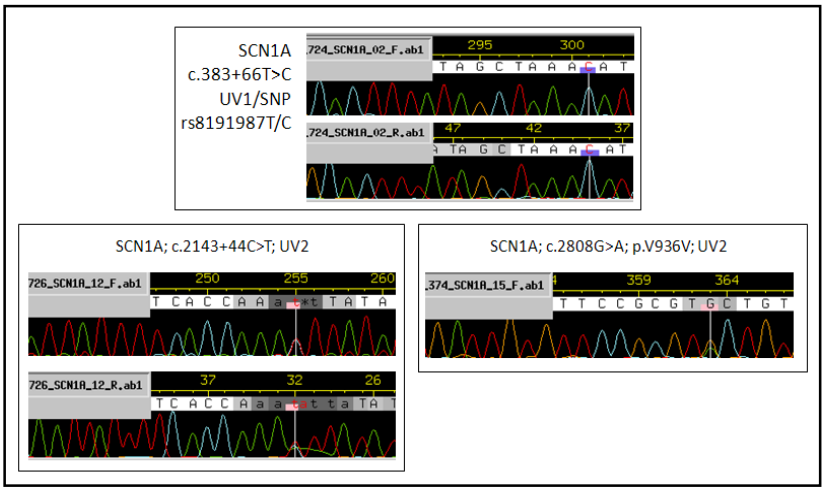

Figure 8. Some of the results of *SCN1A* sequencing.

Analyzing whether this results are real mutation or just SNPs were performed using Alamut software. Some of the results are new SNPs, it might be not recorded yet because mostly Javanese population were sequenced. After comparation from Alamut software that mostly came from Caucasians population, conclusion were drawned.

Table 6. SNPs Results from *SCN1A* screening examination

exon	Intron	SNPs	Protein	RS number	Total From 31	(%)	Caucassian Control (%)
	2	c.383+66 T/C		rs8191987 T/C	12	38,71	66,7
	5	c.603-106 G/T		rs3812719 G/T	2	6,45	
		c.603-91 G/A		rs3812718 G/A	11	35,48	
	6	c.964+116 A/T		rs6750294 A/T	9	29,03	66,7
		c.964+199 T/G		rs6706163 T/G	7	22,58	
	7	c.965-21 C/T		rs994399 C/T	3	9,68	33,3
		c.1028+21 T/C		rs1542484 T/C	13	41,93	
	8	c.1029-68 C/T		rs1461193 C/T	1	3,23	
		c.1170+75 C/A		rs11690962 C/A	7	22,58	33,3
		c.1170+112 C/T		rs11690959 C/T	4	12,9	
	9	c.1212 A/G		rs7580482 A/G	4	12,9	
		c.1377+52 G/A		rs6432861 G/A	4	12,9	
	11	c.1663-47 T/G		rs6753355 T/G	1	3,23	
	12	c.2143+44 C/T		New finding	2	6,45	
13		c.2259 T/C		rs6432860 T/C	5	16,13	33,3
15		c.2808 G/A	p.V936V	New finding	1	3,23	
		c.2913+56 A/G		rs2020318 A/G	4	12,9	
	16	c.3167 G/A		rs2298771 G/A	2	6,45	
	22	c.4305+74 C/T		rs4305294 C/T	3	9,68	
	23	c.4443+33 G/A		rs73969742 G/A	3	9,68	

Two unclassified varients in the intron 12 and exon 15 were found. These variants are believed to be rare nonpathogenic variants, since they did not lead to a change in the amino acid sequence of the protein. Therefore, they are believed not to explain MR and epilepsy from the patients list.

Splice site prediction programs already performed to those SNPs. Yet, no changes were found and proven influencing on gene splicing process.

IV.3.2 *ARX*

Located in Xp.22.13 makes only 19 male patients tested for this gene. It is chosen because of the characteristic of *ARX* that have the widest phenotypic spectrum [79]. The patients list was also not really specific in terms of dysmorphology. No mutation or SNPswere found in the *ARX* gene with sequencing.

IV.3.3 STXBP1

The third one is the *STXBP1* gene. The gene is tested for all patients. It is located in 9q34.1, and it is a good candidate gene for the patients list since Hamdan FF et al.2009 described a screening of this gene in idiopathic MR with epilepsy, and had positive results in 2 out of 95 patients[14]. After sequencing, one possible mutation were found in exon nine that can be seen in the picture below.

