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2 **Running Title:**

3 **Modulating factors in A1555G deafness**

4

5 **Title:**

6 **Molecular characterization of putative modulatory factors in two Spanish families with**
7 **A1555G deafness**

8

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20 KEYWORDS: A1555G, Neurosensory non-syndromic deafness, Mitochondria, TRMU,
21 MTO1, mitochondrial SNPS

22

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26

27 **ABSTRACT:**

28 The aim of this work is to characterize possible modifying factors in two large families
29 carrying the A1555G mitochondrial mutation. The heteroplasmy of the mutation, the
30 presence of aminoglysosides, the co-segregation with other mitochondrial mutations, the
31 proposed linkage in chromosome 8 and the association with TRMU and MTO1 genes were
32 studied. None of the mentioned modifying factors were related with the phenotype
33 presentation of A1555G mutation. <u>However TRMU G28T SNP</u> is present in one of the
34 studied families.

35 **INTRODUCTION**

36 Neurosensory, maternally inherited, non-syndromic deafness is a disease that may be
37 provoked by specific mutations in mitochondrial DNA (mtDNA). The most common of
38 these mutations is the A1555G substitution that arises in the gene encoding the 12S
39 mitochondrial ribosome subunit [Prezant *et al.* 1993]. Although this mutation is considered
40 as the primary cause of neurosensory deafness, biochemical [Guan *et al.* 2001;Guan *et al.*
41 2000] and clinical [el Schahawi *et al.* 1997] evidence suggests that other environmental or
42 nuclear factors might modulate the phenotype of these disorders. Such modulating factors
43 include:

44 ***Exposure to aminoglycosides***. The A1555G mutation was initially described in subjects
45 with both antibiotic-induced and non-syndromic deafness [Prezant *et al.* 1993].
46 Moreover, various patients were later identified that carried this mutation and that
47 developed deafness without having been exposed to aminoglycosides [Li *et al.* 2004;el
48 Schahawi, Lopez, Sarrazin, Shanske, Basirico, Shanske, and DiMauro1997]. Thus, it
49 appears that exposure to these compounds is not required for the appearance of this
50 phenotype [Estivill *et al.* 1998].

51 ***Heteroplasmy of the mutation.*** In the vast majority of studies on A1555G, this mutation
52 was described as homoplasmic but some studies have presented families with different
53 levels of heteroplasmy. These studies have proposed that the level of heteroplasmy could
54 influence the clinical manifestations of the disease [el Schahawi, Lopez, Sarrazin,
55 Shanske, Basirico, Shanske, and DiMauro1997;del Castillo *et al.* 2003].

56 ***Co-segregation with other mtDNA mutations***. Polymorphisms in mtDNA could
57 influence the penetrance of pathogenic mutations responsible for other mitochondrial
58 disorders. For example, it is thought that primary pathogenic mutations exist in Lebers'
59 disease and that secondary mutations, which are not intrinsically pathogenic, influence
60 the expression of the primary mutations [Torroni *et al.* 1997]. It was proposed that

61 various alterations in the mtDNA sequence may modulate the phenotypic expression of
62 the A1555G mutation [Li, Xing, Yan, Cao, Liu, Bu, and Guan2004;Pandya *et al.*
63 1999;Finnila *et al.* 2001]. Other alterations in the mitochondrial genome that cause
64 neurosensory non-syndromic deafness and that could co-segregate with A1555G are the
65 changes A7445G, 7472 insC, T7510C, T7511C [Fischel-Ghodsian 1999] or C1494T
66 [Zhao *et al.* 2004].

67 ***Nuclear genes*** may also be candidates to functionally interact with and modify the
68 mutated 12S rRNA, influencing the phenotypic expression of the A1555G mutation.
69 Genome linkage studies propose that a locus in chromosome 8 might be linked to the
70 disease [Bykhovskaya *et al.* 1998;Bykhovskaya *et al.* 2000], but this linkage has not
71 been replicated and no mutations have been found in the proposed modifying genes.
72 Studies in the yeast *Saccharomyces cerevisae* have proposed the MTO1, MSS1 and
73 MTO2 genes as strong candidates to modulate the effect of the mutation. Accordingly,
74 MTO2 can complement the respiratory-deficient phenotype of yeast cells carrying the
75 C1409G mutation in the 15S rRNA. Indeed, a G28T missense mutation in the TRMU
76 gene (a homologue of MTO2) was identified in 613 subjects with non-syndromic
77 deafness from families of different ethnic origins, altering a conserved amino acid
78 (Alanine). Functionally, this mutation does not affect the import of TRMU precursors but
79 it does lead to a marked failure in mitochondrial tRNA metabolism [Guan *et al.* 2006].
80 These findings support the role of TRMU as a modulating factor of deafness-associated
81 A1555G mutation.
82

83 In the current study, we analyzed possible modifying factors in two families carrying the
84 A1555G mutation in which deafness was observed, in order to explain the differences in the
85 phenotype between the carriers of the mutation. In these families, the heteroplasmy of the
86 mutations was studied, as well as their co-segregation with other mitochondrial mutations in

the 12S gene, and the proposed linkage with a region in chromosome 8, and with the TRMU and MTO1 sequences.

