A Gene for Autosomal Dominant Non-Syndromic Deafness In A Small Family In Malaysia Maps To Chromosome 2

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Abstract
Hearing loss is one of the most frequent sensory disorders in humans. Haplotype reconstruction is one of the methods used in linkage studies, which can be applied to search for the deaf genes in small families. New mutations will arise on a specific chromosomal haplotype, making it easier to track down the genes involved. Thus, haplotype-based methods can offer a powerful approach to disease gene mapping. We investigated a small family of five, consisting of two deaf parents and three deaf male children. As this family is too small to generate a significant lod score, a linkage study in this family was done through haplotyping. Forty one candidate loci for autosomal dominant deafness were tested for linkage in this family. We identified the locus responsible for deafness by using microsatellite markers (10 centiMorgans), then construct a haplotype data for each family member by using the genotypes data obtained. Through haplotyping, we found that the hearing loss is inherited maternally via autosomal dominant non-syndromic deafness. Also, through haplotyping, we found an interesting locus on chromosome 2 (2q32.5-q35) of 27.4 centiMorgans which might be the causative locus for deafness in this family. There are 23 candidate genes residing in the locus and the most probable is MYO1B gene. We have successfully mapped the locus for the autosomal dominant non-syndromic deafness in a small Malaysian family to 2q32.5-q35.

Keywords: Autosomal Dominant, Candidate Gene, Deafness, Haplotype, Locus, MYO1B

Introduction
Hearing impairment is commonly classified by audiometric criteria as conductive, sensorineural or mixed, and quantitatively graded as mild (26-40dB), moderate (41-55dB), moderately severe (56-70dB), severe (71-90dB) or profound (>90dB). In addition, it is described as congenital or late onset, and inherited or acquired. Inherited losses are further subclassified as syndromic or non-syndromic to reflect the presence or absence of co-inherited physical abnormalities [1].

The Department of Social Welfare reported that in 2003, there were only 22, 077 registered deaf people living in Malaysia [2]. We believed that the actual numbers is higher due to the fact that most deaf families in Malaysia still prefer to isolate themselves and live in remote areas. However, it is estimated that the ratio of deaf person is one in every 1, 046 Malaysian citizens. Approximately 50% of all deaf cases are thought to be due to environmental factors leaving another 50% due to genetic etiology with autosomal dominant, autosomal recessive, X-linked or mitochondrial patterns of inheritance [3].

The hearing organ is a complex structure that needs hundreds or maybe thousands of genes for the hearing mechanism to function normally. A mutation in any of these genes may lead to hearing impairment. Due to its heterogeneity, at least 82 loci for non-syndromic hearing impairment have been identified so far through linkage analysis : 41 autosomal dominant (DFNA1-51), 35 autosomal recessive (DFN1-39), 5 X-linked (DFN1-8) and 1 modifier loci (4). As of April 2004, only 41 auditory genes have been cloned which includes 15 genes for autosomal recessive, 14 genes for autosomal dominant, 6 genes that cause both autosomal recessive as well as autosomal dominant hearing loss and 1 gene for X-linked [4]. With more than 400 different syndromes that have hearing impairment as one of the clinical features, only 24 genes have been successfully identified so far. Some of the genes identified are overlapping with those of an autosomal recessive inheritance. For example, the GJB2 gene which is found in autosomal recessive cases is also found in autosomal dominant families [5].

In non-syndromic deafness, the genes that have been cloned and the proteins they encode have been shown to play a vital role in maintaining cellular functions such as ion channel proteins, transcription factors, extracellular matrix proteins, cytoskeletal proteins and synaptic vesicle trafficking proteins [6]. Each of these genes has been shown to be expressed in the cochlea, demonstrating the importance of knowledge of gene expression in the membranous labyrinth to our further understanding of hearing and deafness. Despite these recent successes in...
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discovering hearing loss genes, there are still many non-
syndromic hearing loss genes that remain to be discovered.
This is partially due to the fact that many families in
which non-syndromic hearing loss (NSHL) segregate are
small, with an insufficient number of informative
recombination events to allow narrowing of the genetic
interval to which an NSHL gene maps.

