A Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) Analysis of Connexin 26 (GJB2) Gene Common Mutation (235delC) In Indonesian Patients with Prelingual Nonsyndromic Sensorineural Hearing Loss: A Preliminary Study

Masyita Gaffar[*,1], Budu[2,5], Freddy G. Kuhuwael[3] and Irawan Yusuf[4,5]

[1]*Medical Research Unit, Medical Faculty, Hasanuddin University- Department of Otorhinolaryngology, Labuang Baji Hospital, Makassar, Indonesia*

[2]*Department of Ophthalmology, Medical Faculty, Hasanuddin University, Makassar, Indonesia*

[3]*Department of Otorhinolaryngology, Medical Faculty, Hasanuddin University, Makassar , Indonesia*

[4]*Department of Physiology, Medical Faculty, Hasanuddin University, Makassar, Indonesia*

[5]*Biotechnology Division Research Centre, Hasanuddin University, Makassar, Indonesia*

Abstract: *Background*: Inherited hearing impairment affects about 1 in 1000 newborns. Up to 50 percent of all patients with autosomal recessive nonsyndromic prelingual deafness in many populations have mutations in the gene encoding the gap junction protein connexin 26 (GJB2) at locus DFNB1 (autosomal recessive nonsyndromic deafness) on chromosome 13q11-12. In East Asia, there is a common mutation (235delC) of connexin 26 (GJB2) gene mutation, and a common GJB2 gene mutation (V37I) in Singapore and Malaysia with congenital deafness.

No connexin 26 gene study was done in Indonesia. In this preliminary study, we analyzed 40 Indonesian patients with prelingual nonsyndromic sensorineural hearing loss.

Objective: To detect the common frameshift mutation (235delC) of connexin 26 (GJB2) gene in Indonesian patients by using the PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) analysis.

Materials and Methods: Forty patients with prelingual nonsyndromic sensorineural hearing loss in Makassar Indonesia were identified and genomic DNAs were extracted from peripheral leukocyte blood of each subject. The important region for connexon-connexon interaction of GJB2 gene in the exon 2 was amplified by using PCR. RFLP analysis (ApaI enzyme) was performed to detect the 235delC mutation in all subjects.

Result: The target fragment (722bp) of exon 2 in 40 subjects of prelingual nonsyndromic sensorineural hearing loss were amplified by using PCR method. No 235delC mutation of connexin 26 (GJB2) gene was identified in our RFLP (ApaI enzyme) analysis.

Conclusion: No 235delC of connexin 26 (GJB2) gene mutation was found in our preliminary study in 40 Indonesian patients with prelingual nonsyndromic sensorineural hearing loss.

Keywords: Prelingual nonsyndromic sensorineural hearing loss, PCR-RFLP, connexin 26, 235delC mutation, Indonesia.

INTRODUCTION

Congenital deafness occurs approximately 1 in 1000 live births, of which 50% are hereditary [1].

Nonsyndromic sensorineural prelingual hearing loss is a genetic form of hearing impairment that accounts for at least 60% of cases, where the inner ear appears to be the only affected organ [1].

The molecular basis of deafness is growing rapidly with the identification of 13 nuclear and 2 mitochondrial genes involved in nonsyndromic hearing loss (NSHL) [2,3]. Over 100 genes may be involved in NSHL, but the most recent discovery has been the high incidence of mutations found in the gap junction protein, connexin 26 (GJB2). Connexin 26 (GJB2) is a member of a large family of proteins which forms gap junctions in virtually every cell type. This junction is composed of multimeric connexons, allowing molecules to pass from cell to cell. The connexons are composed of connexins, which vary in their gating properties and cell specificity [4].

The gene encoding gap-junction protein connexin 26 (GJB2), located on chromosome 13q11-12, is reported to have mutations up to 50% of all patients in different populations.

Although several different mutations were identified (35delG, 109G-A, 235delC, V37I, 167delT, etc), but 235delC

*Address correspondence to this author at the Medical Research Unit, Medical Faculty, Hasanuddin University- Department of Otorhinolaryngology, Labuang Baji Hospital, Makassar, Indonesia E-mail: sita-gaffar@lycos.com

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was found to be the most prevalent GJB2 mutation in East Asians populations [5-11].

