RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) AND CONNEXIN-26 GENE SEQUENCE ANALYSIS OF NON-SYNDROMIC HEREDOFAMILIAL HEARING LOSS

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Hearing loss is a relatively common defect of sensorineural function affecting millions of people allover the world. It is estimated that at least 20% of the population develop clinically significant hearing loss at some time during their lives (Stern *et al*., 2002).

Hearing loss is a multifactorial disorder resulting from both genetic and environmental factors. Single gene mutation can lead to hearing loss which might be monogenic with an autosomal recessive, autosomal dominant, X-linked recessive, or manifesting a mitochondrial mode of inheritance. Such monogenic forms could be syndromic (characterized by hearing loss in combination with other abnormalities) or non-syndromic (with only hearing loss) (Flex *et al.*, 2003).

There are about 90 to 100 genes for non-syndromic sensorineural hearing loss, of which 65 loci have been localized [33 autosomal dominant loci (DFNA), 27 autosomal recessive loci (DFNB) and five X-linked loci (DFN)] (Camp and Smith, 2003).

Among different known genes, connexin-26 gene mutations are responsible for half of all hereditary hearing loss in many countries. Connexin-26 gene (GJB2) is one of a family of genes encoding the polypeptide components of gap junctions, which are macromolecular integral-membrane complexes that allow passive diffusion of water and small solutes between adjacent cells. Connexin-26 encodes the gap junction B-2 polypeptide, which is expressed within discrete regions in the cochlea (Goodenough *et al.*, 1996). Connexin-26 gene is located at the chromosomal location 13q11. The GJB2 gene is small, with the entire coding region of 680 base pairs falling within exon 2 (Kenneson *et al.*, 2002). A single known mutation, namely 35 delG, is the most common mutation in connexin-26 gene.

Hearing loss, that occurs before the development of speech (prelingual onset) hamper speech acquisition as well as normal communication and social integration. Early detection is therefore essential for the application of palliative treatment and special education. Apparently, genetic diagnosis and counseling are increasingly demanded (Morell *et al.*, 1998).

A remarkable progress has been

recently achieved in molecular examination, and gene mutations can be easily detected by gene amplification and sequencing. Identification of these mutations and their effects on hearing process may lead to the development of mutation specific therapy that can delay or prevent the development of hearing loss in the future.

The objectives of this study were: (1) To identify the banding patterns resulting from Randomly Amplified Polymorphic DNA (RAPD) analysis, in order to determine specific bands that characterize genes affecting hearing. (2) To detect any mutations in connexin-26 gene resulting in heredofamilial hearing loss.

MATERIALS AND METHODS

Fifteen patients and seven healthy relatives were subjected to RAPD-PCR analysis. Each patient had to fulfill the following criteria: (a) selected patients were of both genders and their ages ranged from 6 months to 32 years old. (b) patients had more than one affected member in the family, or sporadic cases born to consanguineous marriages. (c) no history or manifestations suggestive of environmental causes of hearing loss. (d) no other manifestations suggestive of syndromic hearing loss. All study subjects were subjected to full history, complete general examination and audiological evaluation included otoscopic examination, acoustic immittance measurements, pure tone audiometry, and Acoustic Brain Stem Response (ABR). Audiological evaluation showed that hearing loss was severe to profound (72% of patients), symmetrical (84% of patients), and with had sloping configuration of audiogram (72% of patients).

Total genomic DNA was extracted from blood samples using a modification of Helms method (Helms, 1990). RAPD-PCR analysis was done using five random primers: B01 (5´-GTTTCGCTCC-3'), B05 (5´-TGCGCCCTTC- 3`), B06 (5´-TGCTCTGCCC-3`), B07 (5´-GGTGACGCAG-3`) and B09 (5´ -TGGGGGACTC-3`). RAPD-PCR profiles were analyzed using Gene profiler 3.1 software. The banding profiles were scored in binary manner (1 and 0), where (1) indicates band presence, while (0) indicates band absence. The scored binary profiles were introduced to SPSS statistical software system to estimate similarity and genetic distances of the subjects. A dendrogram was generated by cluster analysis (UPGMA) with the RAPD data.

Eleven patients were subjected to connexin-26 gene sequencing. Amplification of connexin-26 gene (exon 2) was carried out using primers for two interference regions: CX26-ex 2.1 (forward: 5´-CCTATGACAAACTAAGTTGGTTC-3` and reverse: 5´-GACACGAAGATCAGCTGCAG-3`) and CX26-ex 2.2 (forward: 5´-CCAGGCTGCAAGAACGTGTG-3' and reverse: 5´-GACAGCTGAGCACGGGTTGCCTC-3`). Then DNA sequencing reactions were done based on the enzymatic method of Sanger *et al.* (1977), using the previous forward primers. Sequence reactions were done of 25 cycles as: 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for

four minutes.

