RET Cys634Arg mutation confers a more aggressive multiple endocrine neoplasia type 2A phenotype than Cys634Tyr mutation

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Abstract

Objective: Specific germline mutations in the *RET* proto-oncogene are correlated with clinical features in multiple endocrine neoplasia type 2A (MEN2A); however, data are scarce regarding differences in clinical profiles dependent on the type of nucleotide and amino acid substitution at the same codon. We aimed to analyse differences in clinical risk profiles and outcomes among different amino acids encoded by codon 634.

Design: The study was retrospective and multicentric.

Methods: We collected data included in the Spanish Online National Database from patients with MEN2A carrying a *RET* proto-oncogene mutation on codon 634. The mean follow-up time was 7.6 ± 6.9 years (1–32).

Results: Patients ($n=173$) from 49 unrelated families were C634Y carriers, and 26 patients from eight different families had C634R mutation. We found higher penetrance of medullary thyroid carcinoma, phaeochromocytoma and hyperparathyroidism ($P<0.001$, $P=0.007$ and $P<0.001$ respectively) in C634R carriers than in C634Y carriers. The Kaplan–Meier estimate of cumulative lymph node and distant metastases rates showed that these events occurred earlier in patients harbouring the C634R mutation ($P<0.001$). A multivariate adjusted Cox regression analysis indicated that the C634R mutation was an independent factor for persistent/recurrent disease (hazard ratio, 3.17; 95% CI: 1.66–6.03; $P<0.001$).

Conclusions: Our results suggest that there could be clinical differences caused by different amino acid substitutions at codon 634; specifically, the C634R mutation was associated with a more aggressive MEN2A phenotype than the C634Y mutation.

Introduction

Multiple endocrine neoplasia type 2A (MEN2A) is a rare syndrome characterised by the presence of medullary thyroid carcinoma (MTC: 95% of MEN2A patients), phaeochromocytoma (PHEO: 30–50%) and hyperparathyroidism (HPT: 10–20%) (1, 2). This syndrome is caused by a germline activation mutation in the rearranged during transfection (*RET*) proto-oncogene transmitted by an autosomal dominant inheritance (3, 4).

The *RET* proto-oncogene encodes a transmembrane tyrosine kinase receptor that comprises a highly conserved, cysteine-rich, extracellular domain, two intracellular tyrosine kinase domains and an intracellular catalytic core (3, 5). In the majority of MEN2A families (more than 90%), germline mutations affect the cysteine-rich extracellular domain by converting a cysteine into another amino acid, and this mutation determines RET spontaneous dimerisation and activation (1, 2, 3, 4, 5). These mutations are located on codon 634 (exon 11) or codons 609, 611, 618 and 620 (exon 10).

Genotype–phenotype correlation has been described between specific codon mutation and the age of onset, penetrance and clinical aggressiveness of MTC (1, 2, 6, 7). Based on these findings, the American Thyroid Association (ATA) Task Force has published management guidelines (8) on the timing and extension of prophylactic thyroidectomy establishing four different risk categories (from A, the lowest, to D, the highest). There is also a different prevalence of PHEO and HPT according to the type of specific codon mutation (7, 9, 10).

The most common mutation, accounting for 34% of European MEN2A families, affects codon 634 (11). 'In vitro' assays have demonstrated that the codon 634 mutation had a higher probability of transforming NIH3T3 cells than the other *RET* codon mutations (12). This biological characteristic of the highest transforming activity correlates with phenotype because codon 634 mutations are associated with the more severe forms of the MEN2A phenotype and it is classified as class C in the ATA guidelines (8).

There are six different amino acid substitutions for the same cysteine on codon 634, and all of them displayed comparable transforming activity in one 'in vitro' assay (12), indicating that the degree of the activity depends on the position of cysteine mutations rather than the substituted amino acids. However, the assay was performed only in fibroblasts; thus, the biological effects of the substituted amino acids are not known in thyroid C cells, adrenal medullary cells and parathyroid cells. From a clinical viewpoint, few reports have indicated whether there is any difference in the clinical profiles based on amino acid substitutions on codon 634 (4, 6, 9, 10, 13).

The aim of our study was to evaluate any potential differences in clinical risk profiles and outcomes in Spanish patients caused by different mutations on codon 634. This information might be useful for tailoring more accurate clinical care.

