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Maylis Lebeault, Stéphane Pinson, Marine Guillaud-Bataille, Anne Gimenez-Roqueplo, Alain Carrié, et al.. Nationwide French Study of RET Variants Detected from 2003 to 2013 Suggests a Possible Influence of Polymorphisms as Modifiers. *Thyroid*, 2017, 27 (12), pp.1511-1522. 10.1089/thy.2016.0399 . hal-01731580

HAL Id: hal-01731580

<https://amu.hal.science/hal-01731580>

Submitted on 12 Apr 2018

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Nationwide French study of *RET* variants detected from 2003 to 2013 suggests a possible influence of polymorphisms as modifiers

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Short title: *RET* variants in France

Key words: *RET* oncogene, Multiple Endocrine Neoplasia type 2 (MEN2), genetic variants, medullary thyroid cancer (MTC), pheochromocytoma (PHEO)

ABSTRACT

Background: The presence of single nucleotide polymorphisms (SNPs) in the *RET* (*REarranged during Transfection*) gene has been investigated with regard to their potential role in the development or progression of medullary thyroid cancer (MTC) or pheochromocytomas (PHEO) in patients with the multiple endocrine neoplasia type 2 (MEN2) syndrome. The aim of this study was to evaluate the spectrum of *RET* variants in France between 2003 and 2013, and an evaluation of the impact of SNPs on the MEN2 A phenotype.

Methods: In this retrospective cohort study *RET* variants were screened in 5109 index cases and *RET* pathogenic variants were screened in 2214 relatives. Exons 5, 8, 10, 11, 13, 14, 15 and 16 were characterized by Sanger sequencing. *RET* Non-SNP pathogenic variants, *RET* variants with unknown functional significance (VUS), and four *RET* SNP variants, i.e. G691S (rs1799939), L769L (rs1800861), S836S (rs1800862), and S904S (rs1800863) were characterized and are reported in index cases. *In silico* analysis and classification following the recommendation of the ACMG (American College of Medical Genetics and Genomics) was performed for *RET* variants with unknown functional effects. Each patient's age at the time of diagnosis, sex, and the endocrine neoplasias present at molecular diagnosis were recorded.

Results: Twenty-six single non-SNP *RET* variants VUS in *RET* without any well-defined risk profiles were found in 33 patients. Nine of these were considered probably pathogenic, 11 of uncertain significance, and 6 as probably benign. Three double pathogenic variants found in 3 patients were classified as pathogenic. A study of the entire cohort showed that patients carrying non-SNP pathogenic variants or VUS in *RET* together with PHEO were diagnosed earlier than the others. The presence of the G691S SNP, or a combination of SNPs, increased the risk of developing PHEO, but did not modify the date of the diagnosis. No association was found between SNPs and medullary thyroid cancer (MTC) or hyperparathyroidism (HPTH).

Conclusions: Our finding propose a classification of 15 of the 26 non-SNP *RET* variants VUS in *RET* without any well-defined risk profiles and suggest that the G691S SNP, or a combination of SNPs, may be associated with the development of PHEO.

INTRODUCTION

The human gene *RET* (*REarranged during Transfection*) gene encodes the tyrosine kinase transmembrane receptor required for the normal development of cell types derived from the neural crest, e.g. the thyroid C cells or the adrenal chromaffin cells. Gain-of-function variants lead to the activation of the *RET* receptor and to an abnormal proliferation of these cells, which are pathognomonic in patients with multiple endocrine neoplasia type 2 (MEN2). The MEN2 syndrome includes two clinically distinct forms, MEN2A and MEN2B, associated with distinct mutations in the *RET* gene as summarized in the *Revised American Thyroid Association (ATA) Guidelines for the Management of Medullary Thyroid Cancer* (1). MEN2A includes medullary thyroid cancer (MTC) in combination or not with pheochromocytoma (PHEO) and hyperparathyroidism (HPTH). MEN2B is characterized by MTC, PHEO, ganglioneuromatosis, mucosal neuromas and a marfanoid habitus.

Germline gain-of-function *RET* variants mostly occur in exons 5, 8, 10, 11, 13, 14, 15 and 16. Various consortia as well as the ATA have defined the risk profiles of each variant for the risk and aggressivity of MTC, giving estimations of the penetrance of MEN, recommending the optimal age for prophylactic thyroidectomy, and suggesting that asymptomatic patients carrying a germline disease-causing *RET* variant should be screened for PHEO and HPTH (1, 2). Some *RET* variants have not been categorized because of insufficient data. More information about these cases will be needed to provide adequate genetic counseling to patients and families. In 2010, an extensive study of *RET* variants in exon 10 evaluated the risk profiles of these patients and estimated the penetrance of MEN2 (3). The results showed that 50% penetrance was achieved by age 36 for MTC and by age 68 for PHEO. Another study analyzed clinical risk profiles and outcomes for *RET* variants at codon 634. The outcomes varied according to the amino acid substitution and C634R seemed to be associated with a more aggressive MEN2A phenotype (4). However, some of the conclusions concerning *RET* variants are controversial. A recent study focusing on the *RET* Y791F variant, classified as moderate risk by the ATA, reported no association between this variant and susceptibility to MTC (5). The authors found a frequency of 0.008 in the tumor-free cohorts originating in Central Europe. They, and other authors, also

reported subjects harboring *RET* Y791F without the MEN2 syndrome (5, 6). It is therefore important to collect more data about patients in order to update the classification of *RET* variants in MTC.

