Genetic screening of patients with medullary thyroid cancer in a referral center in Greece during the past two decades

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Abstract

Objective: Mutations in the *RET* gene are responsible for hereditary medullary thyroid cancer (MTC) and may vary between ethnic groups. We report the spectrum of mutations detected in patients with MTC in a referral center in Greece.

Patients and methods: Screening for *RET* mutations was performed in 313 subjects from 188 unrelated families: 51 patients had clinical suspicion for familial disease, 133 were apparently sporadic, four patients had only C cell hyperplasia, and 125 were family members. Exons 8, 10, 11, and 13–16 were screened.

Results: A total of 58 individuals (30.85%) were *RET* mutations carriers, 120 (63.8%) were finally classified as sporadic, 13 apparently sporadic cases (9.8%) were identified with *RET* mutation: ten carried the exon 8 at codon 533 mutation (previously reported), two the exon 14 at codon 804 mutation, and one the exon 13 at codon 768 mutation. Six patients (3.19%) with clinical features of multiple endocrine neoplasia type 2A and negative for *RET* mutations were classified as ‘unknown cause’. The mutations of hereditary cases were as follows: 21 cases (36.2%) in exon 8 codon 533, 19 (32.8%) in exon 11 codon 634, nine (15.5%) in exon 10, five (8.6%) in exon 16, three (5.2%) in exon 14 codon 804, and one in exon 13 codon 768 (1.7%).

Conclusion: The spectrum of *RET* mutations in Greece differs from that in other populations and the prevalence of familial cases is higher. The exon 8 (Gly533Cys) mutation was the most prevalent in familial cases unlike other series, followed by exon 11 (codon 634) mutations which are the most frequent elsewhere. The wide application of genetic screening in MTC reveals new molecular defects and helps to characterize the spectrum of mutations in each ethnic group.

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Introduction

Medullary thyroid carcinoma (MTC) constitutes 5–10% of all thyroid malignancies. About 25% of the cases of MTC are familial and the disease is inherited in an autosomal dominant manner. The inherited forms present as multiple endocrine neoplasia (MEN) syndromes MEN2A and MEN2B, which are characterized by occasional coexistence with pheochromocytoma (PHEO) and - in the case of MEN2A - with primary hyperparathyroidism (PHP). Those without further tumors are characterized as familial MTC (FMTC) only (1).

The molecular pathology of inherited MTC is constitutive activation of the *RET* proto-oncogene. The human *RET* gene comprises 21 exons and its size is 53.3 kb. It encodes a tyrosine kinase receptor with a cysteine-rich extracellular domain, a transmembrane domain, and an intracellular tyrosine kinase domain (1, 2).

The *RET* gene plays an important role in cell signaling in neural tissue, especially during the early stages of development. Mutations in the gene lead to abnormalities in cell proliferation and differentiation of tissues derived

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from neural crest cells, such as the C-cells of the thyroid and the adrenal medulla (3). The number and type of recognized *RET* mutations have increased over the last years due to the wider application of genetic screening. Mutations affecting the extracellular domain of the protein in codons 609, 611, 618, 620, 630, and 634 activate the tyrosine kinase receptor by ligand-independent dimerization and cross phosphorylation. Mutations affecting the intracellular domain involve codons 768, 790, 791 (exon 13), 804 (exon 14), 883 and 891 (exon 15), and 918 (exon 16) and these may interfere with intracellular ATP binding of the tyrosine kinase receptor (4).

Carriers of mutations in codon 634 present frequently the full-blown MEN2 syndrome where MTC may be accompanied by PHEO in 50% and PHP in 25% of the cases. Mutations in exon 10 at codons 609, 611, 618, and 620 and in exon 11 at codon 634 (5) are the commonest among the familial cases accounting for 88–98% of the cases. Less frequently, mutations have been reported in the non-cysteine *RET* region such as at codon 804 in exon 14, codon 883 in exon 15, and codon 533 in exon 8 (6, 7). Mutations at codon 918 in exon 16 are found in 95% of MEN2B patients and appear to be specific for this syndrome (5). MEN2 is a rare disease. In recent years, referral centers in several countries have gathered a considerable number of families and published their results which enabled us to correlate *RET* mutations with the phenotype (8, 9).

Currently screening of the *RET* gene is widely applied for the detection of inherited disease, the identification of gene carriers, and thus for preclinical diagnosis of MTC in these families.