Haplotype reconstruction is a method which can be
applied to search for the deaf genes in small families.
Haplotype-based methods offer a powerful approach to
disease gene mapping, based on the association between
causal mutations and the ancestral haplotypes on which
they arose. Haplotype are groups of closely linked alleles
that tend to be inherited together. When a new mutation
arises, it does so on a specific chromosomal haplotype,
thus making it easier to track down the genes involved
[7].

Here we performed linkage analysis through haplotype
reconstruction of a family that segregates in an autosomal
dominant manner for congenital deafness.

Materials and Methods

Families, medical history and general examination

The Institutional Research and Ethical Committee of
Medical Faculty Universiti Kebangsaan Malaysia granted
approval for this study on human deafness in November
2001. A deaf family was identified through collaboration
with a local deaf society. The family consists of two deaf
parents and three equally deaf male siblings. Written
informed consent were obtained for all subjects in the
study. Histories were taken with special attention to any
possible causes of hearing loss and to other symptoms
potentially related to syndromic hearing loss. They were
required to undergo hearing assessments by using Pure
Tone Audiometry (adult) and Otoacoustic Emission (OAE)
for children.

Genomic DNA isolation

Peripheral blood samples were obtained from all
participating subjects. Lymphoprep (AXIS-SHIELD PoC
AS, Oslo, Norway) was used to separate the white blood
cells from the whole blood by using the protocols provided
by the manufacturer. The pelleted white blood cells was
then suspended in 10 ml of normal saline added with 1
ml of 10% sodium dodecyl-sulphate (SDS) and 1 ml of
10mg/ml Proteinase K. After an overnight incubation at
55°C, the mixture was taken out and the volume was
split into two conical tubes (6 ml each). Then, 3 ml of
supersaturated sodium chloride was added to each tube
and the mixture was then inverted carefully for 15 seconds
before being centrifuged at 4, 000 rpm for 15 minutes at
4°C. Later, the supernatant in each tube was added to a
conical tube containing 30 ml of absolute alcohol. After a
while, bubbles could be seen formed at the bottom of the
tube indicating the presence of DNA. After 15 to 30
minutes, DNA strands formed could be seen migrated to
the surface of the absolute alcohol and this DNA was
spooled out by using a micropipette tips and kept in a
screwed vial. Then, 500 µl of 70% alcohol was added to
the spooled DNA to obtain a purer nucleic acid. The
DNA was then left to dry off at room temperature before
being diluted with Tris-EDTA (pH 7.4) or distilled water
and later kept as stock at -20°C for further use.

Table 1: Program Used for GeneAmp PCR System 9700 Thermal Cycler

<table>
<thead>
<tr>
<th>Repetitions of Each Cycle</th>
<th>Cycle Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95°C for 12 minutes</td>
</tr>
<tr>
<td>10</td>
<td>Melt at 94°C for 15 seconds</td>
</tr>
<tr>
<td></td>
<td>Anneal at 55°C for 15 seconds</td>
</tr>
<tr>
<td></td>
<td>Extend at 72°C for 30 seconds</td>
</tr>
<tr>
<td>20</td>
<td>Melt at 89°C for 15 seconds</td>
</tr>
<tr>
<td></td>
<td>Anneal at 55°C for 15 seconds</td>
</tr>
<tr>
<td></td>
<td>Extend at 72°C for 30 seconds</td>
</tr>
<tr>
<td>1 HOLD</td>
<td>Final Extension at 72°C for 10 minutes</td>
</tr>
<tr>
<td></td>
<td>4°C (forever)</td>
</tr>
</tbody>
</table>
Biosystem, Foster City, CA, USA). After denaturation for two minutes at 95°C, the samples were then resolved on a 4.25 % polyacrylamide gel and were detected by using an ABI377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Sizes of marker alleles were defined by the use of GENESCAN software. Analyzed data were imported into the GENOTYPER software for allele-calling procedure to generate a genotyping table.

Haplotype reconstruction

Due to the insufficient number of family members to generate a statistically significant lod score, we decided to reconstruct the most likely haplotype blocks based on an interesting region covered by markers D2S117, D2S235, D2S2382 and D2S126, that we found on the q arm of chromosome 2 (Figure 1). Flanking markers were determined (D2S117 and D2S126) and within the candidate region we genotyped the family further for four markers from the locus using higher density microsatellite markers (5 cM apart); D2S2358, D2S2321, D2S2361 and D2S2382. Haplotypes were reconstructed for all participating subjects using all the 8 markers, and the chromosomal region that might contain the disease gene was traced through the existing pedigree.