The presence of single nucleotide polymorphisms (SNPs) in the *RET* gene has been investigated with regard to their potential role in the development or progression of MTC or PHEO in patients with the MEN2 syndrome. Four SNPs, i.e. G691S (rs1799939), L769L (rs1800861), S836S (rs1800862), and S904S (rs1800863), have been regularly studied in the context of MEN2. For each SNP, the findings were contradictory. Some clinical studies found an increased prevalence of *RET* SNPs in MTC (7-11), whereas others did not (12-14). A meta-analytic study on G691S, combining results from 968 cases of MTC and 2115 controls, found that female patients were at a higher risk of the disease than males (15). Other studies suggest a potential additive effect of these SNPs on the susceptibility to MTC or PHEO (8, 11). Taken together, these findings suggest that the consideration of the possible modifying role of these SNPs may contribute to better clinical and follow-up management.

Here we describe the spectrum of *RET* variants observed in the French population over the last 10 years collected from a nationwide database. We propose a classification of 32 genotypes that have not been classified by the ATA (16). Finally, we explore the effects of multiple *RET* SNPs on the MEN2A phenotype.

Patients and methods

Organization of *RET* gene analysis in France

In France, genetic counseling and *RET* molecular analysis are recommended in the presence of MTC, isolated or in combination with PHEO and HPTH. It is provided free of charge to patients and their next of kin by the French National Cancer Institute (INCa). This institute, created under the French Public Health Act of 2004, plays a pivotal role in the national coordination of actions aimed at fighting cancer. Through its coordinated programs, INCa has funded a total of 11 laboratories across the country to perform *RET* molecular analyses, constituting the nationwide TenGen network.

Database

TenGen, which is affiliated with the French Endocrine Society, has set up an exhaustive national database to collect clinical and genetic information about these patients. The database has been declared to the French National Commission for Computerized Data and Individual Freedom (CNIL) and is registered under N° 91513. The TenGen network has verified all the data included in the *RET* database from 2003 to 2013. This study is nationwide and all centers performing *RET* gene analysis have contributed to the *RET* database.

Patients

Over the ten-year period from 2003 to 2013, 5109 index cases and 2214 relatives underwent *RET* proto-oncogene analysis. Index cases were diagnosed with various combinations of endocrine neoplasias, i.e. MTC, associated or not with PHEO or HPTH. Each index case belonged to apparently unrelated families according to the genetic counseling and the family name. Each laboratory was invited to anonymize and complete a form for each index case. The information collected included each patient's age at the time of diagnosis, sex, the endocrine neoplasias present, and the *RET* genetic variants. For screened relatives only the presence or absence of the *RET* pathogenic variant was available.

Molecular characterization

The germline *RET* variants, including SNP, in exons 5, 8, 10, 11, 13, 14 and 16 were analyzed (Genbank reference NM_020975.4). Genomic DNA was obtained from peripheral blood leucocytes and the genotyping was done using direct sequencing.

We use “variant” as a neutral term, “pathogenic variant” for variants affecting *RET* function, and the term “polymorphism” or “SNP” for a variant found at a frequency above 1% or higher in the general population. The *RET* SNPs studied included G691S (rs1799939), L769L (rs1800861), S836S (rs1800862), and S904S (rs1800863). For variants for which a functional effect was not well-defined and that were not listed in the ATA MTC guidelines (1, 2, 17), we used the term “variant of unknown significance” (VUS). The variants were also classified following the recommendation of the ACMG (16). *In silico* analysis was performed by three different *in silico* bioinformatics algorithms, i.e. the scale-invariant feature transform (SIFT), polymorphism phenotyping (PolyPhen-2) and the MutationTaster. The term “non-SNP *RET* variants” was used to group together “pathogenic variants” and “variants of unknown significance”.

Ethics

Informed consent for genetic analysis and the use of data was obtained from all patients or their legal guardians according to the French governmental regulations.

Statistical analysis

Ordinal or nominal variables were summarized in terms of the frequency of occurrence and percentage, or the mean (\pm sd) for continuous variables. Baseline characteristics were compared using either the Chi Square test for qualitative variables or Student’s t-test for quantitative variables. Kaplan-Meier curves were used for estimating the age-related penetrance of MEN2A according to the presence of PHEO, and comparisons between curves were made with a log-rank test. Statistical analyses were performed using SPSS version 15 (SPSS Inc., Chicago, IL, USA).

RESULTS

Non-SNP Pathogenic variants and VUS in *RET* in France

Over the 10-year period from 2003-2013, 7323 tests were carried out, of which 5109 concerned index cases and 2214 were used for family studies (Table 1). The frequency of non-SNP pathogenic variants and VUS in *RET* was 10.1% in the index cases, i.e. 514 of the 5109 patients tested, and 39.1% in the next of kin, i.e. 867 carriers of *RET* pathogenic variants found in 2214 patients studied. We collected genetic data in 480 of the 514 index cases (94.2%) in which the 8 *RET* exons had been analyzed during the period 2003-2013. The mean age of the patients was 47.3 ± 17.7 years and 59.9% of the patients were women. The clinical feature at the time of diagnosis was MEN2A in 448 patients (93.3%), and MEN2B in 32 patients (6.7%).

Among the 480 index cases, 477 patients had missense single variants (Figure 1). Of the single variants found, 51.6% are located in the extra-cellular domain of the receptor, affecting the cysteine codons. The most frequent variants were V804M (17.2%), C634R (11.0%), C634Y (9.5%), L790F (8.9%) and M918T (6.0%), whereas the other represented less than 5% (as shown in Supplemental Data 1).

Three of the patients had three different double variants, i.e (V804M; R844L); (K603Q; L790F); (D631Y; Y791F). Each of the variants observed was classified as pathogenic by the ATA; however, the double variants were not classified.

Clinical and oncological features of patients carrying ATA classified variants

Following the 2015 ATA Clinical Guidelines for MTC (1), the variants of 444 patients (92.3%) were listed in the guidelines and these patients had a well defined risk profile. Among these, 268 patients in our study (55.7%), could be qualified as carrying a “moderate risk” (corresponding to a combination of the former class A and class B pathogenic variants included in the 2009 ATA clinical guidelines (2), 146 patients (30.4%) as being at “high risk” (former Class C pathogenic variants, that includes patients with the *RET* codon C634 and A883F pathogenic variants), and 30 patients (6.2%) at the “highest risk” (former Class D pathogenic variants that includes MEN2B and *RET* codon M918T) (Figure 1).

