Identification of an ancestral haplotype of the 35delG mutation in the GJB2 (connexin 26) gene responsible for autosomal recessive non-syndromic hearing loss in families from the Eastern Black Sea region in Turkey

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Mutations in the GJB2 gene have been shown to be the major cause of autosomal recessively inherited, prelingual, non-syndromic hearing loss. 35delG was found to be the most frequent mutation among Caucasians. In this study, we performed haplotype analysis of two large families with autosomal recessive non-syndromic hearing loss (totally 33 affected, 37 unaffected) from Trabzon (a city from the Eastern Black Sea region) by using polymorphic markers close to the 35delG mutation region, and identified a common haplotype, "2-6-4". The frequency of the mutant chromosomes having the 2-6-4 haplotype was compared between the Eastern Black Sea region and the other regions of Turkey and the difference was found to be significant (χ^2=5.13/df=1/p=0.023). Also, when the frequency of mutant and wild type chromosomes having the 2-6-4 haplotype was compared in the Eastern Black Sea region, a statistically significant difference was observed in the mutant chromosomes (χ^2=7.46/df=1/p≤0.01). The results of this study demonstrate that the ancestral haplotype of the chromosomes bearing 35delG mutation in the Eastern Black Sea region is "2-6-4".

Key words: prelingual, non-syndromic hearing loss, GJB2, 35delG, ancestral haplotype.

Congenital deafness occurs in approximately 1 in 1,000 live births, and 60% of these cases are hereditary[1,2]. Autosomal recessive non-syndromic hearing loss (ARNSHL) is the most common form of genetic hearing loss, accounting for about 80% of cases[3,4]. In 1991, etiological investigations of children with a high level of sensorineural hearing loss were performed in Turkey and it was accepted that the most important mode of inheritance is the autosomal recessive transmission[5]. Over 30 ARNSHL loci (named DFNB) have been described to date, and 15 genes have been identified (Van Camp G, Smith R. Hereditary Hearing Loss Homepage. World Wide Web URL: http://dnalab-www.uia.ac.be/dnalab/hhl). The GJB2 gene, which encodes the connexin 26 protein, is the first to be associated with DFNB (called as DFNB1). Connexin 26 is a protein component of the gap junctions connecting adjacent cells, allowing small molecules and ions to pass from one cell to the next. Mutations in the *GJB2* gene are responsible for ~50% of all cases of childhood prelingual ARNSHL, the most common of which is 35delG (also referred to as 30delG)[6-9].

It has been determined that more than 50% of autosomal recessive non-syndromic hearing impairments result from the 35delG mutation in Northern and Southern European and American Caucasian populations[10].

According to preliminary studies, high carrier frequency values have been observed in some populations and this indicated that 35delG, which is accepted as a recurrent mutation among populations, occurs in the hot spot region of the GJB2 gene[4,11-13]. After the observation of haplotype diversity in a wide region of 2cM flanking the mutation, different origins of the 35delG were suggested[14].

However, later studies showed that population specific mutations (167delT in Ashkenazi Jews, R143W in Africans and 235delC in Japanese and Koreans, etc.) do occur in the Cx26 gene, which suggests different founding effects. Also in some populations, including African-Americans, Asian Americans and Egyptians, the 35delG mutation could not be determined[14-16]. Different carrier frequencies of 35delG among geographically, ethnically and culturally different populations raised the idea that this mutation is a result of a founder effect[9].

The 35delG mutation in the GJB2 gene is a common cause of prelingual deafness also in Turkey[17]. Initial studies have reported the high frequency of 35delG in Turkey as ranging from 1.0% to 1.8%[17,18]. Although the carrier rate of the 35delG mutation in Turkey is much lower than described for other Mediterranean countries, mutations in other gap- and tight-junction proteins are not a frequent cause of hearing loss[19]. Analyses of two pedigrees from Turkey demonstrated both conserved and different haplotypes, suggesting possible founder effects and multiple origins of the 35delG[17].