As the spectrum of mutations may vary between centers, it is important to characterize the mutations in each population. This information could differentiate the orientation of the genetic analysis to include more than the standard *RET* mutation screening panel which includes only the exons 10, 11, 13–16. In this study, we report the results of *RET* gene screening performed in the Endocrine Units of 'ALEXANDRA' University Hospital, over the last 20 years in subjects diagnosed with MTC after histological confirmation and in family members of *RET*-positive patients with MTC.

Subjects and methods

Subjects

Over a period of 20 years we examined 313 Greek individuals for mutations of the *RET* gene in exons 8, 10,

11, and 13–16 either because they had been diagnosed with MTC, or because they had a relative with MTC. In our center, the majority of newly diagnosed cases from central and southern Greece are referred.

One hundred and eighty-eight of them (120 women and 68 men) were independent individuals belonging to different families. Of these, 133 presented as sporadic MTC cases without other features of MEN2 or family history, 51 patients had clinical suspicion for MEN2 and four patients had only C cell hyperplasia (CCH). Of the 51 suspects for MEN2, 22 had characteristic features of MEN2A syndrome (MTC with PHEO or PHP), five presented with clinical features of MEN2B syndrome, 23 had familial disease, with at least one relative with proven MTC or suspicious for MTC (such as a grandparent having died of 'neck tumor'). One patient who only had bilateral PHEO without MTC was included in the analysis because he was found to carry the exon 11, codon 634 mutation which is the characteristic of MEN2 syndrome. This patient has not developed MTC yet. His latest basal calcitonin (CT) levels were 12 pg/ml and after calcium infusion 128 pg/ml (last screening was at the age of 32 years); thyroid ultrasound was normal. Four patients had only CCH. This group was identified from histological findings after thyroidectomy was performed for multinodular goiter. The remaining 125 individuals were family members of *RET* carriers. Informed consent was given by all subjects participating in the study for *RET* screening. The investigation was approved by the hospital ethics committee.

Methods

DNA extraction, PCR amplification, and sequencing ► A venous blood sample was collected from all patients in EDTA-coated tubes. Genomic DNA was purified from peripheral blood lymphocytes using the QIAMP DNA Mini Kit (Qiagen). *RET* exons 8, 10, 11, and 13–16 were analyzed using PCR and DNA sequencing as follows:

During the first 10 years, we used the manual Sanger sequencing method (Cycle Sequencing Kit, Amersham). More recently, the Mega Bace Sequencing Analyzer was used. The sequencing reaction was performed in a 20 µl final volume containing 5 pmol of the relevant sequencing primers of DYEnamic ET dye terminator kit of Mega Bace, Amersham Biosciences. In 45% of the samples, the results were confirmed with pyrosequencing (Pyrosequencing AB, Uppsala, Sweden).

Specifically, the screening for exon 8 mutation was performed in all our samples by allelic discrimination using Custom TaqMan SNP Genotyping assay in a 7500

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Real-time PCR System (Applied Biosystems). In the ten positive samples and most of the negative for apparently sporadic patients, sequencing was also performed to confirm the results (7).

Results

Genetic screenings of the 188 unrelated patients revealed 13 different *RET* mutations in 58 carriers (Table 1). Of them, 29.3% (17/58) had MEN2A syndrome, 8.6% (5/58) MEN2B syndrome, and 62.1% (36/58) had FMTC (Fig. 1).

Six subjects with MTC who had additional characteristics of MEN2 syndrome (one patient had MTC+PHEO+neurofibromas, two had MTC+PHEO, and three patients had MTC+PHP), all with negative family history, were found *RET* negative and classified as of 'unknown cause'. The four patients with CCH were *RET* negative.

Of the 133 patients considered as sporadic, 13 were found *RET* positive (9.8%): ten of them carried the exon 8 codon 533 mutation as previously reported (7), two were found to carry the exon 14, codon 804 mutation and one had the exon 13, codon 768 mutation. The remaining 120 patients with only MTC and no *RET* mutation were classified as sporadic.

In all cases, MTC was the first manifestation of the syndrome except for a 29-year-old man who presented to our center with bilateral PHEO as the only manifestation. Genetic screening revealed a mutation at codon 634 in exon 11 and the patient was assigned to the MEN2A group. None of his brothers or sisters was found to carry the mutation; the parents were not available to be tested. This patient had not developed MTC at his latest screening.