Ruszymah *et al*. (2003) found that V37I was the commonest connexin 26 (GJB2) mutation in Malaysian patients with congenital deafness [12].

No molecular study of connexin 26 (GJB2) gene was reported in Indonesian patients with prelingual nonsyndromic sensorineural hearing loss.

Therefore, the purpose of this study is to investigate the 235delC mutation in GJB2 gene in Indonesian deafness patients.

MATERIALS AND METHODS

Forty patients with prelingual nonsyndromic sensorineural hearing loss were studied. The subjects were students from two special schools for deaf children in Makassar, South Sulawesi, Indonesia (Sekolah Luar Biasa B Pembina Parang Tambung Makassar and Sekolah Luar Biasa B Cendrawasih Makassar). The patients were from unrelated families with 9 to 24 years old. Most of them are originally from Makassar and Buginese ethnicities. Informed consent was obtained from each subject and from parents of under-aged patients. This study was approved by the Ethical Review Board of Faculty of Medicine Hasanuddin University Makassar.

Clinical Examinations

A detailed history was taken using an extensive and standardized questionnaire including family, natal, peri-natal and post natal histories to exclude environmental causes of deafness. A complete physical examinations were performed of each individual including: general, otorhinolaryngologic, audiologic to exclude syndromic, followed by hearing assessment using Pure Tone Audiometry. The inclusion criteria was the final findings of the examinations with prelingual nonsyndromic sensorineural hearing loss patients.

Audiology

Hearing of all patients was measured by Pure Tone Audiometry (Interacoustics AD 226). Categories of Degrees of Hearing Loss, based on Air Conduction Pure- Tone Average at 500, 1000, and 2000 Hz (Clarke 1981).

Degree of Hearing Loss Category Pure- Tone Average	Range
Normal hearing sensitivity	-10 dB HL to 15 dB HL
Slight hearing loss	16 dB HL to 25 dB HL
Mild hearing loss	26 dB HL to 40 dB HL
Moderate hearing loss	41 dB HL to 55 dB HL
Moderately severe hearing loss	56 dB HL to 70 dB HL
Severe hearing loss	71 dB HL to 90 dB HL
Profound hearing loss	91 dB HL to equipment limits

Molecular Study

Genomic DNAs were extracted from the peripheral venous blood of each subject. Exon 2 of connexin 26 (GJB2) gene was amplified by polymerase chain reaction (PCR) using 1 pair of primer: F (forward), 5-TCT TTT CCA GAG CAA ACC GC-3' and R (reverse), 5-GGG CAA TGC CTT AAA CTG GC-3'.

The amplification of exon 2 was performed using 100-200 µg of genomic DNAs in 20 µl mixture containing 10 mM Tris-HCl (pH 8,3), 50mM KCl, 1,5 mM MgCl2, 200 цM dNTPs and 1,25 U tag DNA Polymerase (Perkin Elmer, Norwalk, CT, USA) and 1-pmol of each primer. The PCR was performed by incubation at 94^{o}C for 15s follow by 35 cycles of 94 ^{o}C for 15 sec (denaturation), of 55^{o}C for 15sec (annealing) and 72^{o}C for 90sec (extension). The PCR products were digested with ApaI enzyme to search the connexin 26 (GJB2) variation (235delC). Wild type PCR product was cut by the enzyme and produce double bands in gel electrophoresis, while 235del C abolish the ApaI enzyme and produce only single band.

RESULT

We collected 40 patients with prelingual nonsyndromic sensorineural hearing loss.

All genomic DNA were amplified and target bands were clearly appear in all subjects (722bp). Incubation of PCR products with ApaI enzyme (Fig. **2**) showed double bands on each subject and represent that there is no 235delC mutation in all study subjects.