Throughout early prehistoric times, Turkey has experienced many migrations of racially, ethnically and culturally distinct populations. When the geographical position is considered, Turkey is within different migration routes. If 35delG is supposed to have originated from a common ancestor, there should be founder haplotypes for the populations.

We have identified two large families with autosomal recessive non-syndromic hearing loss (totally 33 affected, 37 unaffected) from the Eastern Black Sea region having at least three generation origins from the same geographical region. We performed haplotype analysis in these two inbred families and identified the "2-6-4" common haplotype associated with the 35delG mutation. The aim of this study was to determine if the 2-6-4 is an ancestral haplotype for the 35delG mutation in the Eastern Black Sea region.

Material and Methods

Patients

Clinical, audiological and impedance-based examinations revealed no complaints other than hearing impairment among our patients. One hundred and fourteen patients having prelingual nonsyndromic hearing impairment and 106 unrelated control subjects with normal hearing were screened for the 35delG mutation, and haplotype analysis was performed in 94 individuals. Written informed consent was obtained from all participants and from parents of those aged younger than 18 years. The Ethical Committee of Hacettepe University approved the study protocol. The geneology of the patients was questioned for three generations.

35delG Mutation Analysis

Genomic DNA was extracted from peripheral blood according to a standard protocol[20]. The 102 bp region covering the 35delG mutation was amplified by polymerase chain reaction (PCR) and screened by single strand conformational polymorphism (SSCP). Primer sequences were: F: 5' CAT TCG TCT TTT CCA GAG CA 3' and R: 5' TTC CAA TGC TGG TGG AGT G 3'. PCR conditions were 5 min denaturation at 94°C followed by 35 cycles with 30 sec denaturation at 94°C, 1 min annealing at 58°C and 1 min elongation at 72°C. A final extension step was applied for 5 min at 72°C. PCR products were analyzed by polyacrylamide gel electrophoresis (PAGE) (8% non-denaturated gel, 250V, 10mA, 17 hours) and stained with silver nitrate. In each gel, sequence-tested 35delG homozygous/ heterozygous and negative DNA samples were used as controls (Fig. 1).

Haplotype Analysis

Polymorphic markers D13S141, D13S175 and D13S143 were used to perform haplotype analysis. The distances of polymorphic markers to 35delG mutation region were 39.204bp, 84.718bp, and 1.510.295bp, respectively. The alleles of the patients were numerated after comparison with CEPH control DNA (1347-02) and evaluated all together to form the haplotypes.