METHODS

The two families examined in this study were of Spanish origin and they have been partially described in another study [el Schahawi, Lopez, Sarrazin, Shanske, Basirico, Shanske, and DiMauro1997]. All the individuals studied were interviewed at the Neurology Service of the Hospital Donostia. Of 38 individuals in these families (18 male, 20 female), 19 (9 males, 10 females) were diagnosed with neurosensory non-syndromic deafness.

Molecular studies

After obtaining the informed consent of each subject, DNA was extracted from peripheral blood by standard methods [Miller *et al.* 1988]. The control samples were taken from a DNA bank maintained by the Neurology Service of the Hospital Donostia and these controls were from individuals that did not suffer any neurological complaint.

Study of the A1555G mutation

The presence of the A1555G mutation was determined by PCR-RFLP [el Schahawi, Lopez, Sarrazin, Shanske, Basirico, Shanske, and DiMauro1997]. A 94 bp fragment was amplified (see primers in Table I) using a reverse mismatch primer that generated a novel restriction site in conjunction with the mutation. The presence or absence of the mutation could therefore be determined by complete digestion of the PCR product O/N with the restriction enzyme Hae III (*New England Biolabs*), and by analyzing the digestion products by electrophoresis in a 4 % agarose gel. The novel restriction site generated by the presence of the mutated allele (1555G) gives rise to two bands of 64 and 30 bp upon digestion, while the normal allele (1555A) is not digested by the enzyme and thus, a 94 bp band is detected. In this way the carriers of the mutation can be easily differentiated.

111 ***Quantification of the heteroplasmy level***

112 The level of heteroplasmy in mitochondrial mutations has classically been measured by
113 densitometry of the bands obtained in ^{32}P labeled RFLP studies. Since it is not currently
114 clear which is the best technique to measure heteroplasmy [Jacobi *et al.* 2001;Ren-Kui B and
115 Lee-Jun C.Wong 2004], we have used two methods to measure this phenomenon:

116 - The first method involved capillary electrophoresis of the PCR-RFLP products in an
117 ABIPRISM 310 analyzer. As noted above, the reverse mismatch primer creates a new
118 Hae III target when the DNA carries the mutation while the forward primer was labeled
119 with TAMRA. Two different peaks were detected after O/N enzyme digestion, the wild-
120 type peak (94 pb) and the mutated peak (64 pb). In the heteroplasmic subjects, the
121 proportion of the mutated molecules was estimated by the relation between the peak´s
122 areas using the GENESCAN and GENOTYPER softwares (*Applied Biosystems*). Each
123 digestion product was run twice and each digestion was performed three times from three
124 independent PCR reactions. The percentage heteroplasmy was calculated as the mean of
125 the six results.
126 - The other method relied on qPCR SNP detection technology, using a 7300 qPCR genetic
127 analyzer (Applied Biosystems) and a SNP assay with probes specifically designed by
128 Applied Biosystem to detect the A1555G SNP. To establish the level of heteroplasmy,
129 the Ct of each probe (normal and mutated) was compared [Bai and Wong 2004] using
130 SDS v1.3.1 software from Applied Biosystems. We checked each probe in 45 healthy
131 controls to be sure that the mutated probe was sufficiently specific. Each assay was
132 repeated three times and the value presented corresponds to the mean of these three
133 measurements.

134

135 ***Study of the mitochondrial variations***

136 By sequencing a 1141 bp amplicon of mtDNA that includes the MTRNR1 gene (see primers
137 in Table I) we studied an affected individual and a carrier of the mutation in each family
138 (individual III-11 of family 1 and IV-3 of family 2), as well as three control individuals.

139 Likewise, the mitochondrial mutations: 961 insC, G7444A, A7445G, 7472 insC, T7510C,
140 T7511C and C1494T were studied in all the family members (affected and unaffected). The
141 A4336G alteration was analyzed by PCR-RFLP with the enzyme Nla III (*New England*
142 *Biolabs*) rather than by sequencing. The 4336A allele generates a 313 bp fragment while the
143 allele 4336G generates two fragments of 244 bp and 69 bp that can being differentiated by
144 electrophoresis in a 2 % agarose gel.. Each sample was ascribed to one of the following
145 haplogroups, H, U, JT or Y, according to previously published protocols [Otaegui *et al.*
146 2004].