DISCUSSION

Mutations in the GJB2 gene encoding connexin 26 (Cx 26) are a major cause of autosomal recessive and sporadic cases of congenital deafness in most population. More than 90 variants of GJB2 gene have been reported, and many are rare. One variant generally predominates in any given population, such as the most common mutation, 35delG is found in over two-thirds of persons with autosomal recessive nonsyndromic hearing loss and has been reported in persons of Arab, Bedouin, Caucasian, Indian, Israeli, Italian, Pakistanian and Spanish ethnicities. In other populations, other mutations are more common, including the 167delT in Ashkenazi Jewish population [8-11, 13-15]. The 235delC mutation of GJB2 is the most frequent known mutation in some east Asian populations, with a carrier frequency of approximately 1%. 235delC polymorphism was most frequently observed in Japanese, Chinese and Asia populations [5, 6, 16, 17].

We expected that this polymorphism would also be frequently observed in Indonesian population. In order to study the origin of 235delC among east Asians, we analyzed single nucleotide polymorphisms (SNPs) in the coding region of GJB2 of exon 2 and flanking the 235delC mutation. However, of the 40 patients with prelingual nonsyndromic sensorineural hearing loss in our study, no 234delC mutation was identified. Absence of 235delC mutation in our study cannot be understood. As indicated in many studies in the GJB2 gene, there are population differences in the distribution of the various GJB2 alleles. Despite that different alleles predominate in different populations, there are relatively high carrier rate of GJB2 alleles in all describe populations [5, 6, 16, 17]. The other study looked for 35delG variants among 190 African Americans and found none. These studies indicated that 35delG is significantly less common among African American population than it is among the Caucasian population [18]. Likewise, the 235delC variant was not found in this study, which was a most frequently in Japanese,

Table 1. Patients Characteristics, Severity of Hearing Loss, and Result of PCR-RFLP Analysis

Subject	Age	Mean Age	Gender	Level of Hearing Loss		235delC Polymorphism
				Right	Left	
1	14	15,7	female	severe	moderately severe	-
2	15	15,7	female	severe	profound	-
3	15	15,7	female	severe	severe	-
4	15	15,7	female	severe	severe	-
5	15	15,7	female	profound	severe	-
6	14	15,7	female	profound	profound	-
7	16	15,7	female	severe	severe	-
8	18	15,7	female	moderately severe	moderately severe	-
9	14	15,7	female	profound	profound	-
10	14	15,7	female	profound	profound	-
11	24	15,7	female	profound	profound	-
12	15	15,7	female	profound	profound	-
13	20	15,7	female	profound	profound	-
14	18	15,7	female	profound	profound	-
15	18	15,7	female	profound	profound	-
16	13	15,7	female	profound	profound	-
17	9	15,7	female	profound	profound	-
18	19	17,3	male	profound	profound	-
19	12	17,3	male	profound	profound	-
20	12	17,3	male	profound	profound	-
21	15	17,3	male	severe	severe	-
22	19	17,3	male	profound	severe	-
23	14	17,3	male	severe	severe	-
24	20	17,3	male	moderately severe	severe	-
25	14	17,3	male	profound	profound	-
26	14	17,3	male	profound	profound	-
27	20	17,3	male	profound	profound	-
28	19	17,3	male	profound	profound	-
29	13	17,3	male	profound	profound	-
30	24	17,3	male	profound	profound	-
31	14	17,3	male	profound	profound	-
32	20	17,3	male	profound	profound	-
33	18	17,3	male	profound	profound	-
34	15	17,3	male	profound	profound	-
35	16	17,3	male	profound	profound	-
36	20	17,3	male	profound	profound	-
37	20	17,3	male	profound	moderately severe	-
38	28	17,3	male	profound	profound	-
39	16	17,3	male	profound	profound	-
40	16	17,3	male	profound	profound	-

Clinical examination showed all cases presented sensorineural and bilateral hearing loss.