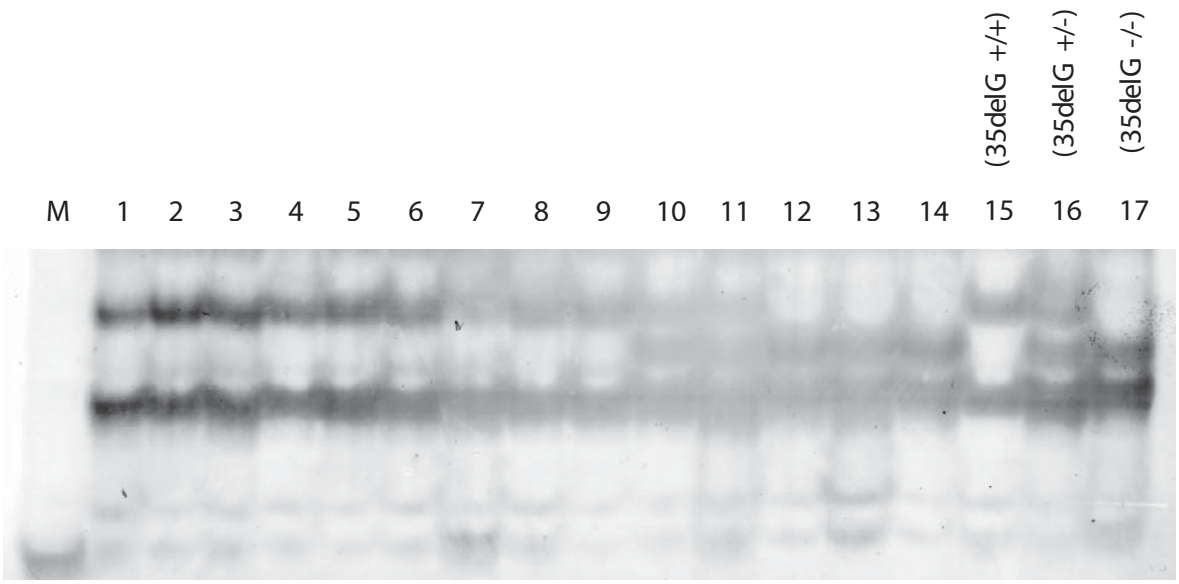

Fig. 1. Single strand conformational polymorphism (SSCP) analysis of the 102 bp region covering 35delG mutation.
M → Molecular weight marker (□x174/*Hae* III)
1, 2, 3, 4, 5, 6, 7, 8, 9 → individuals homozygous for 35delG mutation (35delG+/+)
10, 11 → individuals heterozygous for 35delG mutation (35delG+/-)
12, 13, 14 → individuals not bearing 35delG mutation in either of the alleles (35delG-/-)
15, 16, 17 → Control individuals
(8% nondenaturated polyacrylamide gel, 250V, 10mA, 17 hours)

Statistical Analyses (χ^2 test)

Haplotype frequencies in the Eastern Black Sea region and other regions of Turkey were compared using the χ^2 test (2x2) (STATS Statistics Program, Version 1.1, 1998, Decision Analyst, Inc.).

Results

Hearing loss was inherited in an autosomal recessive mode in two inbred families from Trabzon, which is in the Eastern Black Sea region. Since the first family was very large it was separated into five branches to facilitate the study (Fig. 2a).

Firstly, healthy and affected individuals in these two families were screened for the 35delG mutation. 35delG was observed in homozygous state in all patients (Fig. 2a and 2b) except in one patient in the second family (Fig. 2b/no 12).

Secondly, haplotype analysis was performed. As shown in Figures 2a and 2b, all mutant chromosomes in these two families have the 2-6-4 common haplotype, including four individuals in the first family (Fig. 2a/no 7, mother of no 52, father of no 52 and mother of no 53-55) and two individuals in the second family (Fig. 2b/ mother of no 6-8 and mother of no 3-5). These six individuals are the only non-related individuals who married into the family.

In order to understand whether the 2-6-4 is a founder haplotype for the Eastern Black Sea region, we performed haplotype analysis in eight non-consanguineous families from the same region, where the probands were homozygous for the 35delG mutation (Table I). Two mutant chromosomes having the 2-6-4 haplotype were determined in these patients. The results of these eight families with autosomal recessive non-syndromic hearing loss were evaluated together with the results of the two large families, and the frequency of the mutant chromosomes having the 2-6-4 haplotype was found to be 39.13% in the Eastern Black Sea region.

We also investigated the frequency of the 2-6-4 haplotype associated with the 35delG mutation in families from the other regions of Turkey. Haplotype analysis was performed in 13 families with autosomal recessive non-

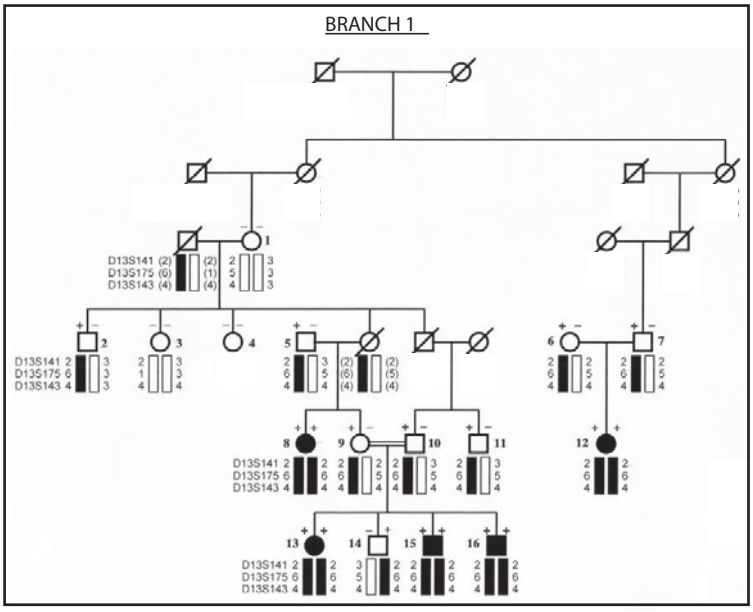
BRANCH 1
D13S141 (2) (2) 2 3
D13S175 (6) (1) 5 3
D13S143 (4) (4) 4 3
1
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Fig. (2a)