Cys634Arg (C634R) is the most common *RET* mutation in European countries (14), except in Italy where Val804Met is more common (15). However, the most common *RET* mutation in MEN2A Spanish patients is Cys634Tyr (C634Y) (16). Consequently, we examined the largest number of patients ever reported with this specific mutation on codon 634, which provided a firmer basis for statistical analyses.

Subjects and methods

The Spanish Group for the Study of MEN and PHEOs/paragangliomas, which depends on the Spanish Endocrinology and Nutrition Society, designed an online national database to collect extensive clinical and genetic information about Spanish patients with MEN1, MEN2A, MEN2B and PHEOs/paragangliomas. Informed consent was obtained for all diagnostic and therapeutic procedures and for the inclusion of family and personal clinical data in the register. The anonymity of patients was not breached; therefore, the study met the conditions required under Spanish law for making medical and or personal data available for scientific research.

Data from patients diagnosed with MEN2A, according to the international clinical guidelines (1), were included in the database from January 2009 to December 2011 and were analysed in the course of 2012. All of these patients were diagnosed and clinically examined by the authors. The study was retrospective and multicentric.

Briefly, the data include sex, birth date, family history and the type of diagnosis (index cases and diagnosis by genetic or biochemical screening), the type of *RET* mutation, and the dates of diagnosis for the syndrome and for each lesion. The age at MEN2A syndrome diagnosis was considered the age at the second manifestation of any clinical features in index cases and the first manifestation or genetic diagnosis in familial cases. The age at diagnosis of clinical disease was considered the age at histological diagnosis for MTC and biochemical diagnosis for PHEO and HPT.

For thyroid disease, the following characteristics were recorded: calcitonin (CT) basal and/or pentagastrin-stimulated levels (positive or negative), extent of surgery and outcome after thyroidectomy. With respect to thyroid histopathology, we recorded the presence or absence of C-cell hyperplasia (CCH) and MTC. For lymph node status, we collected data on lymph node dissection as N0, N1 and Nx (non-conclusive data). For analysis, the N0 classification was used to describe N0 or Nx data. The same approach was applied for distant metastases, which were recorded at thyroid surgery. Tumour stage was assessed according to the 2002 UICC TNM classification (17).

Patients were considered biochemically free of the disease at the last follow-up, according to the international guidelines.

For PHEO, we collected data about the presence or absence of symptoms at diagnosis, uni- or bilateral involvement, type of surgery and clinical outcome.

For HPT, we included the presence or absence of clinical symptoms, type of surgery and clinical outcome.

Statistical analysis

Categorical variables were summarised as frequency counts and percentages. Continuous variables were summarised as the mean ± s.d., unless otherwise specified, and range. For continuous variables, normality was assessed using the Kolmogorov–Smirnov test, and logarithmic transformations were applied as necessary to ensure normal distribution. Baseline characteristics were compared using the two test or Fisher's exact test for qualitative variables, or the Student's *t*-test or Mann–Whitney *U* test for quantitative variables. The Kaplan–Meier estimates for age-related penetrance of MTC, PHEO, HPT, and lymph node and distant metastases and for biochemically free of disease prognosis were compared among the type of amino acid substitution using the long rank test. Risk factors for disease-free survival were assessed via the chi-square test or Fisher's exact test, and we also performed a Cox regression model. Five candidate risk factors were assessed: sex, age at the time of surgery, basal and/or pentagastrin-stimulated CT levels, lymph node metastases and type of amino acid substitution. Associations were expressed as hazard ratios (HRs) with their 95% CI. Results were considered statistically significant at $P<0.05$. All statistics were analysed using the SPSS 20 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

There were clinical and molecular data on 202 patients with MEN2A harbouring a mutation on codon 634 of the *RET* proto-oncogene: 173 patients from 49 unrelated families carried the C634Y mutation, 26 patients from eight unrelated families had the C634R mutation and only three patients harboured the Cys634Ser mutation, who were excluded from the study.

Mean age at MEN2A syndrome diagnosis was significantly higher in patients carrying the C634Y mutation than in patients with the C634R mutation (27.8 ± 18.1 years (age range: 0.5–75) vs 17.3 ± 13.1 years (range: 1.4–44); $P=0.001$). There were fewer female patients

carrying the C634Y mutation (52.3%) vs those carrying the C634R mutation (69.2%; $P=0.07$).