Clinical and oncological features of patients carrying non ATA-classified variants

Among the index cases, 36 patients (7.7%) had variants not classified by the ATA, including 28 patients with 21 different single missense variants, 5 patients with 5 different synonymous *RET* variants, and three patients with double missense variants. Thirty patients were diagnosed with MTC and/or PHEO after age 40, whereas 6 patients were diagnosed before age 40 (Table 2).

Two of the 5 synonymous *RET* variants, i.e. C620C (c.1860C>T) and S699S (c.2097C>T) have never been reported either in the NCBI dbSNP or in the Exome Aggregation Consortium (ExAC) databank: All patients with these variants were diagnosed after age 40.

Of the 21 *RET* single variants not listed in the ATA classification (Table 2), 5 have never been reported either in the NCBI dbSNP or in the ExAC databank. Three of the latter, i.e. C515F (c.1544G>T), C541R (c.1621T>C) and N783S (c.2348A>G), were qualified as “damaging” by three different *in silico* bioinformatics algorithms. The patient who carried the C541R mutation was diagnosed before the age of one year with a suspicion of MEN2B and Hirschsprung’s disease. The parents were asymptomatic, and no genetic analysis was performed for them. Unfortunately, we did not have access to additional clinical data on the patient to confirm MEN2B or Hirschsprung’s disease. The two other variants, V704F (c.2110G>T) and F644V (c.1930T>G), were qualified as “benign” or “damaging” depending on the bioinformatics tool used. The patient carrying the F644V substitution was diagnosed with MTC at age 35, and the patient carrying the V704F variant was diagnosed with HPTH at age 49.

Of the 16 single variants not classified by the ATA listed in the NCBI dbSNP or the ExAC databank, according to the 3 algorithms used, four were qualified as “benign” (one of the patients was diagnosed at age 21), five were qualified as “damaging” or “possibly damaging”, and seven *RET* variants were qualified as being either “benign” or “damaging” according to the algorithm used (two of these patients were diagnosed before age 40) (Table 2).

We used the criteria for classifying these 21 single *RET* variants with the consensus recommendation of the ACMG. Nine variants for 11 patients could be classified as probably pathogenic, six as probably benign, and the others as variants “of unknown significance” (VUS).

Clinical and oncological features of patients carrying *RET* double pathogenic variants

Each single variant found in these cases is already listed in the ATA MTC guidelines with a moderate risk of aggressive MTC but the risks for double mutations have not been reported. One patient with a concomitant V804M and R844L substitution was diagnosed with MTC at age 51, with a metastatic node. Her twin sister was diagnosed with MTC at the same age but without any metastatic nodes. The two pathogenic variants were on the same allele. The patient with K603Q and L790F had MTC alone at age 60. No family study was available for this patient. A 31-year-old male patient, diagnosed with PHEO, harbored D631Y and Y791F. The two variants were in distinct alleles. His father, carrying D631Y, was diagnosed with metastatic bilateral PHEO at age 65. No information was available for his mother.

The non-SNP variant spectrum of the *RET* codon in MEN2A is associated with PHEO and HPTH

We collected complete clinical information at the time of molecular analysis for 366 of the 448 MEN2A patients. Among these patients, 77.3% had MTC alone, 17.5% had MTC and PHEO, 3.3% had MTC and HPTH, and 1.9% had MTC, PHEO and HPTH.

The frequency of patients for whom PHEO was the first appearing lesion was 19.4% (71 of the 366 MEN2A patients). All the variants were pathogenic and already listed in the ATA MTC guidelines, except for G548S and K666T. Sixty-one of the 71 patients with PHEO had predominantly variants in exons 10 or 11 and these mainly concerned cysteine residues for these exons, except for the K666T variant. The mean age at the time of molecular diagnosis of MEN2A was significantly different between patients with or without

PHEO, respectively 41.8 ± 13.0 and 48.7 ± 16.3 years ($p=0.001$, Student's t-test and $p<0.0001$, Log-rank test) (Figure 2).

Codon 634, which was most frequently mutated, was found to be affected in 112 patients among which 55 (49,1%) had PHEO. In this group of 112 patients, 43 had C634R (38,4%), 33 had C634Y (29,5%), 11 had C634W (9,8%), 11 had C634S (9,8%), 6 had C634G (5,4%), 6 had C634F (5,4%), and 2 had C634L (1,8%). The risk of PHEO was about 50% for each variant except for C634F, where it was lower at 17%. The mean age at the time of diagnosis did not significantly differ between patients, with or without PHEO, harboring a codon 634 pathogenic variant.

HPTH was found in 19 of the 366 MEN2A patients (5.2%). All the mutations were pathogenic variants already listed in the ATA MTC guidelines except for V704F. Thirteen of these 19 (68%) patients with HPTH harbored pathogenic variants affecting cysteines in exons 10 or 11. The most frequent was C634R, found in 7 of 19 patients. The other variants were found in the transmembrane domain (S649L in one patient) or the intracellular domain (V704F in one patient, and V804M in four patients).

RET polymorphic alleles

Clinical and molecular data were analyzed according to four SNPs, i.e. G691S (rs1799939), L769L (rs1800861), S836S (rs1800862), and S904S (rs1800863), in 244 of 366 MEN2A patients with clinical information. The frequency of *RET* SNPs was similar to that reported in the literature, i.e. G691S 24.3%, L769L 22.1%, S836S 8.4%, and S904S 19.9%.