147 All the mitochondrial primers (sequences available on request) were designed using the
148 mitochondrial sequence AC_001807.

149 ***Linkage studies***

150 The linkage study was performed using the following markers: D8S277, D8S561, D8S1819
151 and D8S1825 with HEX labeled primers and under the amplification conditions described in
152 the GDB database [2004a]. The PCR products were run in an ABIPRISM 310 genetic
153 analyzer and analyzed with the GENESCAN software. The lod score was calculated using
154 the LCP software package.

155 ***Candidate nuclear gene sequencing***

156 The MTO1 gene was studied by direct sequencing of the coding exons in: one patient (IV-
157 28) from family 1, one asymptomatic individual that is a carrier of the A1555G mutation
158 (III-5) from family 1, one patient from family 2 (III-5) and one healthy control unrelated

159 with either family. The MTO1 primers (available on request) were designed using the
160 sequence ENST00000323606.
161
162 The coding exons of the TRMU gene were also studied by direct sequencing in the following
163 individuals: III-10, III-14, IV-40, V-2 and V-6 from family 1; III-9, IV-3 and IV-4 from
164 family 2. The primers (available on request) were designed from the NC_000022.9
165 sequence. The presence of the G28T mutation was ascertained by PCR-RFLP using
166 previously reported methods [Guan, Yan, Li, Bykhovskaya, Gallo-Teran, Hajek, Umeda,
167 Zhao, Garrido, Mengesha, Suzuki, Del Castillo, Peters, Li, Qian, Wang, Ballana, Shohat, Lu,
168 Estivill, Watanabe, and Fischel-Ghodsian2006]. We examined this mutation in 256 healthy
169 subjects.
170 All sequencing reactions were carried out in a 310 or a 3130 Applied Biosystem genetic
171 analyzer following the manufacturer's recommendations.

172 **RESULTS**

173 ***Heteroplasmy levels***

174 The data from the distinct analyses of heteroplasmy by Genescan qPCR and by
175 electrophoresis were compared (Table 1). The genescan technique detects levels of
176 heteroplasmy between 0.8% and 98 % in both families, finding more heteroplasmy in the
177 family 2. The study in controls with qPCR shows that the mutated probe was highly specific,
178 giving an error of less than 1% (data not shown), this technique detects heteroplasmy only in
179 family 2 (patients II-2 and III-1)
180

181 ***Mitochondrial SNPS***

182 None of the following mitochondrial mutations were found in any of the individuals studied:
183 961 insC, A4336G, A7445G, 7472 insC, T7510C, or the T7511C. Family 1 can be
184 considered within the haplogroup JT, and family 2 in the haplogroup H.

185 We characterized several homoplasmic changes in the 12S mitochondrial gene sequence. At
186 nucleotide 750, we identified the A→G transition in all the samples analyzed (patients and
187 controls). In contrast, a transversion (C→G) was detected at position 1438 in the two
188 families studied and it was not evident in the controls, thereby segregating with the A1555G
189 mutation. Furthermore, a T→C transition was observed at position 1193 in family 2 but not
190 in the other family.

191 ***Linkage and nuclear genes***

192 The linkage analysis generated lod scores between -0.5 and -2.5, ruling out the possible
193 linkage of the chromosome 8 region with the disease (data available on request).

194

195 The sequence of MTO1 coding exons in the families studied did not show any difference in
196 the families when compared with the controls.

197

198 Likewise, no mutations were detected in the TRMU sequences except for the previously
199 reported G28T mutations (see Fig 2). This mutation was only present in family 1, where it
200 was detected in 14 heterozygous individuals, six of whom were deaf and 8 healthy, and in
201 one homozygous-deaf subject. When 256 control subjects were analyzed, the proportion of
202 heterozygotes was 12.1 % and only 1 homozygous subject was identified (0.004%).

203

204 **DISCUSSION**

205 In two Spanish families suffering from maternally inherited neurosensory non-syndromic
206 deafness, we have analyzed different molecular changes that have been proposed as possible

207 modifying factors associated with the A1555G mutation [el Schahawi, Lopez, Sarrazin,
208 Shanske, Basirico, Shanske, and DiMauro1997]. Accordingly, we analyzed the degree of
209 heteroplasmy, the presence of mitochondrial mutations, the proposed linkage to chromosome
210 8 and the role of the MTO1 and TRMU genes in these families and in healthy controls.