CCH was diagnosed only in patients diagnosed by genetic screening; CCH was present in 25 (14.4%) patients with the C634Y mutation at a mean age of 8.4 ± 5.5 years (range: 0.5–28), but only in two (7.6%) patients with the C634R mutation at 1.4 and 2 years of age (Table 1).

MTC was diagnosed in 148 (85.5%) patients carrying the C634Y mutation and in 24 (92%) patients harbouring the C634R mutation. We found a statistically significant difference in the mean age of diagnosis for MTC according to both *RET* mutation types and considering the diagnosis type, e.g. index case, and biochemical or genetic diagnosis (Table 1). Age-related penetrance for MTC was higher in patients with the C634R mutation than in patients with the C634Y mutation: 85 and 46% by age 30 rising to 100 and 85% by age 50 respectively ($P<0.001$) (Fig. 1). This higher age-related penetrance for MTC in patients harbouring the C634R mutation, compared with those with the C634Y mutation, was also present according to the type of diagnosis: index cases ($P=0.03$), biochemically

Table 1 Clinical characteristics and histopathological findings in the 199 thyroidectomised gene carriers with regard to the type of amino acid substitution on codon 634.

	Cys634Tyr $n=173$	Cys634Arg $n=26$	*P* value
CCH (%)	25 (14.53%)	2 (7.6%)	0.5
Mean age (years)	8.4±5.5	1.4; 2	
Range	(0.5–28.6)		
MTC (%)	148 (83.7%)	24 (92.3%)	0.54
Mean age	32.7±17.1	19.4±12.7	<0.01
Range	(4–75.1)	(1.5–44)	
Index cases (%)	28 (18.9%)	6 (25%)	
Mean age	43.7±15.4	28.3±12.6	0.039
Range	(14.7–75.1)	(13.1–43)	
Biochemical screening (%)	27 (18.3%)	13 (54.1%)	
Mean age	37.8±8	20.5±11	0.001
Range	(11.5–72)	(6.7–44)	
Genetic screening (%)	93 (63.2%)	5 (20%)	
Mean age	27.8±15.4	6.2±4.6	<0.001
Range	(4.0–72.4)	(1.5–13.9)	
Nodal and distant metastases (%)	31 (20.9%)	7 (29.1%)	0.28
Mean age	38.8±15.2	24.6±.5	0.008
Range	(16.5–75.1)	(13.1–41.5)	
Nodal metastases (%)	26 (17.5%)	4 (16.6%)	0.76
Mean age	37.8±15.8	23.41±2.4	0.094
Range	(16.5–75.1)	(13.1–41.5)	
Distant metastases (%)	5 (3.3%)	3 (12.5%)	
Mean age	43.7±11.8	26.3±5.9	0.03
Range	(34–63)		

CCH, C-cell hyperplasia; MTC, medullary thyroid carcinoma.
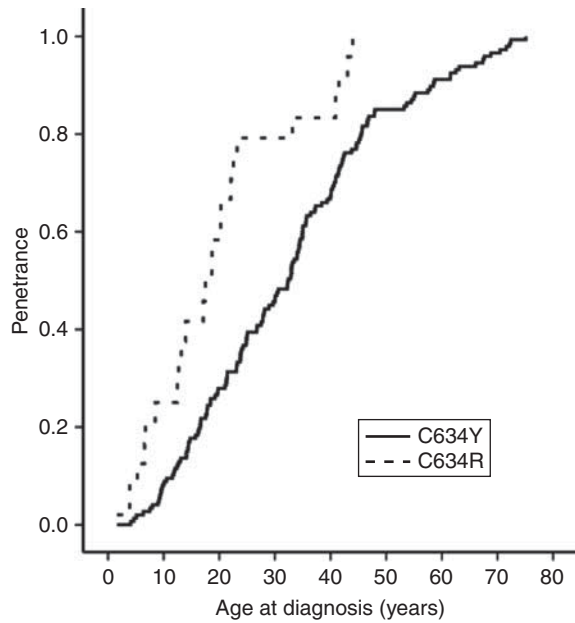
Figure 1

Age-related penetrance of MTC according to the specific codon 634 mutation. The log rank was used to compare curves (*P*<0.001).

diagnosed patients (*P*<0.001) and genetically diagnosed patients (*P*<0.001).