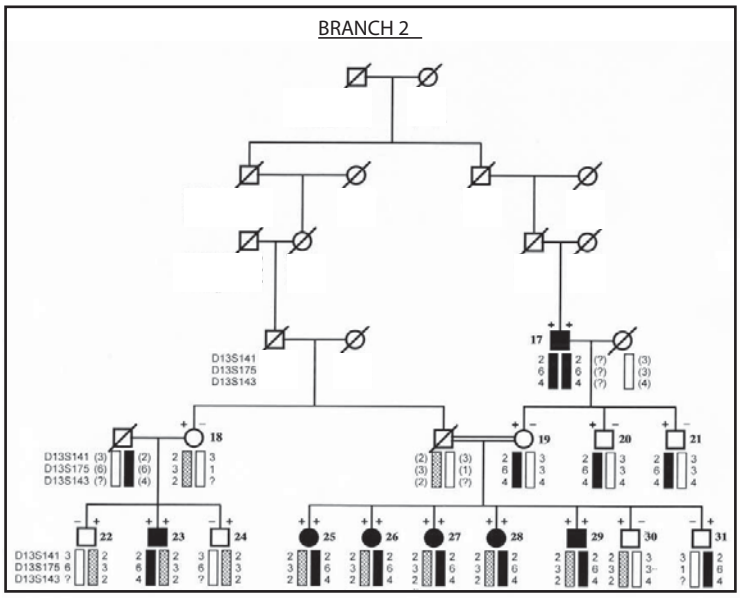
BRANCH 2
D13S141
D13S175
D13S143
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Fig. (2a)

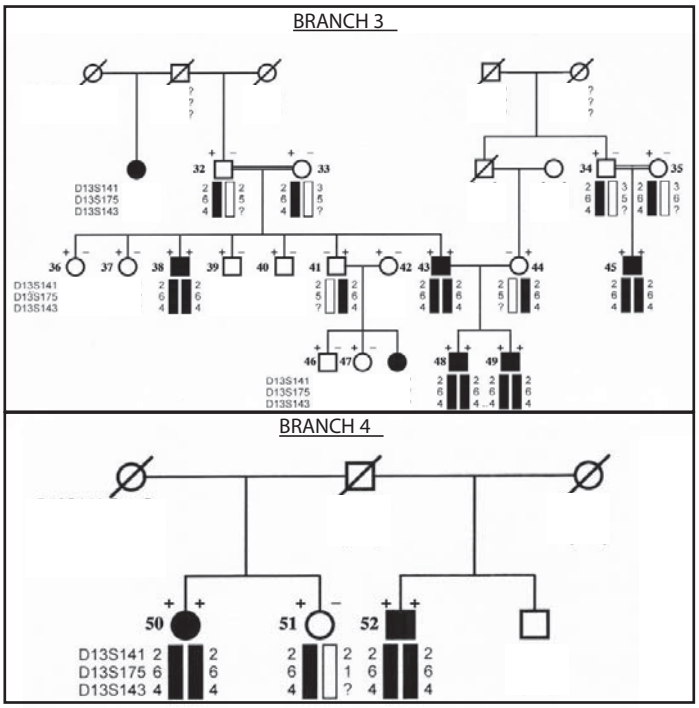


Fig. (2a)