The presence of the G691S SNP was associated with a higher risk of developing PHEO ($p=0.036$) (Figure 3A). The same tendency was observed when in analyses of the two groups of the ATA classification « high risk » group (patient carrier of variant in 634 codon) and « moderate risk » group (patient carrier of variant other than 634), without reaching the significance threshold for both group ($p=0.07$). However, for the patient with PHEO, statistical testing was not able to detect a difference in age at the time of molecular analysis in groups with or without G691S (46.7 ± 1.5 years with G691S and 44.3 ± 1.0 years without, $p = 0.8$). We examined whether the G691S SNP would change the age at which

PHEO might have been diagnosed according to the 3 ATA categories of pathogenic variants. No difference between the different risk levels was found.

We also examined whether the presence of one, two, three or four *RET* SNPs might increase the risk of PHEO. The distribution of the number of polymorphisms was significantly different between the four groups of patients ($p=0.04$). Patients with PHEO were found to have a higher SNP number (1.38 ± 1.06) than patients without PHEO (1.05 ± 0.95) (Figure 3).

DISCUSSION

We report the rate of new cases carrying pathogenic variants or VUS in non-SNP *RET* per year and their distribution in the French population studied between 2003 and 2013. As recommended by INCa and based on the literature, the exons bearing activating mutations (exons 5, 8, 10, 11, 13, 14, 15 and 16) were analyzed in all patients. In our 480 index cases, we found 74 different pathogenic variants or VUS non-SNP. Codon 634 was the most frequently altered codon, as already described (18, 19). However, the frequency spectrum of these variants is not universal and there are differences among ethnic groups, presumably caused by founder effects. For instance, in Greece, the G533C variant, with a prevalence of 36.2%, appears to be characteristic of a specific ethnic group (20). This variant has rarely been found in other series (18, 19, 21, 22), and in our study the frequency amounts to only 0.6% of patients. We found 8.9% of the patients to be carriers of L790F, a pathogenic variant that seems to be present more often in Germany and Slovenia but rarely in other European countries (Supplemental Data 2) (22, 23). Our spectrum of *RET* variants is similar to that found in Germany, but quite different from that in Italy, Spain, or Greece (18-21).

The accumulation of genotype-phenotype data over the last decade has made it possible to define risk profiles, to estimate the penetrance of MEN for index cases, and to recommend the optimal age for a prophylactic thyroidectomy or PHEO and HPTH screening in asymptomatic carriers of *RET* pathogenic variants (1, 2, 24).

In our study, 92.3% of the patients carrying pathogenic *RET* variants received such recommendations. However, for 36 patients carrying *RET* variants not classified by the ATA, the data were not sufficient to support a recommendation. The majority of these patients had been diagnosed after age 30, but two patients had been diagnosed at the age of one year and 21 years. The patient diagnosed at the age of one year had a C541R variant associated with a MEN2B-like phenotype and Hirschsprung's disease. Unfortunately, there is no current available clinical data to further confirm the clinical diagnosis. This variant concerns one of the extracellular cysteine residues. The C541R variant is predicted to be deleterious by the three bioinformatics algorithms used. Based on the consensus recommendation of the ACMG for the characterization of variants, the substitution is considered as probably pathogenic. A nonsense change in codon 541 in patients with Hirschsprung's disease has been described in the literature but there is no precedence of a codon 541 variant with an activating mutation (25). The functional impact of C541R awaits confirmation by further studies. The patient diagnosed at age 21 had a V648I variant. With functional tests, this variant is qualified as having a low oncogenic potential (26), and the three bioinformatics algorithms have predicted it to be a tolerable substitution. This variant has been reported in trans with C634R for a patient with MTC and bilateral PHEO but his two children with the V648I variant had no evidence for the presence of tumors (27). Considering all these arguments, we consider this variant to be probably benign. The M918T pathogenic variant is qualified as the most aggressive variant in the ATA classification. In our study, we found another type of substitution at codon 918, i.e. M918V. Although this variant was also predicted to be deleterious by the three bioinformatics algorithms used, functional testing showed that it has a low oncogenic potential (26). As reported in a recent study showing a moderate risk of MTC associated with the M918V variant, our patient was diagnosed with MTC only at age 56 (28). The M918T pathogenic substitution has been shown to modify the three-dimensional structure of the protein, creating new hydrogen bonds and stabilizing its close conformation (29, 30). Our unpublished data show that valine, a small amino acid that does not create new hydrogen bonds, does not alter the conformation of the protein. Based on these arguments, we believe that this variant is probably pathogenic. In contrast to M918T, the M918V variant is associated with a moderate risk of aggressive MTC in our patient.

Concerning the combined mutations, each variant was classified as moderate risk by the ATA. For the concomitant presence of V804M and R844L, or K603Q and L790F, there is no clinical evidence that suggests a need for upgrading the risk from “moderate” to “high”. For the patient carrying D631Y and Y791F, the diagnosis of PHEO was made earlier than in his father who carried D631Y alone. However, in the absence of information about his mother, and because of the controversy about the pathogenicity of Y791F, there is insufficient data to support a change in risk category.

Overall, we have classified nine variants as probably pathogenic and six as probably benign. It is known that MTC occurs at a variable age and aggressivity depending on the specific pathogenic variant. The clinical follow-up of these patients and further phenotype-genotype information will be required to specify the MTC risk profile. As recommended for the patients carrying pathogenic *RET* variants, serum calcitonin assays and ultrasound examinations can serve as an indicator for MTC in patients carrying variants not yet classified by the ATA (1, 31, 32)

Our results show that the distribution of mutations was significantly different between patients with or without PHEO. Patients with PHEO had predominantly mutations in exons 10 or 11 and these concerned cysteine residues. These tumors are known to produce catecholamines, and about one-third of the tumors are bilateral at initial diagnosis. The more dramatic clinical behavior explains why the mean age of diagnosis is lower when PHEO is the first manifestation of MEN 2A. Thus, the ATA recommended that screening for PHEO should begin by age 11 for children carrying a 634 pathogenic variant and by age 16 in children with other pathogenic variants.