Lymph node metastases were present in 26 (17.5%) patients carrying the C634Y mutation and in four (16.6%) patients with the C634R mutation. Distant metastases were diagnosed in five (3.3%) C634Y patients and three (12.5%) C634R patients. Kaplan–Meier estimates of cumulative lymph node and distant metastasis rates demonstrated that these events occurred earlier in patients harbouring the C634R mutation than in those with the 634Y mutation (*P*<0.001) (Fig. 2).

Follow-up data were available after thyroidectomy in 178 patients; 21 patients were lost to follow-up after surgery and ten patients were excluded from the analysis because the follow-up period was <1 year. Thus, we analysed the results of thyroidectomy in 168 patients: 148 patients carried the C634Y mutation and 23 harboured the C634R mutation (Table 2). The mean follow-up time was 7.6±6.9 years (range: 1–32). During the follow-up, two patients with the C634Y mutation died: one of them died as a consequence of catecholaminergic crisis, and the other because of a metastatic MTC.

At the last follow-up, statistically significant number of patients with the C634Y mutation (92 out of 148 patients: 63.4%) were biochemically free of disease after

thyroidectomy, in contrast to only six out of 23 (26.1%) patients with the C634R mutation (*P*=0.001). The estimated cumulative frequency of being biochemically free of disease by Kaplan–Meier curves was lower in C634R carriers than in C634Y carriers (*P*<0.001) (Fig. 3). Kaplan–Meier curves, generated according to the type of diagnosis, demonstrated that index cases carrying the C634R mutation had a lower probability of being biochemically free of disease than C634Y index cases (*P*=0.03), but there was no difference in biochemically (*P*=0.16) or genetically diagnosed patients (*P*=0.72). We analysed patients who underwent a thyroidectomy before 20 years of age, and Kaplan–Meier curves demonstrated that C634R patients had a lower probability of being biochemically free of disease than C634Y patients (*P*<0.001).

We analysed five risk factors associated with the biochemically free of disease prognosis. Univariate analysis showed that the following factors were significantly associated with biochemically persistent or recurrent diseases: increased age at thyroidectomy (*P*=0.001), presence of lymph node metastases at surgery (*P*=0.001), preoperative elevated basal and/or pentagastrin-stimulated CT levels (*P*=0.01) and type of amino acid substitution (*P*=0.001). Basal or pentagastrin CT levels were not included in the multivariate analysis because they were abnormal in
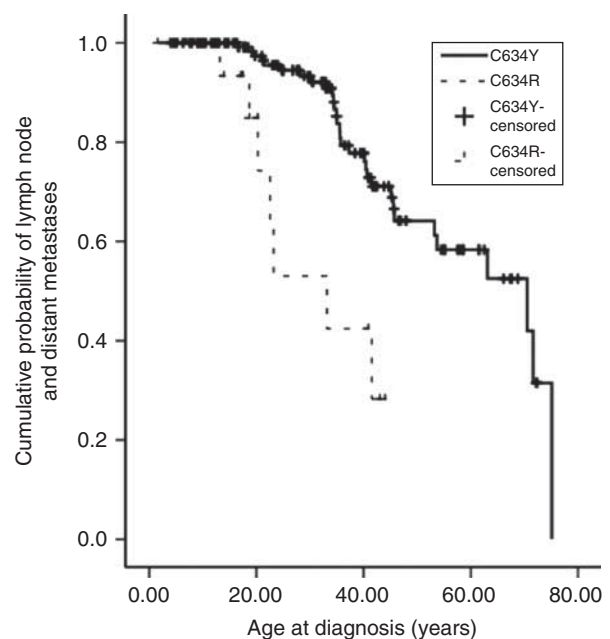
Figure 2

Kaplan–Meier estimates of the proportion of carriers with C634R and C634Y mutations with lymph node and distant metastases at the moment of diagnosis (*P*<0.001).

Table 2 Comparison of patients biochemically free of disease or not according to the type of amino acid substitution on codon 634.