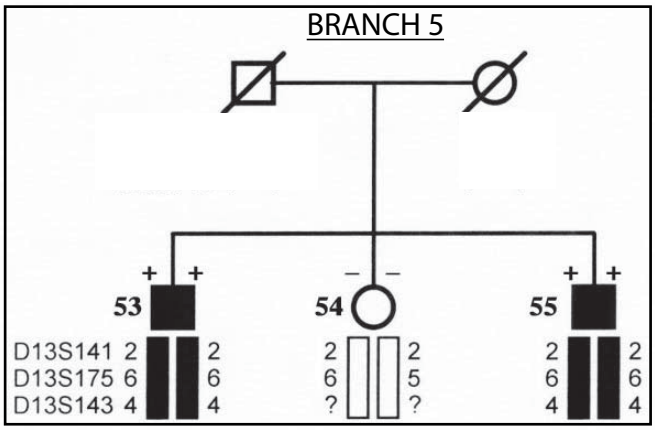


Fig. (2a)

Figure 2. (a) Haplotype analysis of the first family from the Eastern Black Sea region.

→ Chromosomes bearing 35delG mutation with 2-6-4 haplotype

→ Chromosomes bearing 35delG mutation with different haplotype

→ Chromosomes not bearing 35delG mutation

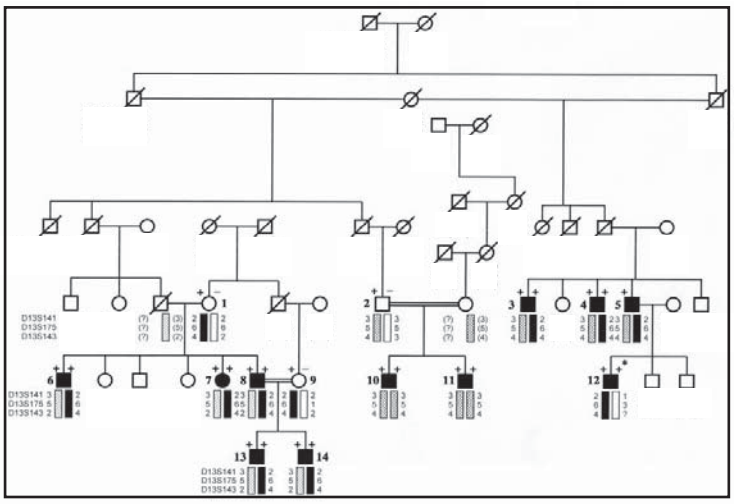
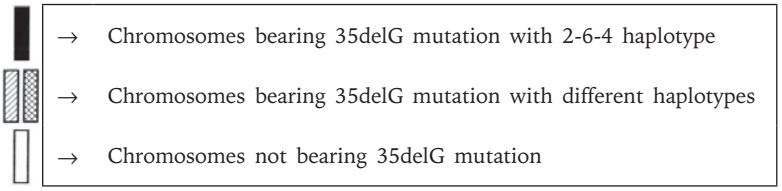


Figure 2. (b) Haplotype analysis of the second family from the Eastern Black Sea region.

* Patient No 12 is a compound heterozygote (35delG/184-186delGAG in Cx26 gene)



+/+ = individuals homozygous for 35delG mutation
+/– = individuals heterozygous for 35delG mutation
–/– = individuals not bearing 35delG mutation in either of the alleles

• In order to follow the haplotypes more easily, carrier individuals are depicted with +/– symbols.

Table I. Haplotype Analysis of 8 Probands Belonging to 8 Non-Consanguineous Families from the Eastern Black Sea Region and Homozygous for the 35delG Mutation

	Markers	Patients							
		1	2	3	4	5	6	7	8
Haplotypes	D13S141	2 3	3 3	3 2	3 2	2 3	2 3	2 2	2 2
	D13S175	6 5	5 5	6 5	6 1	5 5	6 5	5 5	3 3
	D13S143	4 4	4 4	4 4	4 4	4 4	4 4	4 4	4 4
Consanguineous marriages		–	+	–	–	+	–	+	+

syndromic hearing loss, where the probands were homozygous/heterozygous for 35delG mutation. The 2-6-4 haplotype was observed in only one chromosome of two patients from the Central Anatolian region. The frequency of the mutant chromosomes having the 2-6-4 haplotype was determined as 9.5% in the general patient population. Haplotypes of these 13 patients are shown in Table II.