211

212 ***Heteroplasmy***

213 Although the A1555G mutation is usually described as displaying a homoplasmic
214 inheritance, individuals with heteroplasmic mutations have been described [del Castillo,
215 Rodriguez-Ballesteros, Martin, Arellano, Gallo-Teran, Morales-Angulo, Ramirez-Camacho,
216 Cruz, Solanellas, Martinez-Conde, Villamar, Moreno-Pelayo, Moreno, and del, I2003;el
217 Schahawi, Lopez, Sarrazin, Shanske, Basirico, Shanske, and DiMauro1997]. Indeed, it was
218 established that the mutated copies ranged between 3.75 and 96.60 % in these heteroplasmic
219 individuals and a pathogenic threshold was proposed between 20 and 52 % . According to
220 these conclusions, individuals with less than 20 % of mutated copies would be asymptomatic
221 while the individuals with more than 52 % would be deaf. However, these affirmations do
222 not correlate with the existence of healthy homoplasmic carriers of the mutation, as occurs
223 both in our families and in families reported elsewhere.

224

225 Instead of the classical densitometry methods, we have used two different techniques to
226 measure the degree of heteroplasmy that avoid the use of radioactivity. A technical
227 comparison of these two methods showed slight differences between the percentages
228 obtained by genescan or by qPCR. In the genescan approach, we use a restriction enzyme
229 and a mismatch primer and these two conditions increase the variability of the technique,
230 particularly in the values close to the extremes (0 and 100). Such problems were not
231 associated with the use of qPCR, which displays less variability between replicates than
232 genescan. However, based on the control results qPCR may have an error of 1% in terms of

233 the estimation of the percentage level of mutation. Nevertheless, we believe that these two
234 approaches are more realistic and more efficient than the densitometry approach.
235
236 In family 1 the mutation always appears as homoplasmic and while the pattern is clearly
237 maternal, we found several carrier individuals without symptoms (III-10, III-21, III-15, IV-9,
238 V-1 y V-8 in Fig 2A). Such asymptomatic carriers have also been found in other families,
239 sustaining the existence of modulating factors. In contrast, heteroplasmic individuals were
240 identified in family 2, the degree of heteroplasmy ranging between 71.186 (II-2) and 93.214
241 % (III-1). Similarly, asymptomatic homoplasmic carriers were identified (IV-4) which
242 makes it impossible to establish a pathogenic threshold. The III-9 individual in this family
243 should be a carrier of the mutation but does not present the A1555G mutation. The genotype
244 of this individual could be explained by non-equitable mitochondrial segregation during
245 oogenesis in the mother which produces two or more ovule types with high levels of the
246 mutation or with no mutated copies. This phenomenon, named "bottle neck" could be
247 defined as the minimal number of mitochondrial genetic copies which occurs during oocyte
248 formation and early development which maximizes genetic drift for a heteroplasmic mother.
249 It has been described in other mitochondrial diseases, and non-equitable A1555G inheritance
250 in the offspring of the carrier mothers was described by Del Castillo et al, supporting the
251 possible occurrence of such a bottle neck. Moreover, we must take into account that our
252 study was carried out on blood and thus, the values obtained might not be representative of
253 the affected tissue. Although several authors have tried to explain the mechanisms behind
254 heteroplasmic inheritance, they still remain unclear [Battersby B *et al.* 2003;Jenuth JP *et al.*
255 1996].
256 In conclusion, the two families studied here carry a homoplasmic A1555G mutation on the
257 one hand and in the other, the A1555G mutation is clearly heteroplasmic. In both families
258 homoplasmic carriers were identified that did not display any symptoms.

259 ***Mitochondrial mutations***

260 The MTRNR1 gene, in which the A1555G mutation is located, encodes the ribosomal 12S
261 subunit. Hence, any other alteration in the sequence of this gene could affect the spatial
262 conformation of this subunit and act in conjunction with the A1555G mutation to modulate
263 its effects. Several different polymorphisms in this gene have been proposed as factors that
264 might modulate this disease (see introduction) and our data clearly indicate that the transition
265 at nucleotide 750 can be considered as a polymorphism. This alteration appears with a
266 similar frequency in both the healthy group of subjects and in the patients, and as such it is
267 most likely to be a polymorphism rather than being related with the disease.