We had the opportunity to examine one large kindred with 27 members (family number 1) through four generations (Fig. 2). The index case was a 32-year-old woman originally characterized as FMTC (Fig. 2, C10). Twenty-one out of 27 family members screened in our center were found to carry the 620 TGC/TAC mutation in exon 10. After 8 years of follow-up, one MTC woman in the third generation developed PHEO (Fig. 2, C4); recently, her daughter also developed PHEO (Fig. 2, D3). Interestingly, a young girl in the fourth generation of another branch of the same family developed Hirschsprung's disease (HIRSC; Fig. 2, D1). Thus, during follow-up, this family was reclassified as MEN2A (Table 1).

Distribution of *RET* mutations

In the group of 58 *RET* positive patients, the most prevalent mutation was the Gly533Cys of exon 8

(36.2%, 21/58) followed by mutations at codon 634 at a percentage of 32.8% (19/58). The Cys634Arg substitution was present in 57.9% of codon 634 mutations (11/19), while its prevalence in *RET* positive cases was 19% (11/58). A lower prevalence of mutations was found at exon 10 (15.5%, 9/58); exon 16, codon 918 (8.6%, 5/58); exon 14, codon 804 (5.2%, 3/58); and exon 13, codon 768 (1.7%, 1/58) (Fig. 3). We did not find mutations in codons 609 and 611 of exon 10 or in codons 790/791 of exon 13 in our patients (Fig. 3).

Family members

We examined 125 family members belonging to the 58 *RET*-positive families: 61 of them (48.8%) were found to carry a *RET* mutation and 64 were negative (Table 1).

Discussion

The *RET* mutation testing for inherited MTC is a unique paradigm of the value of genetic screening in the early diagnosis, prevention, and treatment of cancer, which has been of great importance for the management of patients with MTC. The indications for screening and intervention follow a mutation-based risk classification for MTC patients and carriers according to the guidelines of the American Thyroid Association (ATA) (10) and the European Thyroid Association (ETA) (11). However, the spectrum of mutations is not the same in all ethnic groups. The first International Consortium for *RET* mutation analysis identified only eight hot spots for mutations and described their distribution in the subtypes of MEN2 syndromes (MEN2A, MEN2B, and FMTC) (4). However, in the two largest series that have been presented recently, several new mutations have been described and a large variability in the mutation distribution in the MEN2 population in different countries in Europe and worldwide has been shown. Forty different mutations are mentioned in both reports: one report from three European countries; Germany, Italy, and France (8) and the other from Italy (9).

Variability of mutations spectrum in different ethnic groups

The exon 8 Gly533Cys mutation appears to be the characteristic of our ethnic group with a prevalence of 36.2% in hereditary cases. This mutation appears to be specific to the Mediterranean region (7, 12) and has rarely been found in other series. Interestingly, the first

Table 1 Clinical characteristics of the index cases in each of the families diagnosed with hereditary MTC in our center. The affected codon, the resulting amino/acid change, and relative exon are also given.