	Cys634Tyr $n=148$	Cys634Arg $n=23$	P value
Biochemically cured (%)	92 (63.4%)	6 (26.1%)	0.003
Time of follow-up after surgery (years) (range)	6.9±6.3 (1–32)	11.6±8.6	0.01
Biochemically cured	6.2±5.3 (1–25)	10.8±10.8 (1–27)	0.3
Not biochemically cured	8.2±7.7 (1–32)	12±8.1 (1–25)	0.78
Mean age at surgery (years) (range)	29.1±18.1 (0.5–75)	17.5±13.2 (6.7–44)	<0.001
Biochemically cured	24.4±5.7 (0.5–61.5)	8.3±5.5 (1.4–17.1)	<0.001
Not biochemically cured	40.5±17 (14.3–75.1)	23.4±12.3 (6.7–44)	0.009
Lymph node metastases	23 (16.4%)	4 (20%)	0.75
Biochemically cured	7 (30.4%)	0	0.54
Not biochemically cured	16 (69.5%)	4 (100%)	

all patients with a persistent or recurrent disease. A Cox regression analysis, after adjusting for age and lymph node metastases, demonstrated that the type of amino acid substitution has an independent effect on being biochemically free of disease for a long term after surgery (HR, 3.17; 95% CI: 1.66–6.03; $P<0.001$).

PHEO was present in 70 (40.6%) patients carrying the C634Y mutation and 15 (57.6%) patients with the C634R mutation at a mean age of 40.6±15.2 years (range: 16.4–77.3) and 33.0±16.2 years (range: 14.5–65) respectively ($P=0.1$). Age-related penetrance was higher in C634R patients than in C634Y patients (50% vs 16% by age 30 and 83% vs 57% by age 50 ($P=0.001$)), and it was higher in C634R index cases and in C634R biochemically- diagnosed patients compared with C634Y index cases ($P=0.04$) C634Y and biochemically- diagnosed patients ($P<0.001$). Patients diagnosed by genetic screening were not analysed, because only one C634R genetic carrier was diagnosed with PHEO.

We did not find any statistically significant difference regarding the prevalence of symptoms, bilateral or unilateral adrenal involvement or recurrence rate after surgery between the two amino acid substitutions.

HPT was diagnosed in four (2.2%) patients with the C634Y mutation and in five (19.2%) patients carrying the C634R mutation ($P=0.002$) at a mean age of 40.1±26 years (range: 18–73) vs 32.5±14.9 years (range: 17.7–54.5) respectively ($P=0.62$). By age 30, HPT penetrance was 1% in C634Y carriers and 21% in C634R carriers, and, by age 50, HPT penetrance remained constant at 1% in C634Y carriers and reached 50% in C634R carriers ($P=0.001$).

Discussion

Our findings from documented clinical data suggest that there could be clinical differences between the C634R and C634Y mutations, because we found that carriers with the

C634R mutation had higher penetrance of MTC, PHEO and HPT than those with the C634Y mutation. Furthermore, compared with C634Y carriers, C634R mutation carriers developed MTC, and lymph node and distant metastases at an earlier age. These results confirm and extend previous suggestions by Puñales *et al.* (13) that substitution of amino acid Arg for Cys on codon 634 of the *RET* proto-oncogene might confer more aggressive behaviour than Tyr amino acid substitution.

In contrast, another study did not find any differences in the mean age of developing CCH, MTC and lymph node metastases with respect to different amino acid substitutions at codon 634 (18), but the study included only asymptomatic
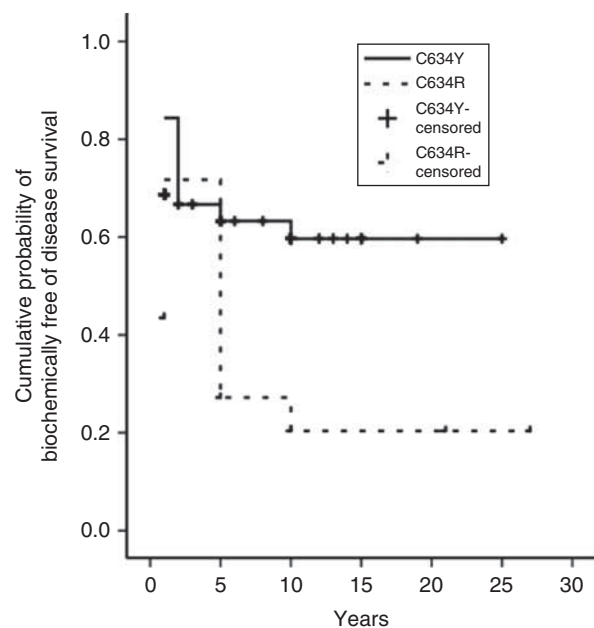
Figure 3