The polymorphic *RET* alleles G691S (rs1799939), L769L (rs1800861), S836S (rs1800862), and S904S (rs1800863) have sometimes been considered as disease modifiers. The role that the *RET* G691S polymorphism plays in the susceptibility to MTC has been investigated and a meta-analysis based on 11 studies, combining 968 MTC cases and 2115 controls, confirms the involvement of this *RET* variant in MTC (15), but no involvement in risk of PHEO has been reported. *In silico* analysis has shown that the G691S SNP changes the phosphorylation pattern that may enhance signal transduction.

Moreover, two functional studies (33, 34) have demonstrated that two pathogenic variants (S891A and K666Q), qualified as “moderate” risks according to the ATA classification, display a higher oncogenic potential when they are associated with the G691S substitution. We investigated whether G691S modifies the age at diagnosis for patients carrying pathogenic variants with low oncogenic potential, but no modifier effect was found. However, we found that the G691S variant increases the risk of PHEO independently of the 634 *RET* pathogenic variant. According to some studies, G691S should be described as a risk modifier in MEN2. The additive effect of *RET* polymorphisms on sporadic MTC and PHEO has been investigated including multiple *RET* variants, i.e. G691S, L769L, S836S, and S904S (8, 11). The results showed that three or more polymorphic alleles were not associated with age at diagnosis or tumor size, but increased the risk of lymph node and distant metastases at diagnosis of sporadic MTC. In the case of PHEO, the presence of two *RET* variants was associated with increased susceptibility to PHEO and age-related penetrance. We found no link between the number of SNPs and the age at diagnosis of MTC, but the susceptibility for PHEO increased. Our study is retrospective and evaluated the potential role of the 4 *RET* SNPs found in the 8 analyzed exons. Our results suggest that *RET* G691S or a combination of SNPs may affect the development of PHEO in MEN2. These results need to be confirmed by family screening to identify an intra-familial co-segregation of these SNPs with PHEO.

The main limitations of our work may be related to the retrospective nature of the collection of data from multiple centers and the heterogeneity of the observation due to the varying method of patient management over the ten-year period of the study. Moreover, it is likely that subjects carrying mutations with a low transforming activity may not have been diagnosed if they had no family histories and had not yet developed MEN2.

Finally, our results offer a more comprehensive vision of the distribution of *RET* variants in France and, to our knowledge, this is the first exhaustive study of *RET* screening over a 10-year span in France. We report the clinical description of 36 patients carrying non-ATA-classified *RET* variants, and classify them according to the recommendations of the ACMG. Our results suggest that *RET* SNPs such as G691S, or a combination of SNPs, may be associated with the development of PHEO. This finding may have significant

implications in the management of patients, particularly for the diagnosis of PHEO in patients carrying the G691S SNP. With the advent of next-generation sequencing, comprehensive analysis of the *RET* exome or the entire locus may be extended to all patients with MEN2 so that phenotype-genotype correlations for rare or unknown variants could be usefully implemented for the management of patients.

ACKNOWLEDGMENT

We are grateful to Mr Kanaya Malkani and Ms Caroline Jacques for their critical reading of the manuscript.

DISCLOSURE

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

FUNDING

This work was supported by the University Hospital of Angers, INCa, Groupes d'étude des tumeurs endocrines (GTE), OncoGENetic on NeuroEndocrine Tumors (TENGEN) Société Française d'endocrinologie (SFE), INSERM, CNRS.

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Table 1: RET molecular analysis performed and patient carriers of RET germline non-SNP pathogenic variants or VUS found in France for index cases or screened relatives (2003-2013).

	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Total 2003-2013
Total Index Cases MEN2 (N)	894	454	386	383	449	492	429	390	378	385	469	5 109
Patient with RET variants (N)	66	42	40	47	55	43	43	56	24	42	56	514
%	7,4%	9,3%	10,4%	12,3%	12,2%	8,7%	10,0%	14,4%	6,3%	10,9%	11,9%	10,1%
screened relatives (N)	217	264	203	200	205	206	179	210	147	150	233	2 214
Screened relatives with RET variants (N)	87	106	81	83	51	86	57	77	75	63	101	867
%	40,1%	40,1%	40,0%	41,5%	24,9%	41,7%	31,8%	36,7%	51,0%	42,0%	43,3%	39,1%
Total Patient with RET variants (N)	153	148	121	130	106	129	100	133	99	105	157	1381

Report completed by the INCa each year for index cases and screened relatives

Table 2: Rare RET VUS non-SNP variants non ATA-classified: clinical feature, population database report, *in silico* predictive algorithms and proposition of classification following ACMG