Family no.	Age	Exon	Codon	Base change	Amino/acid change	Clinical diagnosis	GC/total family members
1	32	10	620	TGC/TAC	CYS/TYR	MEN2A (MTC + PHEO + HIRSC)	21/27
2	36	10	618	TGC/CGC	CYS/ARG	MEN2A (MTC + PHEO)	1/7
3	35	10	620	TGC/CGC	CYS/ARG	FMTC	2/5
4	30	10	620	TGC/CGC	CYS/ARG	FMTC	3/3
5	31	10	618	TGC/TAC	CYS/TYR	FMTC	1/4
6	50	10	620	TGC/CGC	CYS/ARG	FMTC	2/3
7	20	10	618	TGC/TCC	CYS/SER	FMTC	1/1
8	60	10	618	TGC/CGC	CYS/ARG	MEN2A (MTC + PHEO)	1/1
9	35	10	620	TGC/TTC	CYS/PHE	FMTC	1/1
10	29	11	634	TGC/CGC	CYS/ARG	MEN2A (MTC + PHEO)	3/6
11	41	11	634	TGC/TAC	CYS/TYR	FMTC	2/3
12	34	11	634	TGC/CGC	CYS/ARG	MEN2A (MTC + PHEO + PHP)	4/8
13	50	11	634	TGC/CGC	CYS/ARG	MEN2A (MTC + PHEO)	6/8
14	28	11	634	TGC/CGC	CYS/ARG	FMTC	3/6
15	20	11	634	TGC/TAC	CYS/TYR	MEN2A (MTC + PHEO)	2/3
16	31	11	634	TGC/CGC	CYS/ARG	FMTC	1/3
17	25	11	634	TGC/TAC	CYS/TYR	FMTC	2/3
18	30	11	634	TGC/TAC	CYS/TYR	FMTC	1/1
19	25	11	634	TGC/TTC	CYS/PHE	MEN2A (MTC + PHP)	4/7
20	42	11	634	TGC/TAC	CYS/TYR	MEN2A (MTC + PHEO)	4/7
21	35	11	634	TGC/TAC	CYS/TYR	MEN2A (MTC + PHEO)	1/7
22	25	11	634	TGC/CGC	CYS/ARG	MEN2A (PHEO BILAT)	1/1
23	33	11	634	TGC/CGC	CYS/ARG	MEN2A (MTC + PHEO)	2/3
24	29	11	634	TGC/TAC	CYS/TYR	FMTC	1/1
25	23	11	634	TGC/CGC	CYS/ARG	MEN2A (MTC + PHEO + PHP)	1/2
26	21	11	634	TGC/CGC	CYS/ARG	MEN2A (MTC + PHEO)	2/3
27	22	11	634	TGC/CGC	CYS/ARG	FMTC	2/2
28	36	11	634	TGC/CGC	CYS/ARG	FMTC	1/1
29	36	8	533	GGC/TGC	GLY/CYS	FMTC	1/1
30	40	8	533	GGC/TGC	GLY/CYS	FMTC	1/1
31	50	8	533	GGC/TGC	GLY/CYS	FMTC	2/3
32	51	8	533	GGC/TGC	GLY/CYS	FMTC	2/3
33	28	8	533	GGC/TGC	GLY/CYS	FMTC	1/1
34	62	8	533	GGC/TGC	GLY/CYS	FMTC + PTC	1/1
35	38	8	533	GGC/TGC	GLY/CYS	FMTC	3/3
36	39	8	533	GGC/TGC	GLY/CYS	FMTC	1/1
37	55	8	533	GGC/TGC	GLY/CYS	FMTC	4/4
38	50	8	533	GGC/TGC	GLY/CYS	MEN2A (MTC + PHEO BILAT)	1/1
39	68	8	533	GGC/TGC	GLY/CYS	FMTC	1/1
40	72	8	533	GGC/TGC	GLY/CYS	FMTC	1/3
41	43	8	533	GGC/TGC	GLY/CYS	FMTC	1/1
42	36	8	533	GGC/TGC	GLY/CYS	FMTC	1/1
43	58	8	533	GGC/TGC	GLY/CYS	MEN2A (MTC + PHEO)	1/2
44	40	8	533	GGC/TGC	GLY/CYS	FMTC	2/2
45	56	8	533	GGC/TGC	GLY/CYS	MEN2A (MTC + PHP)	1/1
46	18	8	533	GGC/TGC	GLY/CYS	FMTC	4/6
47	46	8	533	GGC/TGC	GLY/CYS	FMTC	1/1
48	47	8	533	GGC/TGC	GLY/CYS	FMTC	1/2
49	37	8	533	GGC/TGC	GLY/CYS	FMTC	3/5
50	15	16	918	ATG/ACG	MET/THR	MEN2B	1/4
51	15	16	918	ATG/ACG	MET/THR	MEN2B	1/1
52	15	16	918	ATG/ACG	MET/THR	MEN2B	1/1
53	16	16	918	ATG/ACG	MET/THR	MEN2B	1/1
54	6	16	918	ATG/ACG	MET/THR	MEN2B	1/1
55	28	13	768	GAG/GAC	GLU/ASP	FMTC	1/1
56	50	14	804	GTG/ATG	LEU/MET	FMTC	1/1
57	43	14	804	GTG/ATG	LEU/MET	FMTC	1/1
58	48	14	804	GTG/ATG	LEU/MET	FMTC	1/1

MTC, medullary thyroid cancer; GC, gene carriers; FMTC, familial MTC; MEN2B, multiple endocrine neoplasia type 2B; PHP, primary hyperparathyroidism; PHEO, pheochromocytoma; HIRSC, Hirschsprung's disease; PTC, papillary thyroid cancer.

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Gly533Cys-positive family recently reported in USA was of Greek ancestral origin (13). This rare mutation has occasionally been identified in other Mediterranean regions; at least one Gly533Cys family has been reported in South Portugal and a few families in the French registry (V Leite and D Prunier 2014, personal communication). One patient of Slovenian origin with the same mutation has also been reported in Ireland (14). The fact that not every *RET* gene screening program includes exon 8 might be the reason for the rarity of Gly533Cys-positive families outside Western Europe.