Cumulative probability of biochemically free- of- disease survival in patients with C634R and C634Y mutations ($P<0.001$).

patients, below the age of 20 years and without distant metastases at the moment of diagnosis. Conversely, our investigation and the previous study by Puñales *et al.* (13) analysed data from both index patients, those diagnosed by screening, any age and with or without distant metastases at the time of thyroidectomy; consequently, we have been able to observe the natural course of this disease.

The C634R mutation also increased the risk for more aggressive disease behaviour in the long term. Specifically, the percentage of patients biochemically free of disease after thyroidectomy was significantly lower in the C634R patients than in the C634Y patients, despite undergoing surgery at an earlier age than C634Y patients. This result was confirmed by Kaplan–Meier curves and multivariate analysis in which the C634R mutation was found to be an independent risk factor for persistent or recurrent diseases. Notably, to our knowledge, this is the first time that a study has shown this result. Few studies examined the long-term outcome after thyroidectomy in MEN2A patients (19, 20, 21, 22); moreover, only one of them (21) analysed differences in the long-term outcome between the different variants of codon 634 mutations, and that study did not find any differences. This previous study had analysed a smaller number of patients than our investigation, and patients were below the age of 20 years. In contrast, in our study, the C634R mutation conferred a lower probability of being biochemically free of disease after thyroidectomy even in patients below the age of 20 years who were the subject of the previous study.

PHEO is expressed differently between different *RET* codon mutations (1, 2, 7, 10); even one previous report (10) described variable rates of PHEO with different amino acid substitutions at codon 618, but not at 634. That previous study found a similar frequency of PHEO in C634R and C634Y carriers to that found in our data, but the age-related PHEO penetrance was not determined. In agreement with our results, the MEN Consortium of Japan (23) reported a very similar age-related PHEO penetrance in C634R and C634Y genetic carriers. That Japanese study (23) observed a statistically significant difference between age-related PHEO penetrance in C634R carriers versus C634G/C634F carriers combined, but, unlike our study, there was no specific comparison of the C634R mutation with C634Y.

There is controversy regarding whether the C634R mutation confers a greater risk of developing parathyroid disease (4, 7, 9, 24, 25). Our investigation showed a significantly higher HPT prevalence in C634R carriers than in those with the C634Y mutation, despite the fact that more patients above the age of 40 years harboured the C634Y

mutation and that the risk of developing HPT increases beyond this age. Furthermore, the mutation-specific penetrance data in our study also showed higher penetrance of the C634R mutation than the C634Y mutation. Our data regarding HPT prevalence and age-related penetrance in C634R carriers agree with a previous report (24), which found higher HPT age-related penetrance in C634R carriers than in patients with any other amino acid exchange at codon 634; an individual comparison was not performed between specific amino acid substitutions at codon 634.

Our investigation has several limitations, including the most critical limitation, the retrospective study design; thus, it has the drawbacks associated with this type of research. Another limitation is the small number of patients and families with the C634R mutation, but the results of the statistical analysis were very strong. Indeed, our findings were reinforced by the statistical analysis in index patients, which was statistically significant. This point is an important issue, because index patients facilitate observation of the natural course of the disease without any medical interference. Moreover, as we have discussed earlier in this study, our data extended and confirmed previous suggestions. Furthermore, our data agree with previous reports on patients from Europe, North and South America, and Japan regarding prevalence and age-related neoplastic risk profiles. In addition, a founder effect or other molecular events in C634R and C634Y genetic carriers could be ruled out as a cause of our results.

Taking into account the findings of previous studies (13, 23, 24), and our own findings, we hypothesise, in agreement with others (10), that specific amino acid substitutions could change the protein biochemistry altering its function; thus, the C634R mutation could confer a higher penetrance of the MEN2A phenotype and more aggressive behaviour of MTC than the C634Y mutation.

It will be useful to continue collecting clinical data to confirm or refute these observations and to extend research to other amino acid substitutions at codon 634, in order to tailor more precise genetic counselling, diagnosis and treatment, based on the nature of the codon mutation.

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