268 The C1438G transversion was also present in both families but it was not identified in the
269 control subjects. Moreover, this alteration has been described as a polymorphism in the
270 MITOMAP database [2004b]. This transversion was also identified in another study on the
271 relationship between mitochondria and deafness both in, patients and control subjects
272 [Yamasoba *et al.* 2002]. Accordingly, its potential implication in this disease remains
273 unclear. Indeed, this substitution could be a population polymorphism that is found at greater
274 frequency in carriers of the A1555G mutation. In order to evaluate this possibility, it will be
275 necessary to determine the presence of this polymorphism in a larger number of A1555G
276 carriers.

277 The nucleotide change 1193(T→C) appears exclusively in family 2 and it is not included in
278 the databases. It occurs at a nucleotide that is not highly conserved across evolution, and that
279 is found between structural helix 32 and 33 of the 12S subunit [Hickson R *et al.* 1996]. This
280 could be a rare polymorphism or alternatively, a cofactor that modulates the effects of the
281 A1555G mutation in an analogous manner to that seen for the secondary mutations in
282 LEBER disease. However, the fact that the polymorphism segregates with the mutation does
283 not explain the existence of asymptomatic carriers.

284

285 In summary, all the mitochondrial changes encountered appear to be polymorphisms that are
286 not related with the incomplete penetrance of the A1555G mutation. However, it will be
287 important to carry out an analysis in larger series of patients to study the effects of these
288 changes on the expression of the A1555G mutation.

289 ***Linkage mutations and candidate genes***

290 The proposed positive linkage of the 8q23 region [Bykhovskaya *et al.* 2001] has not been
291 verified either by our data or in an earlier study [Finnila and Majamaa 2003], so the linkage
292 of this region with the disease remains unclear.

293 The MTO1 gene, located in chromosome 6 has been proposed as a candidate gene to
294 modulate the effects of the A1555G mutation. When mutated it produces a deficit in the
295 production of the respiratory channel, but only when the mtDNA of this cells carries the
296 P^{R}_{454} mutation, equivalent to the human A1555G mtDNA mutation [Colby *et al.* 1998]. Like
297 two other studies [Finnila and Majamaa2003;Bykhovskaya *et al.* 2004], we found no
298 mutations in this gene in our families, and this gene could thus be discarded as an factor
299 influencing this disease.

300 In 2006, it was proposed that the G28T mutation in the TRMU gene was associated with this
301 disease [Guan, Yan, Li, Bykhovskaya, Gallo-Teran, Hajek, Umeda, Zhao, Garrido,
302 Mengesha, Suzuki, Del Castillo, Peters, Li, Qian, Wang, Ballana, Shohat, Lu, Estivill,
303 Watanabe, and Fischel-Ghodsian2006]. This variant was found in around a 25% of two
304 cohorts carrying the A1555G mutation (one Arab-Israeli and one European), while it was
305 only present in 10% of Jewish and white controls. The mutation is not present in either
306 controls or families suffering deafness in the Chinese population. Biochemically, the G28T
307 mutation was shown to aggravate the mitochondrial dysfunction caused by the A1555G

308 mutation [Guan, Yan, Li, Bykhovskaya, Gallo-Teran, Hajek, Umeda, Zhao, Garrido,
309 Mengesha, Suzuki, Del Castillo, Peters, Li, Qian, Wang, Ballana, Shohat, Lu, Estivill,
310 Watanabe, and Fischel-Ghodsian2006]. According to this hypothesis, the G28T mutation
311 acts in synergy with the mitochondrial mutation, lowering the mitochondrial translation
312 process below the pathogenic threshold and thereby causing the phenotype.

313 In our cohorts, both of Spanish origin, we found the G28T mutation in 15 out of 28 members
314 in family 1. The mutation appears more frequently in this family than in normal controls,
315 consistent with the proposed modifying role. However, in this family we did not find a
316 correlation between the genotype and phenotype, since we found heterozygous individuals
317 carrying the mutation with and without deafness. In some families, including one presented
318 here, this mutation seems to be a marker for the pedigree, but it not seems to be a modifying
319 factor. More genes related to the complex set of factors involved in the mitochondrial
320 translation process should be studied in relation with this phenomenon.

321 In our families the A1555G mitochondrial mutation seems to be necessary but not sufficient
322 to cause clinical symptoms. It therefore seems necessary to identify more susceptibility
323 factors, probably SNPS in nuclear genes related with mitochondrial metabolism, to explain
324 the inheritance of this disease.