Two other countries are reported to have a high prevalence of unusual mutations: a large multicenter study from Italy (ItaMEN study) (9) reported that the rare mutation Val804Met in exon 14 is found with a prevalence of 19.6%, especially in Sardinia 59%, followed by the Cys634Arg substitution in exon 11 (13.6%) (9, 15). At codons 790/791 in exon 13, another mutations' spot appears with high prevalence in Germany at 13 and 7% respectively (8).

Apparently, founder effects are responsible for this variability in the frequency of mutations. Notably, the codon 533 in exon 8 mutation is found especially in the southern part of central Greece and the south east region of Peloponnese (7). So far, no exon 8 mutations have been reported at a referral center of Northern Greece (K Pazaitou-Panayiotou 2014, personal communication). Studies underway indicate that the Greek families may share a common haplotype with the first described family carrying this mutation that was of Brazilian/Spanish origin (R M Maciel and M Alevizaki 2014, unpublished).

The exon 11 mutations that are the most frequent in the literature (4, 8, 9) come in second range in our center with a percentage of 32.8% (19/58). Eleven of these cases carry the Cys634Arg (TGC/CGC) mutation, which is also the commonest in other series.

In recent years several other groups have published the genetic findings in small series of MTC patients with familial disease. Mutations in both exons 10 or 11 (Spain, Portugal, India, Saudi Arabia, Korea, and Czech Republic) and only 11 (Turkey and Taiwan) have been reported (16, 17, 18, 19, 20, 21, 22, 23). Especially in the Czech Republic a higher than usual prevalence of mutations in exons 13 and 14 (17.45 and 13.0% respectively) has been reported (20).

The compiled data from three European countries that have recently been published show the distribution of different *RET* mutations as follows: 34.2% at codon 634, 17.2% at codon 804, 10.4% at codon 918, 7.6% at codon 790, 7.2% at codon 891, 7.0% at codon 620, 6.4% at codon 618, 3.2% at codon 791, 2.6% at codon 768,

1.6% each at codons 609 and 611, and 1.0% at codon 630. In this large series, there is no report of mutation at codon 533 in exon 8 (8).

One MTC family with mutation in exon 8 but at the 515 codon (not 533) was reported in Italy by Fazioli *et al.* (24). A comparison of our data with those of other European cohorts is given in Table 2. Mutations in codons 609, 611, and 790/791, occasionally found in Europe, were not identified in our center. From this table, it is obvious that, with a few exceptions, the highest prevalence still concerns codon 634 in exon 11, which is associated with a more dramatic clinical picture, more frequent PHEO co-existence, and more aggressive MTC and is thus more frequently identified to represent familial disease.

Phenotype of hereditary cases in our center: the majority are FMTC

In our center, we have observed one of the highest percentages of hereditary forms of MTC (30.8%, 58/188) compared with 25% that are globally reported (11). One cannot exclude a bias, as this was the first center performing *RET* screening in our country. In a study in the Iranian population, the depicted 13 *RET*-positive families represent 24.5% (13/53) while in a study from

Figure 1

Distribution of the hereditary (*RET* positive) cases of medullary thyroid cancer (MTC) after the genetic screening of apparently sporadic cases. One FMTC family has been re-classified as MEN2A after the appearance of pheochromocytoma in the third generation. FMTC, familial MTC; MEN2A, multiple endocrine neoplasia syndrome type 2A.

* Index case

Figure 2

One large kindred followed in our center for four generations. The index case is shown with an asterisk. The age at which the MTC diagnosis was made in each of the RET positive cases is

shown in parenthesis. PHEO, pheochromocytoma; HIRSC, Hirschsprung's disease.

the Czech Republic they represent 21.7% (23/106) of MTC patients (20, 25). The relevant percentage in Spain is 22.8% (26/114) (22).

The presence of only CCH was not found to be associated with genetic disease in our series, in agreement with the report by Scheuba *et al.* (26). Thus, incidental CCH is probably not a strong indication for genetic screening.

In our center, the prevalence of inherited MTC without any features of MEN2A was higher (62.1%) compared with the 10–20% mentioned in the first Consortium for MEN2 (4) or the 35–60% reported recently by Raue & Frank-Raue (27). Our findings are closer to the 57.6% reported in the ItaMEN study (9). It should be noted that in the 'FMTC' group, we have included the cases where pathogenic *RET* mutation was found even when the

family history was negative, because on many occasions the relatives were not available for the study.