Exon	genotype	Protein change	N Patient	dbSNP/NCBI database (Number of report)	ExAC (frequency)	SIFT	Polyphen2	Mutation Taster	oncogenic potential in vitro (references)	Age at diagnosis	Clinical phenotype	References	Classification following ACMG
SINGLE VARIANTS (missense)													
5	c.0884C>T	T295M	1	not reported	8.765e-06	T	T	T		49	MTC		VUS
5	c.1013C>T	T338I	1	rs377767433 (3)	4.32e-05	T	T	T	low or No (26)	42	ND	18-26-35	VUS
8	c.1529 C>T	A510V	2	rs201745826 (5)	0.0003	T	T	T	potential (36)	75	ND	36	VUS
										69	MTC		
8	c.1531G>A	E511K	2	rs201553718 (8)	0.0002	T	T	D	potential (36)	73	MTC	36	VUS
										38	MTC		
8	c.1544G>T	C515F	1	not reported	not reported	D	D	D		49	MTC		likely pathogenic
8	c.1591T>C	C531R	2	rs377767390 (1)	not reported	D	P	D	potential (36)	59	MTC	36	likely pathogenic
										58	MTC		
8	c.1621T>C	C541R	1	not reported	not reported	D	D	D		0,12	MEN2B-Hirsprung		likely pathogenic
8	c.1642G>A	G548S	2	rs374461212 (3)	4.555e-05	T	D	D		53	MTC and PHEO		VUS
										53	MTC		
11	c.1930T>G	F644V	1	not reported	not reported	T	T	D		35	MTC		VUS
11	c.1942G>A	V648I	3	rs77711105 (6)	9.076e-05	T	T	T	low or No (26)	65	MTC	26-27	Likely benign
										68	MTC		
										21	MTC		
11	c.1997A>C	K666T	1	rs377767439 (2)	8.242e-06	D	D	D		41	MTC and Pheo		likely pathogenic
11	c.1998delinsT TCT		2	rs377767440 (1)	8.242e-06	T	D	D		35	ND	37	likely pathogenic
										58	MTC		likely pathogenic
11	c.1998 G>C	K666N	1	rs146646971(5)	not reported	T	D	D		63	MTC	36	likely pathogenic
11	c.1998G>T	K666N	1	rs146646971 (5)	2.473e-05	T	D	D	potential (36)	59	MTC	36-44	likely pathogenic
11	c.2110G>T	V704F	1	not reported	not reported	T	D	D		49	HPT		VUS
13	c.2342A>G	Q781R	1	rs377767416 (2)	not reported	D	D	D		73	MTC	38-39	VUS
13	c.2348A>G	N783S	1	not reported	not reported	D	D	D		57	MTC	40	VUS
13	c.2371T>A	Y791N	1	rs377767417 (3)	2.503e-05	D	P	D		58	MTC and CCH	41	VUS
15	c.2647G>A	A883T	1	rs377767428 (1)	not reported	T	D	D		68	MTC	42	likely pathogenic
15	c.2657G>A	R886Q	1	rs373594744 (1)	3.377e-05	T	D	D		84	ND		VUS
16	c.2752A>G	M918V	1	rs377767442 (1)	not reported	D	D	D	low or No (26)	56	MTC	26-28	likely pathogenic
SINGLE VARIANTS (synonymous)													
8	c.1539G>A	A513A	1	rs761430718 (1)	8.271e-06					60	MTC		Likely benign
			1	not reported	not reported					70	MTC and PHEO		Likely benign
10	c.1860C>T	C620C											
11	c.2037C>T	P679P	1	rs55862116 (9)	1.128e-03					45	MTC		Likely benign
	c.2052G>A	P648P	1	rs145122337 (2)	2.718e-04					75	MTC		Likely benign
	c.2097C>T	S699S	1	not reported	not reported					62	MTC		Likely benign
DOUBLE VARIANTS													
14;14	c.[2410G>A (:)c.2531G>T]	[V804M(;)R844L]	1							51	MTC	43	Pathogenic
10;13	c.[1807 A>C (:) c.2370 G>T]	[K603Q(;)L790F]	1							60	MTC		Pathogenic
11;13	c.[1891G>T(;)c.2372A>T]	[D631Y(;)Y791F]	1							31	MTC and PHEO		Pathogenic

T tolerated; D deleterious; P possibly damaging; ND not done; CCH C Cell Hyperplasia

```

graph TD
    A[7323 RET molecular analysis] --> B[5109 Index cases]
    A --> C[2214 Screened relatives]
    B --> D[514 Patients with RET variants]
    B --> E[34 Patients with no data]
    D --> F[480 Patients with genetic data available 74v]
    D --> G[3 Patients with Double variants 6v]
    F --> H[477 Patients with Single variant 71v]
    F --> I[472 Patients with Missense variants]
    H --> J[5 patients with Synonymous variants]
    I --> K[30 pat. Highest risk 1v]
    I --> L[146 pat. High risk 9v]
    I --> M[268 pat. Moderate Risk 35v]
    J --> N[28 pat. 21v]
    J --> O[5 pat. 5v]
    N --> P[9 v<sup>s</sup> 9v]
    N --> Q[11 v<sup>s</sup> 11v]
    N --> R[1 v<sup>s</sup> 1v]
    O --> S[5 v<sup>s</sup> 5v]
    P --> T[Likely Pathogenic 11 pat.]
    Q --> U[Uncertain significance 14 pat.]
    R --> V[Likely Benigns 8 pat.]
    S --> W[Likely Benigns 8 pat.]
    G --> X[3 pat. 6v]
    X --> Y[Pathogenic Variants]
    Y --> Z[444 Patients with ATA classification]
    T --> AA[36 Patients with no ATA classification]
    U --> AA
    V --> AA
    W --> AA
    
```

V : pathogenic variants or VUS in RET; Pat. : patient;
 Blue variants classified in ATA; Orange variants not classified in ATA; ^s : classification following the recommendation of the ACMG

Figure 1 : _Pathogenic variants or VUS in_RET for index cases and screened relatives from 2003 to 2013. Classification by risk groups following the 2015 ATA clinical Guidelines for MTC and proposition of classifying variants not found in the Guidelines following the consensus recommendation of the ACMG.