Results

Medical history and general examination

Medical history investigation revealed subject I-1 as having acquired postlingual hearing loss due to viral infection at the age of seven. The rest of the family suffered from congenital hearing loss. With this information, we excluded I-1 as the affected haplotype provider and that the disease phenotype portrayed by II-1, II-2, and II-3 was inherited from I-2 (Figure 1). The diagram shows the region on chromosome 2 that could be the site of an autosomal dominant locus. It is hard to determine the mode of inheritance of deafness in this family that we were studying due to the fact that both parents and siblings of I-2 are not affected and if it were autonomic recessive, it would be more convincing to see I-2 having the same haplotype on both strands, or the best way is to examine markers that are more closely spaced than the previous markers (less than 1 cM apart). However, not being able to show homozygosity of a haplotype would give weight to the possibility that the disorder is dominant, and that individual I-2 might have sporadic germ-line mutations.

Audiological examination

Pure-tone audiometry was performed with air conduction at 250, 500 1000, 2000, 4000 and 8000 Hertz (Hz). Audiograms were only available for 3 family
From the audiogram results, it may be concluded that individual I-1 has high frequency, profound hearing loss with 15 dB hearing loss difference between the mean of 500 Hz and 1000 Hz and the mean of 4000 Hz and 8000 Hz (gentle slope). Individual I-2 has severe hearing loss with less than 15 dB difference between the mean of 250 and 500 Hz thresholds, the mean of 1000 and 2000 Hz and the mean of 4000 and 8000 Hz (flat audiogram). Individual II-3 also has high frequency, profound hearing loss with gently sloping audiograms. Whereas otoacoustic emission (OAE) results obtained for the other two younger family members showed that both II-4 and II-5 failed the OAE test which means both of them might have profound hearing loss. Audiograms and OAE test results obtained from all participants are simplified as in Table 2.

### Haplotype analysis

The exact location of the disease gene is not known, however the markers that flank the interval that contains these genes are known. The markers between these intervals were used to reconstruct haplotypes. Genetic analysis indicated that the hearing loss in this family segregated with eight polymorphic markers of the 2q32.3-gene 35 region where at least 23 candidate genes in the vicinity of 27.4 cM reside. The locus is flanked by markers D2S117 and D2S126. The list of candidate genes and its location are as shown in Figure 2. So far, there is no study reporting these genes to be the underlying cause of deafness though the function of each genes shows positive relations to the inner ear.

### Candidate gene analysis

In the vicinity of the disease locus interval, we have encountered more than 200 potential genes encoding expressed sequence tags, though half of them are with unknown protein products or functions [8]. To identify the candidate genes for human hearing disorders and to understand better human hearing at the molecular level, we referred to the Human Cochlear cDNA Library, an expression database developed from human fetal inner ear tissue constructed by Skvorak et al [3]. With the information, we narrowed the list of possible candidate genes to 23. The most likely genes to be responsible for causing deafness as seen in the family are MYO1B (OMIM : 606537), BMPR2 (OMIM : 600799) and IGFBP5 (OMIM : 146734) (Figure 2).

**Table 2: Audiometric Evaluation of Family AA**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Sex</th>
<th>Severity</th>
<th>Audiograms</th>
<th>Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-AA1</td>
<td>37</td>
<td>Male</td>
<td>Profound</td>
<td>High Frequency :</td>
<td>Gently Sloping</td>
</tr>
<tr>
<td>I-AA2</td>
<td>37</td>
<td>Female</td>
<td>Severe</td>
<td>High Frequency :</td>
<td>Flat</td>
</tr>
<tr>
<td>II-AA3</td>
<td>10</td>
<td>Male</td>
<td>Profound</td>
<td>High Frequency :</td>
<td>Gently Sloping</td>
</tr>
<tr>
<td>II-AA4</td>
<td>7</td>
<td>Male</td>
<td>Failed OAE</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>II-AA5</td>
<td>5</td>
<td>Male</td>
<td>Failed OAE</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2:** The diagram shows the region of interest on the q arm of chromosome 2 of 27.4 cM area in which lies the 3 most probable candidate genes. Also shown is two of the previously reported locus on the q arm of chromosome 2; designated as DFNA16 and DFNB27 which are not overlap with the locus found in this study.