RESULTS

By analysis of RAPD-DNA band profiles from 22 individuals studied (15 patients and 7 relatives), all primers were amplified successfully. The five primers did not react with DNA of all individuals, as individual number 11 (patient) and 93 (relative) (Fig. 1).

The number of amplified fragments per primer varied between two (primer B05) and ten (primer B09), with an average 7.2 bands per primer, with total number of 397 bands were amplified (281 bands in patients and 116 bands in relatives). Only one band (485 bp) being monomorphic (shared by all examined individuals), and the other 35 bands were polymorphic with a polymorphism ratio of 97.3%. Bands 965, 950, 730 and 80 bp were absent in all seven relatives, while present in some of the affected patients. These bands can be used as diagnostic bands, as their presence can identify patients with non-syndromic heredofamilial hearing loss. On the other hand, bands 440, 310, 205 and 175 bp were present in all seven healthy relatives, but absent in some patients (band 175 bp was absent in one patient, and bands 440, 310 and 205 bp were absent in two patients). These bands could have a diagnostic value, as their presence suggested non-affection.

The dendrogram, that followed RAPD analysis, classified patients into three groups (Fig. 2). The first group contains one subject, the second compromises three subjects, and the third includes ten subjects suggesting the presence of three possible mutations responsible for this type of hearing loss.

Gene sequencing of connexin-26 revealed mutation in only one patient, while the other examined ten patients revealed no mutations. Analysis of connexin-26 gene sequence of the patient with mutant gene, revealed one frame shift mutation, 35 delG (a deletion of a guanine base in a stretch of six guanines beginning at position 30 and ending at position 35) (Fig. 3). This type of mutation was reported to be the commonest mutation (76%) in connexin-26 gene.

DISCUSSION

Several different forms of non-syndromic hearing loss were discriminated on the basis of detailed history and audiometric findings, the validity of these results as a guide to recognize genotypes remained unproven (Salvinelli *et al.*, 2004; Camp and Smith, 2003; Marazita *et al.*, 1993; Bragagnolo and Brunoni, 2000; Arnos *et al.*, 2002; Nekahm *et al.*, 2000).

The present study is the first attempt that provides information about the prevalence of connexin-26 gene mutations and DNA molecular differences in Upper Egypt. Sequencing analysis of connexin-26 gene in Lower Egypt revealed that 35delG mutation was present in 10.8% of examined subjects (Snoeckx *et al.*, 2005). Morell *et al.* (1998) suggested that the 35 delG mutation was recurrent and occurred at a mutation hot

spot, so they advised for screening of this mutation in all suspected subjects.

After sequencing of (5') end of ex.2 in eleven patients, mutation were detected in only one subject (9%), while no mutations in this gene were detected in the other ten subjects. This is significantly different from connexin-26 gene mutations found in other ethnic groups (59% in Italy and Spain, 50% in USA, 61.3% in Israel, 43% in Lebanon, 35% in China, 40% in Norway, and 53% in Japan) (Denoyelle *et al.*, 2001; Mustapha *et al.*, 2001). However, the reduction of the Connexin-26 mutation observed in the present study may be due to the small size of the sample examined. The type of mutation found in connexin-26 gene reveals one frame shift mutation, 35 delG, which reported to be the commonest mutation (76%) in connexin-26 gene (Frei *et al.*, 2002).

Results of this study supports the theory of extraordinary genetic heterogeneity of non syndromic hearing loss reported by Kenneson *et al.* (2002), who suggested that mutations rather than connexin-26 mutations were mostly responsible for hearing loss. These different findings suggest that population differences existed between Egyptians and other populations in the distribution of genetic mutations.

To support the theory of genetic heterogeneity in non-syndromic hearing loss, RAPD-PCR was performed using five random primers. After estimation of genetic similarity, cluster analysis, and establishment of dendrogram, subjects were classified into three distinct groups. Therefore, three mutations are probably responsible for non-syndromic hearing loss in examined subjects, which was supported by the observed low incidence (9%) of connexin-26 gene mutations. Complete analysis of the resulted bands revealed further interesting findings. Band 485 bp was the only band existing in all examined patients and relatives, while the other 35 bands are polymorphic. Moreover, bands 965, 950, 730 and 80 bp were absent in all seven relatives, but present in some of the affected patients. These bands can be used as diagnostic bands.

So, RAPD-PCR can be used as a diagnostic tool for patients with non-syndromic heredofamilial hearing loss. It is simple, easy, rapid and cheap method for diagnosis of affected individuals.