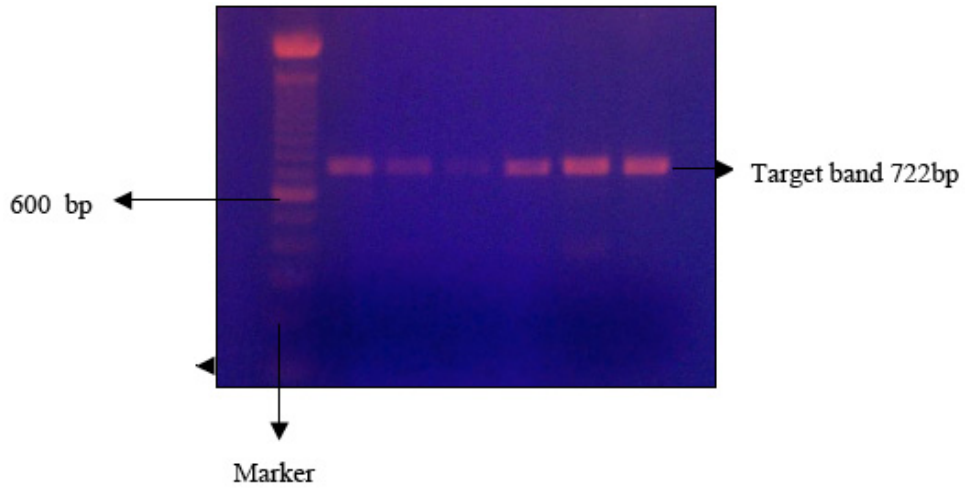

Fig. (1). Agarose gel patterns of amplificated target band (722 bp) of exon 2 in 6 of 40 patients enrolled in this study.

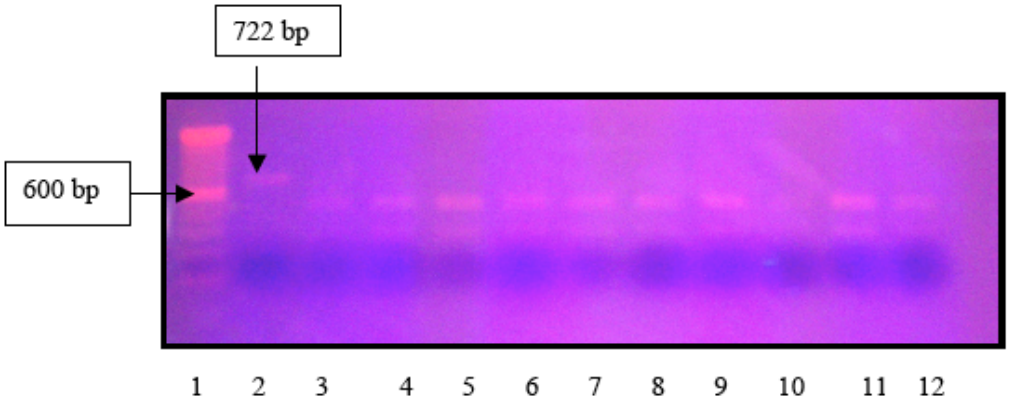

Fig. (2). Restriction enzyme analysis of PCR product by ApaI enzyme. Lane 1= DNA marker, lane 2= Single band of PCR product (control sample) with no enzyme (722 bp), and lane 3-12= double bands of wild type samples cutting by ApaI enzyme.

Chinese, Thailand and east Asian [5, 6, 16, 17]. The possible explanation for this data (1) the 235delC variant is not a cause of prelingual nonsyndromic sensorineural hearing loss in Indonesia, (2) the proportion of cases of prelingual nonsyndromic sensorineural hearing loss attributed to 235delC variant is lower in Indonesian than east Asian populations, and/ or (3) the others variation of connexin 26 (GJB2) than 235delC might be play a significant role in Indonesian population and we proposed to analyzed those variations with direct DNA sequencing. Interestingly, this result show a similar result with a study in Malaysia which did not find the 235delC variant [12]. It might be the 235delC rarely found in Malayan ethnic. It is thought that the ethnic backgrounds more close between Indonesian and Malaysian population than other Asian populations.

Some authors have also reported the possibility of other genes to involved in pathogenesis of prelingual nonsyndromic sensorineural hearing loss, such as GJB6, GJB3 [1]. Those also need to be explorated. To get more reliable results, it is proposed to increase the number of samples to participate in the future studies.

CONCLUSION

The 235delC mutation described in this study is not found in our 40 patients. The 235delC mutation might be rare in Indonesian patients with prelingual nonsyndromic sensorineural hearing loss.

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