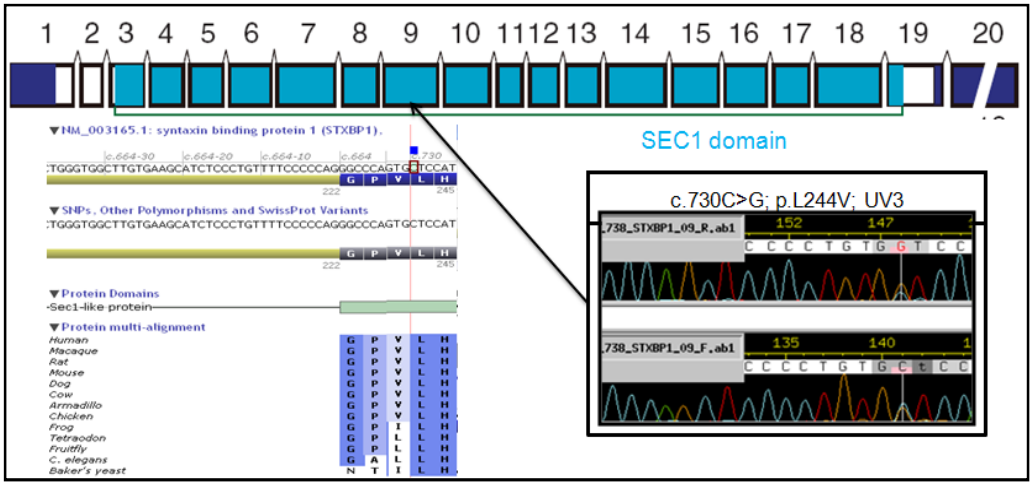

Figure 9. Results of *STXBP1* sequencing compared with Alamut software and pointed to the location of UV in exon nine.

From the mutation screening result, decission to predict pathogenicity of that mutation using prediction programs that based on amino acid changes were made. By using alamut conservation program, Leucine as highly conserved amino acid, also located in the sec1 domain, can be analyzed. Grantham distance between Leucine and Valine is 32. Not really big differences

if the comparation were resulted from the range of zero until 215. The conclussion was that it has no effect on splicing processin Alamut Splicing Predictions program.

In PolyPhen Prediction, this variant is predicted to be benign, probably because Valine and Leucine has the same type which is non polar. On the other hand, the SIFT prediction softwareshows that Valine is predicted to be not tolerated. The prediction is mainly based on the fact that Valine on this position in the protein is fully conserved in evolution. In Poly Phen, black colour means that protein is non polar, green: uncharged polar, red: basic, and blue: acidic. Seq rep is the fraction of sequences that contain one of the basic amino acid. Low seq rep means it has low fraction, it means also that the position severely gapped or unalignable or has a little information. Since the prediction software shows contradictory results, and the mutation concerns a highly conserved amino acid in an important domain of the protein. The hypothesis was that this might be the causative mutation in the the patient.

The clinical features of the patient that has this possible mutation is described as follows. He was examined when he was 11 years old and he originated from Bandung special school. His family history is normal, also prenatal, natal and perinatal history. No congenital anomalies. His developmental milestones was normal. No behavioral problems in this patient. From the measurement he has microcephaly and has prominent ears. He is severely mentally retarded, and has also clinodactyly on the fourth right toe. From the neurological examination,he had epilepsy with general partial type of the seizures since he was 6 months old.

Comparation ofthe clinical features of the patients with previous reported cases by Saitsu et al in 2008, who described four patients with a mutation in *STXBP1* were done [80, 81]. All of them are missense mutation, located separately in different exons (5, 7, 15, and 18). Three of

them are *de novo* mutation, and most likely causing loss of function. Possible pathogenic mutation in exon 9 that haven't reported before was confirmed by testing the parents.

Further mutation screening to confirm the mutation

The result in *STXBP1* gene (c.730C>G/ p.Leu244Val) need to be confirmed. This is where genetic counseling take the most important role.

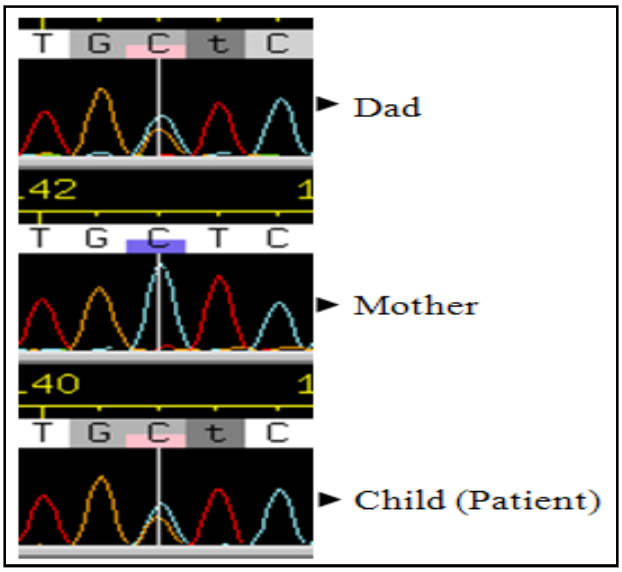

Figure 10. Result of verification

Thisvariant was also present in the healthy father of patient. That result proved that this UV is not the cause of the MR and epilepsy in this patient, because the healthy father also had the exactly same mutation.

No pathogenic mutation were found in *STXBP1* gene sequencing in thepatients list.

IV.3.4 *LGI1*

LGI1 or known as leucine-rich gene, gliomainactivated-1 gene is studied due to its relationship with Autosomal dominant lateral temporal lobe epilepsy (ADLTE). *LGI1* mutations is one of familial epileptic syndrome that characterized by the auditory ictal manifestation and

generalized seizures.Anypathogenic mutation in *LGI1* gene were did not found in sequencingofthe patients list.