325

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335 Table 1

FAMILY	sample	GeneS	qPCR	Gel	Clinical Symptoms
FAMILY 1	III-8	98.00	100.00%	+	YES
	III-10	96.00	100.00%	+	NO
	III-11	91.29	100.00%	+	NO
	III-12	1.00	0.10%	-	NO
	III-15	95.00	100.00%	+	NO
	IV-1	95.70	100.00%	+	YES
	IV-2	95.36	100.00%	+	YES
	IV-3	0.00	0.00%	-	NO
	IV-4	92.78	100.00%	+	YES
	IV-5	96.70	100.00%	+	YES
	IV-6	96.67	100.00%	+	YES
	IV-8	96.56	100.00%	+	YES
	IV-11	95.91	100.00%	+	YES
	IV-12	1.40	0.05%	-	NO
	IV-13	96.14	100.00%	+	NO
	IV-14	1.44		-	NO
	IV-15	95.14	100.00%	+	YES
	IV-18	93.67	100.00%	+	YES
	IV-20	94.89	100.00%	+	YES
	IV-21	2.29	0.00%	-	NO
	IV-24	3.00	0.06%	-	NO
	V-1	96.00	100.00%	+	NO
	V-2	96.43	100.00%	+	YES
	V-3	94.78	100.00%	+	NO
	V-4	6.20	0.00%	-	NO
	V-5	1.25	0.07%	-	NO
	V-6	1.00	0.03%	-	NO
	V-7	2.29	0.04%	-	NO
	V-8	6.14	0.13%	-	NO
FAMILY 2	II-2	81.67	71.19%	±	NO
	III-1	67.71	93.22%	±	YES
	III-5	78.50	99.97%	+	YES
	III-7	91.25	N.A	+	YES
	III-8	0.88	0.26%	-	NO
	IV-3	N.A	100.00%	+	YES
	IV-4	N.A	100.00%	+	¿?
	IV-5	N.A	100.00%	+	YES
	IV-6_	95.43	100.00%	+	YES

336 *Heteroplasmy observed using two techniques: GeneScan (GeneS) and quantitative PCR*
337 *(qPCR), and compared with the electrophoresis results and the status of the individuals. The*
338 *results of the RFLP in agarose gels are represented, whereby: + indicates the presence of*
339 *the mutation; a negative result is represented by -; and both symbols indicate that the RFLP*
340 *indicates the existence of hetroplasmy. ND: Not available.*

341

342 Table 2.

Change	Nucleotide	Fam 1, III-11	Fam 2, IV-3	Controls	MITOMAP
A→G	750	YES	YES	YES	Polymorphism
C→ G	1438	YES	YES	NO	DM associated polymorphism
T→C	1193	NO	YES	NO	