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SUMMARY

Hearing loss is a multifactorial disorder resulting from both genetic and environmental factors. About 90 to 100 genes were identified for non-syndromic heredofamilial sensorineural hearing loss. Among different known genes, Connexin-

26 gene mutations are reported to be responsible for half of all hereditary hearing loss in many countries. A single known mutation, 35 delG, is the most common mutation in Connexin-26 gene. The present study aimed to identify the patterns resulting from Randomly Amplified Polymorphic DNA (RAPD) analysis, to search for specific band(s) that characterize genes affecting hearing, and to detect mutations in Connexin-26 gene resulting in heredofamilial hearing loss.

DNA isolation was carried out for fifteen patients and seven healthy relatives and subjected to RAPD-PCR analysis. Eleven patients were subjected to Connexin-26 gene sequencing. Gene sequencing of Connexin-26 reveals mutation in only one patient, which, suggests that mutations other than Connexin-26 genes may be implicated in non-syndromic heredofamilial hearing loss in Upper Egypt. The dendrogram, that followed RAPD analysis, classified patients into three groups, suggesting the presence of three possible mutations responsible for this type of hearing loss. Complete analysis of the obtained bands revealed that band 485 bp is the only one existing in all examined patients and relatives, while the other 35 bands are polymorphic. Some bands (965, 950, 730 and 80 bp) are absent in all seven relatives, but present in some of the affected patients. These bands can be used as diagnostic bands, as their presence can identify patients with non-syndromic heredofamilial hearing loss.

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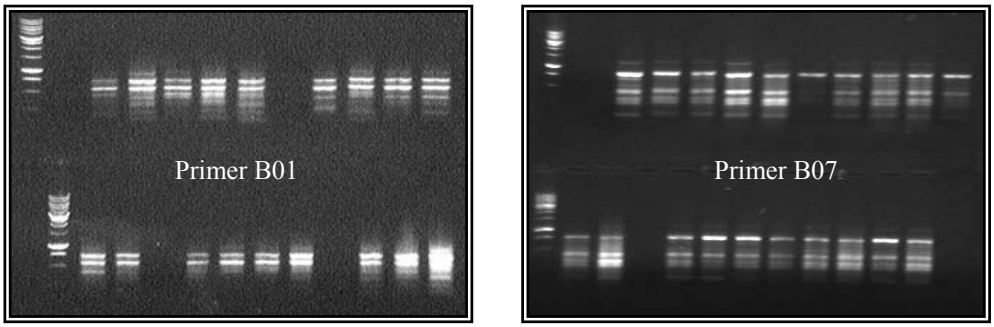

Fig. (1): RAPD-DNA band profiles from 22 individuals studied.

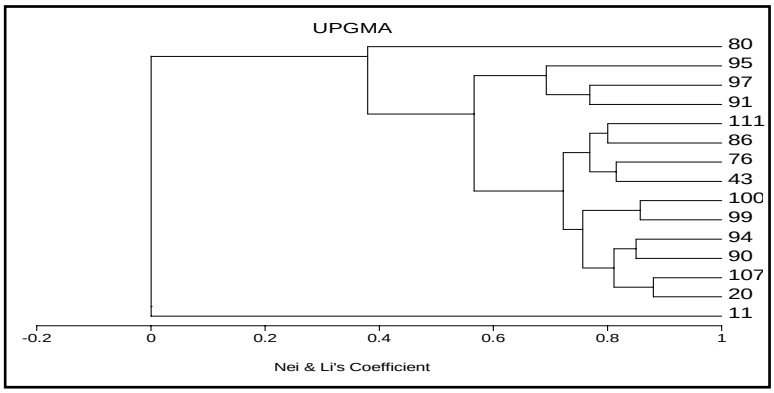

Fig. (2): UPGMA dendrogram of the fifteen patients based on values of genetic distances calculated from data of all five primers.

(Patient 113):

TGNCTTTNCCTCGTTTTGGTGAGGTTGTGTAAGAGTTGGTGTTTGCTCAGGAAGA
GATTTAAGCATGCTTGCTTACCCAGACTCAGAGAAGTCTCCCTGTTCTGTCCTAGC
TAGTGATTCCTGTGTTGTGTGCATTCGTCTTTTCCAGAGCAAACCGCCCAGAGTA
GAAGATGGATTGGGGCACGCTGCAGACGATCCT**GGGGG**TGTGAACAAACACTCC
ACCAGCATTGGAAAGATCTGGCTCACCGTCCTCTTCATTTTTCGCATTATGATCCT
CGTTGTGGCTGCAAAGGAGGTGTGGGGAGATGAGCAGGCCGACTTTGTCTGCAA
CACCCTGCAGCCAGGCTGCAAGAACGTGTGCTACGATCACTACTTCCCCATCTNC
CACATCCGGCTATGGGCCCTGCAGCTGATCTTCGTGTNAA

Fig. (3): Gene sequence of the mutant Connexin-26 revealed 35 delG mutation.