Many investigators consider FMTC as a subtype of MEN2A, given the fact that in some MTC families PHEO or other MEN2A manifestations may appear in the third or fourth generation or later, as in family number 1 in our series. Moers *et al.* (28) have also reported a large MEN2A family followed through several generations, in which only two subjects developed PHEO in the fourth generation and two developed PHP among the 150 screened family members.

In family number 1 of our series, HIRSC developed for the first time in the fourth generation (Fig. 1, D1). HIRSC in the context of MEN2A syndrome has invariably been associated with exon 10 mutations, which is in agreement with our results (27). Interestingly, 7.5% of exon 10

Figure 3

The prevalence (%) of different *RET* mutations in our cohort of 58 Greek families with hereditary medullary thyroid cancer (left). The position of mutations (codon and exon) in the *RET* gene in our cohort is shown in a schematic representation on the right side of the picture.

mutations carriers are reported to have HIRSC (27). For this reason, Fugazzola *et al.* (29) recommend screening of exon 10 in all individuals with HIRSC. We have considered the possibility that an anticipation phenomenon might have occurred in family number 1. The mean age at diagnosis in the second generation was 68.7 years dropping to 30 years in the third generation and 8.8 years in the fourth generation. However, TNM stage did not substantially change between second and third generations. It should be taken into account that once the mutation was characterized in a family member, the next generation was tested and operated at a much earlier age.

As concerns the clinical presentation, in our series there is a preponderance of rather mild disease. This is due to the following reasons: the high percentage of FMTC in

which MTC appears usually at later age and is less aggressive and also to the high prevalence of the Gly533Cys mutation which is associated with a milder form of the disease.

Conclusions

Genetic screening is a very useful diagnostic tool and is recommended by ATA (10) for all MTC patients. The ETA guidelines also recommend genetic screening in all apparently sporadic cases (11). This strategy is expected to increase the probability of identifying new MEN2 kindreds. Thus, the mutations associated with lower penetrance are likely to be enriched in other series as well in the future. The reason why the 634 mutation appears to be the most prevalent in practically all the previous publications is obviously the fact that the earlier onset of disease and the frequent combination with PHEO made the referral and diagnosis easier. The identification of the rare occurrence of PHEO in members of families, which for generations were classified as FMTC, stresses the need for continuous awareness for PHEO in such families as well. The recognition of the specific pattern of *RET* mutations for each country will help toward a more focused screening which is expected both to speed up the diagnosis and also to lower the cost. This is more helpful in areas where there is a high prevalence of rare *RET* mutations, not included in the commonly screened panel (exons 10, 11, 13–16) which is likely to be replaced in the future with whole-gene screening when next generation sequencing becomes more widely used.

Table 2 The spectrum of *RET* mutations identified in families with hereditary medullary thyroid cancer in our center in Greece compared with those described by other European groups. The prevalence (*n*(%)) of each mutation is shown (data from references (8, 20, 22)).

Codon	Germany	Italy	France	Spain	Czech Republic	Greece
533	0	0	0	0 (0)	0	21 (36.2)
600	0	0	0	1 (3.85)	0	0
609	1 (0.5)	6 (3)	1 (1)	1 (3.85)	1 (4.2)	0
611	6 (2.9)	1 (0.5)	1 (1)	0	1 (4.2)	0
618	11 (5.4)	15 (7.6)	6 (6.2)	1 (3.85)	0	4 (6.9)
620	14 (6.8)	9 (4.5)	12 (12.4)	1 (3.85)	0	5 (8.6)
630	1 (0.5)	4 (2)	0	0	0	0
634	73 (35.6)	52 (26.3)	46 (47.4)	17 (65.4)	11 (45.8)	19 (32.8)
768	2 (1)	9 (4.5)	2 (2.1)	1 (3.85)	1 (4.2)	1 (1.7)
790	26 (12.7)	8 (4)	4 (4.1)	0	0	0
791	14 (6.8)	2 (1)	0	0	3 (12.5)	0
804	19 (9.3)	52 (26.3)	15 (15.5)	0	3 (12.5)	3 (5.2)
883	0	0	0	0	0	0
891	6 (2.9)	23 (11.6)	7 (7.2)	1 (3.85)	0	0
918	32 (15.6)	17 (8.7)	3 (3.1)	3 (11.5)	4 (16.6)	5 (8.6)
Total	205	198	97	26	24	58

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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