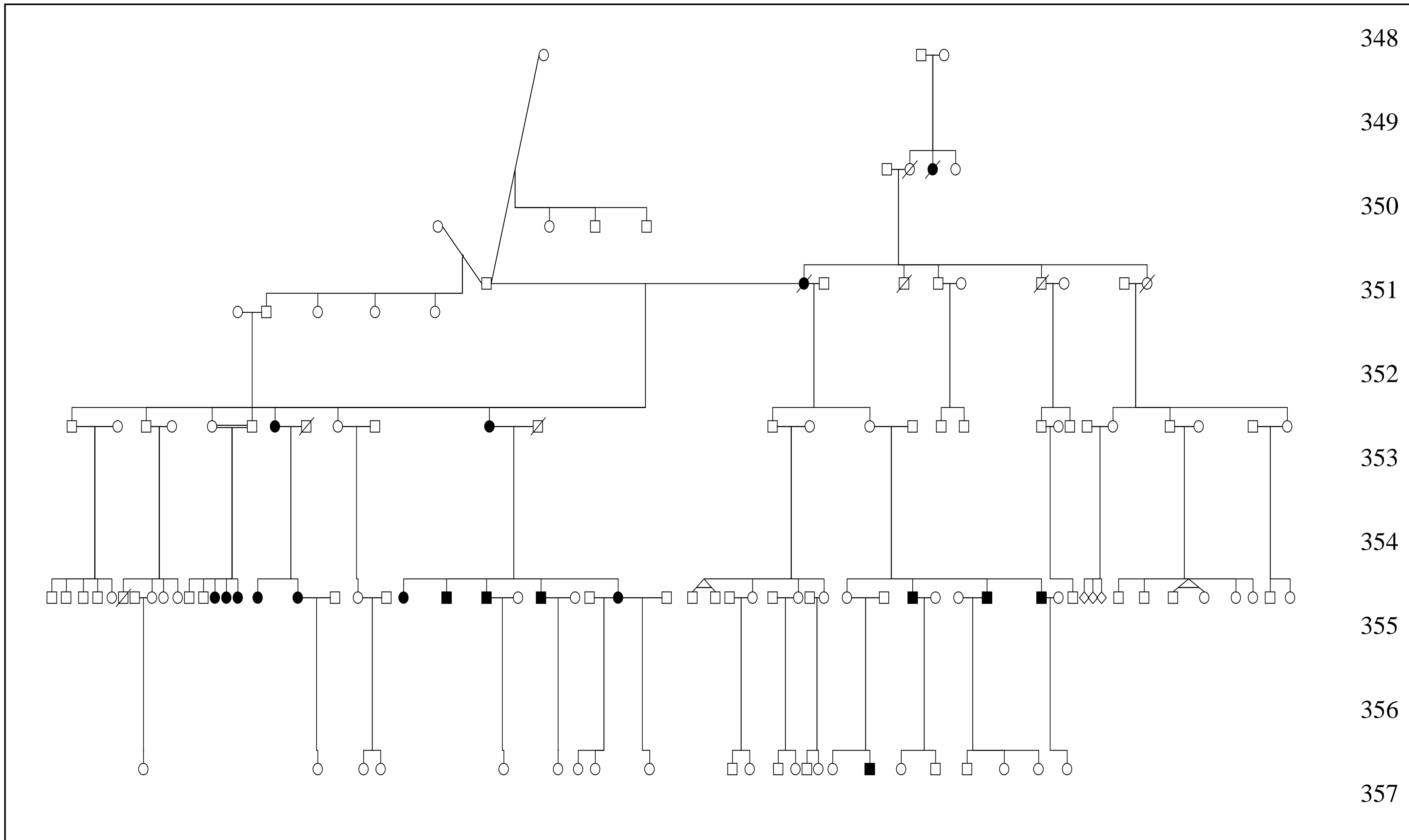
343

344 *Changes in the 12S mitochondrial gene. DM: Diabetes Mellitus*

345

346

347



358 *Family 1. Deaf individuals are indicated by black symbols*

A

B

359

+: A1555G carrier, - : No A1555G carrier, _/_: TRMU ntd 28 genotype. A: Family 1 abbreviated. B: Family 2 showing the percentage of heteroplasmy, all the members are carriers of the G/G genotype at ntd 28. Deaf individuals are indicated by black symbols

360 Reference List
361

362 www.mitomap.org. 2004b.

363 www.gdb.org. 2004a.

364 Bai R, Wong L-J. Detection and quantification of heteroplasmic mutant mitochondrial
365 DNA by real-time amplification refractory mutation system quantitative PCR analysis:
366 A single-Step Approach. Clin Chemistry 2004; 50: 996-1001.

367 Battersby B, Loredo-Osti JC, Shoubridge E. Nuclear genetic control of mitochondrial
368 DNA segregation. Nat Genet 2003; 33: 183-186.

369 Bykhovskaya Y, Estivill X, Taylor K *et al*. Candidate locus for a nuclear modifier gene
370 for maternally inherited deafness. Am J Hum Genet 2000; 66: 1905-1910.

371 Bykhovskaya Y, Mengesha E, Wang D *et al*. Human mitochondrial transcription factor
372 B1 as a modifier gene for hearing loss associated with the mitochondrial A1555G
373 mutation. Mol Genet Metab 2004; 82: 27-32.

374 Bykhovskaya Y, Shohat M, Ehrenman K *et al*. Evidence for complex nuclear
375 inheritance in a pedigree with nonsyndromic deafness due to a homoplasmic
376 mitochondrial mutation. Am J Med Genet 1998; 77: 421-426.

377 Bykhovskaya Y, Yang H, Taylor K *et al*. Modifier locus for mitochondrial DNA
378 disease: linkage and linkage disequilibrium mapping of a nuclear modifier gene for
379 maternally inherited deafness. Genet Med 2001; 3: 177-180.

380 Colby G, Wu M, Tzagoloff A. MTO1 codes for a mitochondrial protein required for
381 respiration in paromomycin-resistant mutants of Saccharomyces cerevisiae. J Biol
382 Chem 1998; 273: 27945-27952.

383 del Castillo FJ, Rodriguez-Ballesteros M, Martin Y *et al*. Heteroplasmy for the
384 1555A>G mutation in the mitochondrial 12S rRNA gene in six Spanish families with
385 non-syndromic hearing loss. J Med Genet 2003; 40: 632-636.

386 el Schahawi M, Lopez DM, Sarrazin AM *et al*. Two large Spanish pedigrees with
387 nonsyndromic sensorineural deafness and the mtDNA mutation at nt 1555 in the 12s
388 rRNA gene: evidence of heteroplasmy. Neurology 1997; 48: 453-456.

389 Estivill X, Govea N, Barcelo E *et al*. Familial progressive sensorineural deafness is
390 mainly due to the mtDNA A1555G mutation and is enhanced by treatment of
391 aminoglycosides. Am J Hum Genet 1998; 62: 27-35.