When the frequencies of the mutant chromosomes having the 2-6-4 haplotype was compared between the Eastern Black Sea region (39.13%) and the other regions of Turkey (9.5%), a statistically significant difference was observed between these two groups ($\chi^2=5.13/df=1/p=0.023$). The frequencies of the other mutant chromosome haplotypes were not found to be statistically different between these two groups (Table III).

In addition, haplotype analysis was performed in 50 unrelated healthy individuals from the Eastern Black Sea region, and the frequency of the 2-6-4 haplotype in the wild type chromosomes was determined as 14.3%. When

Table II. Haplotypes of the Chromosomes Bearing 35delG Mutation in 13 Patients from the Other Regions of Turkey

Mutant chromosome haplotypes	No. of chromosomes (n=21)
2-6-4	2
2-5-4	8
3-5-4	5
3-5-2	2
3-4-4	1
2-5-1	1
3-1-1	1
1-5-4	1

Table III. Frequencies of the Haplotypes of the Chromosomes Associated with the 35delG Mutation in the Eastern Black Sea Region and the Other Regions of Turkey

	No. of chromosomes	
Haplotypes of the chromosomes bearing 35delG mutation	Eastern Black Sea region	Other regions of Turkey
2-6-4	9 (34.5%)	2 (9.52%)
2-5-4	4 (16.7%)	8 (38.1%)
3-5-4	6 (23.1%)	5 (23.8%)
3-5-2	1 (3.8%)	2 (9.52%)
3-4-4	- (0%)	1 (4.8%)
2-5-1	- (0%)	1 (4.8%)
3-1-1	- (0%)	1 (4.8%)
1-5-4	- (0%)	1 (4.8%)
3-6-4	2 (7.7%)	- (0%)
2-1-4	1 (3.8%)	- (0%)
2-3-4	2 (7.7%)	- (0%)
2-3-2	1 (3.8%)	- (0%)
Total	26	21

the frequency of mutant (39.1%) and wild type (14.3%) chromosomes having the 2-6-4 haplotype was compared in the Eastern Black Sea region, a statistically significant difference was observed in the mutant chromosomes ($\chi^2=7.46/df=1/p\leq0.01$).

Discussion

Turkey, one of the largest countries of the Middle East, has experienced many migrations since early prehistoric times. Geographically, Turkey is located at a crossroad between Asia and Europe.

As hypothesized before, the 35delG mutation might have originated from the Middle East and spread out to Europe via two different routes. The first migration route follows the coast of the Mediterranean and the second follows the Danube and Rhine Valleys[21]. Turkey is located between these two migration routes for the 35delG mutation. Therefore it may be considered that Turkey has played an important role in the dissemination of the 35delG mutation among populations.

Trabzon, which is an important seaport city in the East Black Sea region, is located in the second migration route of the 35delG mutation. As it was subjected to the external migrations of many populations in history, it is possible that Trabzon is one of the regions from which the 35delG was introduced into the Anatolian gene pool.

We performed haplotype analysis in two large inbred families with autosomal recessive non-syndromic hearing loss from Trabzon, which is in the Eastern Black Sea region, and identified the 2-6-4 common haplotype associated with the 35delG mutation. The aim of our study was to determine if the 2-6-4 is an ancestral haplotype for 35delG mutation in the Eastern Black Sea region.