### Myosin

Myosins are divided into subclasses on the basis of comparisons of motor domains and tails. There are two types of myosin; conventional myosin is of class II and other 13 classes (I, III-XIV) are unconventional myosin. Myosins are a family of actin-based molecular motors that use energy from hydrolysis of ATP to generate mechanical force. The classic, two-headed filament-forming myosins that provide the basis for muscle contraction are referred to as conventional myosins. The other member of the myosin super family which is...
unconventional myosin, has functions that are less well understood but in some cases are thought to mediate intracellular trafficking events [9], where one or several myosins are presumably responsible for modulating the tip-link tension that controls the gating of cation-transduction channels [10]. There are several myosin genes that were found to be the cause of deafness. Mutations in myosins are important causes of deafness. This has been proven with the discovery of 4 different types of myosin in non-syndromic hearing loss; MYO7A (both in autosomal dominant and recessive cases), MYO15 and MYO3A (autosomal recessive), MYO6 (autosomal dominant). The first gene to affect the sensory hair cells directly also encoded an unconventional myosin, Myosin VIIA, in the shaker-1 mouse mutant and Usher Syndrome Type 1B (USH1B) [11]. A mutation in myosin VIIA has also been found in a Japanese family with autosomal dominant non-syndromic progressive hearing loss (DFNA11) [12]. The identification was followed rapidly by the discovery of the second myosin, Myosin VI, as responsible for the deafness in the Snell’s Waltzer mouse mutant [4].

Recently, one myosin gene from class I (MYO1A) was discovered to be responsible for autosomal dominant non-syndromic deafness in a large Italian family [13]. With a maximum lod score of 3.31, the novel locus, which is designated as DFNA48 was mapped to 2q13-q14. This study has proven that Myosin Class I is another class of myosin to be considered as an important causal gene for deafness. Isozymes of this class have been implicated in various motile processes, including organelle translocation, ion-channel gating, and cytoskeleton reorganization [14]. MYO1B is the favored candidate for the role of adjusting the tension on the tip link of the sensory hair cells [15]. Myosin 1B is an unconventional myosin which is widely distributed in many tissues and is highly expressed in the inner ear sensory epithelial [16], thus making it the most likely candidate gene to be responsible in causing deafness in this small Malaysian family.

**IGFBP-5**

Though IGFBP-5 is expressed in the cochlea, the role of this gene in causing deafness remains unclear. However, recent studies using insulin-like growth factor I (IGF-I) knockout mice demonstrated that IGF-binding protein (IGFBP)-5, an important bone formation regulator, is a growth factor with cellular effects not dependent on IGFs [17]. If this is true, then, IGF-binding protein might also have the same role of IGF-I in modulating inner ear proliferation, differentiation and survival [18]. With that, IGFBP5 might also be a candidate gene in causing deafness in the family that we are studying.

**BMPR2**

This gene encodes a member of the bone morphogenetic protein (BMP) receptor family of transmembrane serine/threonine kinases. BMPs are involved in endochondral bone formation and embryogenesis. BMPs act on osteoblasts and chondrocytes as well as other cell types, including neurocells, and they play important roles in embryonic development. Mutations in this gene have been associated with primary pulmonary hypertension [19]. The role of this gene in causing hearing loss is unknown.

**Discussion**

In usual practice, candidate gene approach is always followed by positional cloning strategy. However, in this study, the defined chromosomal candidate gene interval was too large for us to undertake the search for the corresponding gene exclusively by positional cloning. Thus, a candidate gene approach based on the isolation of genes specifically or preferentially expressed in the inner ear had to be done, as the proteins encoded by these genes are likely to have a role in auditory function [20]. This approach, the selection of a gene for in-depth analysis based on its chromosomal location and purported function, is known as the ‘positional candidate gene approach to gene identification [21].