A

Age at first diagnosis: years (Mean±sd)						
	Patients with pheochromocytoma		Patients without pheochromocytoma		P (student's t-test)	
	N		N		N	
Patients with MEN2A	366	41.8±13.0	71	48.7±16.3	295	0.001
Patients with mutation in codon 634	112	38.6±14.5	55	39.8±11.0	57	0.376
Cys634Arg	43	36.96 ±12.18	24	39.08 ±12.70	19	
Cys634Tyr	33	42.10±11.03	15	37.36±18.09	18	
Cys634Trp	11	38.93±10.40	6	39.50±11.95	5	
Cys634Ser	11	49.69±6.43	5	42.65±13.58	6	
Cys634Gly	6	39.73±2.17	3	40.80±7.67	3	
Cys634Phe	6	35.03	1	37.26±19.47	5	
Cys634Leu	2	35.85	1	21.54	1	

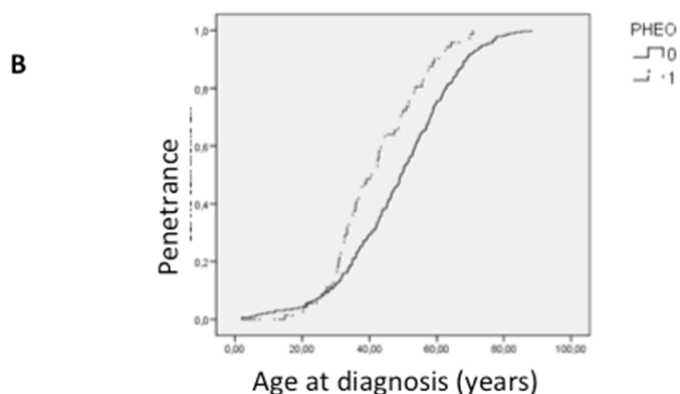


Figure 2: Patients with MEN2A carrying RET germline pathogenic variants or VUS non-SNP variants (N=366 Index cases). (A) Age at the time of MEN2A diagnosis according to the presence or absence of PHEO for 366 patients and for 112 patients with a RET 634 variant. Student's-t-test was used to compare mean of age. (B) Age-related penetrance of MEN2A according to the presence or absence of PHEO for 366 patients. Kaplan–Meier estimates for the proportion of MEN2 patients who developed PHEO. The log-rank test was used to compare curves.

A

SNP	Patients with pheochromocytoma (n=53)	Patients without pheochromocytoma (n=191)	p (Fisher Chi Square test)
G691S	26	63	0.036
L769L	23	58	0.098
S836S	7	24	1
S904S	17	56	0.730

Number of SNPs per Patient	Patients with pheochromocytoma (n=53)	Patients without pheochromocytoma (n=191)	p (student test)
None	14	74	
One	13	45	
Two	19	61	
Three	6	10	
Four	1	1	
Mean±sd of SNP	1.38±1.06	1.05±0.95	0.004

B

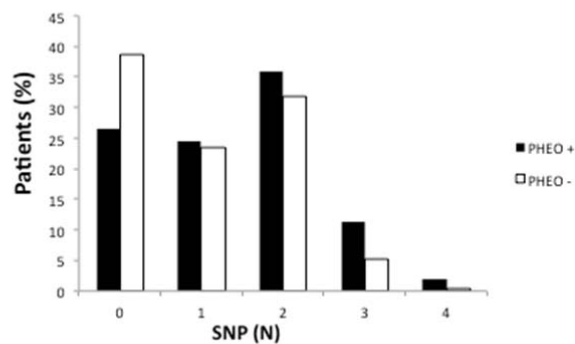


Figure 3 : Additive effect of *RET* polymorphisms on PHEO in patients with MEN2A (N=244 index cases). (A) Distribution of each of the four studied SNPs, i.e. G691S (rs1799939), L769L (rs1800861), S836S (rs1800862), and S904S (rs1800863) and number of SNP per MEN2A patient according to the presence or absence of PHEO. (B) Proportion of MEN2A patients with zero, one, two, three or four SNPs according to the presence or absence of PHEO.

Supplemental data 1: RET pathogenic variants or VUS found in France between 2003 and 2013 in index cases.