Since the 35delG mutation is very old, haplotype sharing can only be observed in a very small chromosomal interval by using polymorphic markers close to the mutation region. Likewise, a common haplotype was observed in a ~70 kb region among the patients from Belgium, the United Kingdom

and the United States, and an association of the mutation with one SNP allele (731 bp far from the 35delG) was found[21]. In our study, due to the difficulties of studying SNP polymorphic markers, D13S141, D13S175 and D13S143 (CA)n polymorphic markers close to the Cx26 gene were used. The distances of the markers to the 35delG mutation region are 39.204bp, 84.718bp, and 1.510.295bp, respectively. To evaluate for founder effects, Tekin et al.[17] also used the same polymorphic markers, D13S141 and D13S175, in their studies. Since the rate of consanguinity in Turkey is very high (20-25%)[22], it may also be possible to observe a common haplotype in a 1.471.091bp region flanking the 35delG mutation. In addition, parental consanguinity was noted in 34% of 35delG homozygotes in Turkey[23].

As a result of haplotype analysis, we investigated the frequency of the 2-6-4 haplotype associated with the 35delG mutation in the Eastern Black Sea region. When we compared the frequency of the chromosomes having the 2-6-4 haplotype between the Eastern Black Sea region and the other regions of Turkey, a statistically significant difference was observed. Also, the frequency of the 2-6-4 haplotype was found to be higher in mutant chromosomes than wild type chromosomes in this region. Therefore, it was thought that 35delG might have been introduced to the gene pool of the Eastern Black Sea region with this haplotype thus creating a region specific profile. After that it might have spread out to other regions of Turkey. In the mean time, the inclusion of many different ethnical origins might have decreased the frequency of the 2-6-4 haplotype among the mutant chromosomes in the other regions of Turkey.

The mutant chromosome haplotypes other than 2-6-4 observed in the other regions of Turkey might have been introduced to our population from different regions with different ancestors. This possibility remains to be verified with detailed analysis of different chromosome haplotypes.

Furthermore, if the 35delG is considered to be an old mutation[21], the possibility of the 2-6-4 haplotype's having changed as a result of chromosomal recombinations should be taken into consideration. The detection of other mutant chromosome haplotypes such as 3-6-4, 3-5-4 and 2-5-4 in the other regions of Turkey makes this possibility stronger.

In the other regions of Turkey, 35delG haplotypes other than 2-6-4 were observed frequently among wild type chromosomes, while the 2-6-4 haplotype was rare. In addition, the 2-6-4 haplotype was also not detected among the chromosomes bearing other GJB2 gene mutations. These additional findings together with the original results prove that the haplotype of the founder chromosomes bearing the 35delG mutation in the Eastern Black Sea region is 2-6-4.

Although the 35delG mutation occurs in a hot spot region, it is not possible to obtain the definitive evidence that it is a recurrent mutation. The mutation might be introduced in one population's gene pool as a result of a founder effect while it can also be introduced to another population independently. Therefore, observation of the same mutation with different haplotypes in geographically, religiously and culturally distinct populations does not always prove that the mutation is recurrent.

Performing haplotype analysis in many different populations and genotyping of mutant and wild type chromosomes with polymorphic markers will provide new hints about the origin of the 35delG mutation. Furthermore, the 2-6-4 haplotype should be analyzed in other populations located in the second migration route. This would also help us to support the idea that 2-6-4 could be a founder haplotype for 35delG.

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REFERENCES

1. Morton NE. Genetic epidemiology of hearing impairment. Ann NY Acad Sci 1991; 630: 16-31.
2. Cohen MM, Gorlin RJ. Epidemiology, etiology and genetic patterns. In: Gorlin RJ, Toriello HV, Cohen MM Jr (eds). Hereditary Hearing Loss and Its Syndromes. Oxford: Oxford University Press; 1995: 9-21.
3. Van Camp G, Willems PJ, Smith RJ. Nonsyndromic hearing impairment: unparalleled heterogeneity. Am J Hum Genet 1997; 60: 758-764.
4. Denoyelle F, Weil D, Maw MA, et al. Prelingual deafness: high prevalence of a 30delG mutation in the connexin 26 gene. Hum Mol Genet 1997; 6: 2173-2177.