In identifying the gene responsible for causing hearing impairment in this family, we have encountered several major setbacks. First of all, none of the markers listed in the 41 autosomal dominant loci for deafness gave positive results for disease linkage, instead, the chromosomal interval that possibly contained the gene of interest is situated near the DFNA16 locus (2q22.3-2q24.3) which is 2q32.5-q35. This further strengthened the fact that hearing impairment is highly heterogenous, with many genes and different mutations involved in causing a similar phenotype. Even routine molecular diagnosis with known deaf genes might be stressful because the genes involved are sometimes large with many exons and no mutational hotspots [22]. Furthermore, different regions and different origins often display different mutations. Unlike cases of migration histories, mutations found in deaf people are the same as those of their ancestral land. For example, the GJB2 gene where 35delG is the commonest mutation is only found in those of Caucasian ancestry [23].

A thorough clinical investigation has to be conducted and must be included in routine screening. Patients who participate in the study have to undergo a complete medical check up which includes vestibular studies, CT scans, heart disease studies (e.g.: electrocardiogram, thyroid and renal function tests, visual function tests and dermatological observations) [24]. Most of the tests mentioned are biochemical and radiological tests which cannot be detected during a physical check-up and clinicians cannot rely fully on verbal investigation. Even those who are under investigation might be unaware of the status of their body functions because some of the syndromes which are related to deafness are usually progressive in nature.
Other than clinical studies, medical and family histories are equally important. In this case, like I-1, I-2 also has a negative family history of hearing impairment. But unlike I-1, I-2 did not acquire hearing loss but instead is congenitally deaf. I-2 did mention that her mother had a high fever during the gestational period. However, that was the only information that she could give us during the interview session. Insufficient information regarding the treatment of the high fever, has hampered our understanding of the cause of deafness. Information on the antibiotics treatment taken and the stage of pregnancy when the incidence occurred are very important, because, antibiotics like aminoglycoside are ototoxic drugs which have great potential in causing mitochondrial mutations. This type of mutation is usually maternally inherited and appears as non-syndromic in many patients. This might be the case of the family that we are studying. In narrowing down the list of candidate genes in the linked chromosomal interval, information regarding the stage of pregnancy might help to identify the genes involved in embryonic development.

Most of the families asking for genetic diagnosis are small in number, with only one or two affected members, which makes linkage analysis inconclusive. Their extended families are usually not interested to join in the research because they are afraid of the outcome, for example, the news that they are carrying the diseased gene and they might pass it on to their next generation. This is particularly true in hearing impairment with autosomal recessive inheritance. That is why mapping gene loci responsible for deafness remain a challenge. Most non-syndromic hearing loss is recessive and single families of a size suitable for conventional linkage analysis are uncommon. Small families of non-consanguineous unions contain insufficient information to determine gene location, and pooling of multiple families is precluded by the inability to reliably subclassify non-syndromic hearing loss by audiometric criteria [25]. Most researchers favor analysis of large and highly consanguineous families affected by deafness and living in geographically isolated region for several generations, as this minimize the risk of several deafness genes segregating within the same family. This is because the major pitfall of family linkage studies is that by including several families, there is a risk that not all problems are due to mutations at the same genetic locus and statistically significant results about gene localization may be obscured. To a certain degree, careful selection of families for entry into the study and well-defined clinical criteria help overcome this hurdle and this is particularly true of syndromic conditions.

As the linked chromosomal region contains many genes, identification of the specific disease causing gene can be difficult and time consuming. Prioritizing genes in the linked interval for further studies is easier if complete clinical studies, medical and family history are obtained. Hopefully, identification of the diseased gene should further improve our understanding of one type of non-syndromic hearing loss and may offer future opportunities for counseling as well as intervention with rational therapy to mitigate further hearing loss.

Conclusion

In conclusion, the candidate gene approach to determining the disease-causing gene for specific hereditary conditions is only one method amongst many. However, selection of disease causing genes for mutation screening based on chromosome location, protein expression and functions will become an even more important approach especially for a small affected family. Although we did not do the mutational screening using markers of all the candidate genes located in the chromosomal interval that we found, we believe that MYO1B is the most possible candidate gene in causing autosomal dominant non-syndromic deafness in this family.

Acknowledgement

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