392 Finnila S, Autere J, Lehtovirta M *et al*. Increased risk of sensorineural hearing loss and
393 migraine in patients with a rare mitochondrial DNA variant 4336A>G in tRNAGln. J
394 Med Genet 2001; 38: 400-405.

395 Finnila S, Majamaa K. Lack of a modulative factor in locus 8p23 in a Finnish family
396 with nonsyndromic sensorineural hearing loss associated with the 1555A>G
397 mitochondrial DNA mutation. Eur J Hum Genet 2003; 11: 652-658.

398 Fischel-Ghodsian N. Mitochondrial deafness mutations reviewed. Hum Mutat 1999;
399 13: 261-270.

400 Guan MX, Fischel-Ghodsian N, Attardi G. A biochemical basis for the inherited
401 susceptibility to aminoglycoside ototoxicity. Hum Mol Genet 2000; 9: 1787-1793.

402 Guan MX, Fischel-Ghodsian N, Attardi G. Nuclear background determines biochemical
403 phenotype in the deafness-associated mitochondrial 12S rRNA mutation. Hum Mol
404 Genet 2001; 10: 573-580.

405 Guan MX, Yan Q, Li X *et al*. Mutation in TRMU related to transfer RNA modification
406 modulates the phenotipic expression of the deafness-associated mitochondrial 12S
407 ribosomal RNA mutations. Am J Hum Genet 2006; 79: 291-302.

408 Hickson R, Simon C, Cooper A, Spicer G, Sullivan J, Penny D. conserved sequence
409 motifs, alignment,a nd secondary structure for the third domain of animal 12S rRNA.
410 Mol Biol Evol 1996; 13: 150-169.

411 Jacobi F, Leo-Kottler B, Mittelviefhaus K *et al*. Segregation patterns and heteroplasmy
412 prevalence in Leber´s hereditary optic neuropathy. Investigative Ophtalmology &
413 Visual Science 2001; 42: 1208-1214.

414 Jenuth JP, Peterson AC, Shoubridge EA. Random genetic drift in the female germline
415 explains the rapid segregation of mammalian mitochondrial DNA. Nat Genet 1996; 14:
416 146-151.

417 Li R, Xing G, Yan M *et al*. Cosegregation of C-insertion at position 961 with the
418 A1555G mutation of the mitochondrial 12S rRNA gene in a large Chinese family with
419 maternally inherited hearing loss. Am J Med Genet 2004; 124A: 113-117.

420 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA
421 from human nucleated cells. Nucleic Acids Res 1988; 16: 1215.

422 Otaegui D, Saenz A, Martinez-Zabaleta M *et al*. Mitochondrial haplogroups in Basque
423 multiple sclerosis patients. Mult Scler 2004; 10: 532-535.

424 Pandya A, Xia XJ, Erdenetungalag R *et al*. Heterogenous point mutations in the
425 mitochondrial tRNA Ser(UCN) precursor coexisting with the A1555G mutation in deaf
426 students from Mongolia. Am J Hum Genet 1999; 65: 1803-1806.

427 Prezant T, Agapian J, Bohlman M *et al*. Mitochondrial ribosomal RNA mutation
428 associated with both anticbiotic-induced and non-syndromic deafness. Nat Genetics
429 1993; 4: 289-298.

430 Ren-Kui B, Lee-Jun C.Wong. Detection and quantification of heteroplasmic mutant
431 mitochondrial DNA by Real-Time amplification refractory mutation system quantitative
432 PCR analysis: A single-step Approach. Clinical Chemistry 2004; 50: 996-1001.

433 Torroni A, Petrozzi M, D'Urbano L *et al*. Haplotype and phylogenetic analyses suggest
434 that one European-specific mtDNA background plays a role in the expression of Leber
435 hereditary optic neuropathy by increasing the penetrance of the primary mutations
436 11778 and 14484. Am J Hum Genet 1997; 60: 1107-1121.

437 Yamasoba T, Goto Y, Oka Y, Nishino I, Tsukuda K, Nonaka I. Atypical muscle
438 pathology and a survey of cis-mutations in deaf patients harboring a 1555 A-to-G point
439 mutation in the mitochondrial ribosomal RNA gene. Neuromuscul Disord 2002; 12:
440 506-512.

441 Zhao H, Li R, Wang Q *et al*. Maternally inherited aminoglycoside-induced and
442 nonsyndromic deafness is associated with the novel C1494T mutation in the
443 mitochondrial 12S rRNA gene in a large Chinese family. Am J Hum Genet 2004; 74:
444 139-152.
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