5. Belgin E, Akdaş F, Böke B, Cağlar A. The children population with sensory-neural hearing loss in Türkiye. In: Proceedings of the 2nd International Meeting in Audiology for Mediterranean Countries, Thesseloniki-Greece, 5-9 October 1991: 914-919.
6. Gasparani P, Estivill X, Volpini V, et al. Linkage of DFNB1 to non-syndromic neurosensory autosomal recessive deafness in Mediterranean families. Eur J Hum Genet 1997; 5: 83-88.
7. Wilcox SA, Sounders K, Osborn AH, et al. High frequency hearing loss correlated with mutations in the GJB2 gene. Hum Genet 2000, 106: 399-405.
8. Kelsell DP, Dunlop J, Stevens HP, et al. Connexin 26 mutations in hereditary non- syndromic sensorineural deafness. Nature 1997**;** 387: 80-89.
9. Rabionet R, Zelante L, Lopes-Bigas N, et al. Molecular basis of childhood deafness resulting from mutations in the GJB2 (connexin 26) gene. Hum Genet 2000; 106: 40-44.
10. Gasparini P, Rabionet R, Barbujani G, et al. High carrier frequency of the 35delG deafness mutation in European populations. Eur J Hum Genet 2000; 8: 19-23.
11. Carrasquillo MM, Zlotogora J, Barges S, Chakravarti A. Two different connexin 26 mutations in an inbred kindred segregating non-syndromic recessive deafness: implications for genetic studies in isolated populations. Hum Mol Genet 1997; 6: 2163-2172.
12. Kelley PM, Harris DJ, Comer BC, et al. Novel mutations in the connexin 26 gene (GJB2) that cause autosomal recessive (DFNB1) hearing loss. Am J Hum Genet 1998; 62: 792-799.
13. Lench NJ, Markham AF, Mueller RF, et al. A Moroccan family with autosomal recessive sensorineural hearing loss caused by a mutation in the gap junction protein gene connexin 26 (GJB2). J Med Genet 1998; 35: 151-152.
14. Morell RJ, Kim HJ, Hood LJ, et al. Mutations in the connexin 26 gene (GJB2) among Ashkenazi Jews with nonsyndromic recessive deafness. N Engl J Med 1998; 339: 1500-1505.
15. Abe S, Usami S, Shinkawa H, Kelley PM, Kimberling WJ. Prevalent connexin 26 gene (GJB2) mutations in Japanese. J Med Genet 2000; 37: 41-43.
16. Brobby GW, Müller-Myhsok B, Hortstmann RD. Connexin 26 R143W mutation associated with recessive nonsyndromic sensorineural deafness in Africa. N Engl J Med 1998; 338: 548-549.
17. Tekin M, Akar N, Cin S, et al. Connexin 26 (GJB2) mutations in the Turkish population: implications for the origin and high frequency of the 35delG mutation in Caucasians. Hum Genet 2001; 108: 385-389.
18. Barıs I, Kılınç MO, Tolun A. Frequency of the 35delG mutation in the connexin 26 gene in Turkish hearing-impaired patients. Clin Genet 2001; 60: 452-455.
19. Uyguner O, Emiroglu M, Uzumcu A, et al. Frequencies of gap- and tight-junction mutations in Turkish families with autosomal-recessive non-syndromic hearing loss. Clin Genet 2003; 64: 65-69.
20. Milder SA, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nuc Ac Res 1988; 28: 1215.
21. Van Laer L, Coucke P, Mueller RF, et al. A common founder for the 35delG GJB2 gene mutation in connexin 26 hearing impairment. J Med Genet 2001; 38: 515-518.
22. Tuncbilek E. Clinical outcomes of consanguineous marriages in Turkey. Turk J Pediatr 2001; 4: 277-279.
23. Tekin M, Duman T, Boguclu G, et al. Spectrum of GJB2 mutations in Turkey comprises both Caucasian and Oriental variants: roles of parental consanguinity and assortative mating. Hum Mutat 2003; 5: 552-553.