Exon	N(%)	type of chan	genotype	N(%)	codon	N(%)	Protein change	ATA risk level (2015)	ATA risk level (2009)
5	2 (0,4)								
5		substitution	c.0884C>T	1 (0,2)	295	1 (0,2)	T295M	Not classified	
5		substitution	c.1013C>T	1 (0,2)	338	1 (0,2)	T338I	Not classified	
8	17 (3,5)								
8		substitution	c.1529 C>T	2 (0,4)	510	2 (0,4)	A510V	Not classified	
8		substitution	c.1531G>A	2 (0,4)	511	2 (0,4)	E511K	Not classified	
8		substitution	c.1543T>A	3 (0,6)	515	4 (0,8)	C515S	Moderate risk	A
8		substitution	c.1544G>T	1 (0,2)	515		C515F	Not classified	
8		substitution	c.1591T>C	2 (0,4)	531	2 (0,4)	C531R	Not classified	
		dup		1 (0,2)		1 (0,2)			
8			c.1594dupAGGAGTGTG		529-531		531/9 base pair duplication	Moderate risk	A
8		substitution	c.1597G>T	3 (0,6)	533	3 (0,6)	G533C	Moderate risk	A
8		substitution	c.1621T>C	1 (0,2)	541	1 (0,2)	C541R	Not classified	
8		substitution	c.1642G>A	2 (0,4)	548	2 (0,4)	G548S	Not classified	
10	78 (16,2)								
10		substitution	c.1807A>C	1 (0,2)	603	1 (0,2)	K603E	Moderate risk	A
10		substitution	c.1826G>A	3 (0,6)	609	5 (1,0)	C609Y	Moderate risk	B
10		substitution	c.1826G>T	1 (0,2)			C609F	Moderate risk	B
10		substitution	c.1827C>G	1 (0,2)			C609W	Moderate risk	B
10		substitution	c.1831T>G	2 (0,4)	611	12 (2,5)	C611G	Moderate risk	B
10		substitution	c.1832G>A	7 (1,5)			C611Y	Moderate risk	B
10		substitution	c.1833C>G	3 (0,6)			C611W	Moderate risk	B
10		substitution	c.1852T>A	12 (2,5)	618	29 (6,0)	C618S	Moderate risk	B
10		substitution	c.1852T>C	6 (1,2)			C618R	Moderate risk	B
10		substitution	c.1853G>A	1 (0,2)			C618Y	Moderate risk	B
10		substitution	c.1853G>C	8 (1,7)			C618S	Moderate risk	B
10		substitution	c.1853G>T	1 (0,2)			C618F	Moderate risk	B
10		substitution	c.1854C>G	1 (0,2)			C618W	Moderate risk	B
10		substitution	c.1858T>C	10 (2,1)	620	31 (6,4)	C620R	Moderate risk	B
10		substitution	c.1858T>G	5 (1,0)			C620G	Moderate risk	B
10		substitution	c.1859 G>A	8 (1,7)			C620Y	Moderate risk	B
10		substitution	c.1859G>C	1 (0,2)			C620S	Moderate risk	B
10		substitution	c.1859G>T	3 (0,6)			C620F	Moderate risk	B
10		substitution	c.1860C>G	4 (0,8)			C620W	Moderate risk	B
11	158 (32,9)								
11		substitution	c.1888 T>C	1 (0,2)	630	1 (0,2)	C630R	Moderate risk	B
11		substitution	c.1900T>A	12 (2,5)	634	144 (29,9)	C634S	High risk	C
11		substitution	c.1900T>C	53 (11,0)			C634R	High risk	C
11		substitution	c.1900T>G	7 (1,5)			C634G	High risk	C
11		delins	c.1901_1902delGCinsTG	2 (0,4)			C634L	High risk	B
11		substitution	c.1901G>A	46 (9,5)			C634Y	High risk	C
11		substitution	c.1901G>C	3 (0,6)			C634S	High risk	C
11		substitution	c.1901G>T	9 (1,9)			C634F	High risk	C
11		substitution	c.1902C>G	12 (2,5)			C634W	High risk	C
11		substitution	c.1930T>G	1 (0,2)	644	1 (0,2)	F644V	Not classified	
11		substitution	c.1942G>A	3 (0,6)	648	3 (0,6)	V648I	Not classified	
11		substitution	c.1946C>t	2 (0,4)	649	2 (0,4)	S649L	Moderate risk	A
11		substitution	c.1996A>G	1 (0,2)	666	7 (1,5)	K666E	Moderate risk	A
11		substitution	c.1997A>C	1 (0,2)			K666T	Not classified	
11		substitution	c.1998 G>C	1 (0,2)			K666N	Not classified	
11		delins	c.1998delinsTTCT	2 (0,4)			K666	Not classified	
11		substitution	c.1998G>T	1 (0,2)			K666N	Not classified	
11		substitution	c.2110G>T	1 (0,2)	704	1 (0,2)	V704F	Not classified	
13	66 (13,7)								
13		substitution	c.2304G>C	10 (2,1)	768	14 (2,9)	E768D	Moderate risk	A
13		substitution	c.2304G>T	4 (0,8)			E768D	Moderate risk	A
13		substitution	c.2342A>G	1 (0,2)	781	1 (0,2)	Q781R	Not classified	
13		substitution	c.2350A>G	1 (0,2)	783	1 (0,2)	N783S	Not classified	
13		substitution	c.2370G>C	5 (1,0)	790	43 (8,9)	L790F	Moderate risk	A
13		substitution	c.2370G>T	38 (7,9)			L790F	Moderate risk	A
13		substitution	c.2371T>A	1 (0,2)	791	7 (1,5)	Y791N	Not classified	
13		substitution	c.2372A>T	6 (1,2)			Y791F	Moderate risk	A
14	95 (19,8)								
14		substitution	c.2410G>A	83 (17,2)	804	95 (19,7)	V804M	Moderate risk	A
14		substitution	c.2410G>C	3 (0,6)			V804L	Moderate risk	A
14		substitution	c.2410G>T	9 (1,9)			V804L	Moderate risk	A
15	24 (5,0)								
15		substitution	c.2647_2648delGCinsTT	2 (0,4)	883	3 (0,6)	A883F	High risk	D
15		substitution	c.2647G>A	1 (0,2)			A883T	Not classified	
15		substitution	c.2657G>A	1 (0,2)	886	1 (0,2)	R886Q	Not classified	
15		substitution	c.2670T>G	20 (4,1)	891	20 (4,1)	S891A	Moderate risk	A
16	32 (6,7)								
16		substitution	c.2735G>C	1 (0,2)	912	1 (0,2)	R912P	Moderate risk	A
16		substitution	c.2752A>G	1 (0,2)	918	30 (6,2)	M918V	Not classified	
16		substitution	c.2753T>C	30 (6,0)			M918T	Highest risk	D
Double variants	3 (0,8)								
10;13		double	c.[1807 A>C(;):c.2370 G>T]	1 (0,2)	603-790	1 (0,2)	K603Q;L790F	Not classified	
11;13		double	c.[1891G>T(;):c.2372A>T]	1 (0,2)	631-791	1 (0,2)	D631Y;Y791F	Not classified	
14;14		double	c.[2410G>A (;):c.2531G>T]	1 (0,2)	804-844	2 (0,4)	V804M;R844L	Not classified	
synonymous	5 (1,0)								
8		substitution	c.1539G>A	1 (0,2)	513	1 (0,2)	A513A	Not classified	
10		substitution	c.1860C>T	1 (0,2)	620	1 (0,2)	C620C	Not classified	
11		substitution	c.2037C>T	1 (0,2)	679	1 (0,2)	P679P	Not classified	
		substitution	c.2052G>A	1 (0,2)	684	2 (0,4)	P684P	Not classified	
		substitution	c.2097C>T	1 (0,2)	699	1 (0,2)	S699S	Not classified	

Supplemental data 2: Distribution of *RET* germline pathogenic non SNP variants in European families. The table is modified from the 2013 manuscript by Machens et al. (19)

	Italy (n=244)) (18)	German (n=205) (19)	Spain (n=25) (21)	Greece (n=58) (20)	France (n=480)
Variants non SNPs RET classify in ATA					
532 duplication					1 (0.2)
C515S	1(0.4)				3 (0.6)
G533C				21 (36.2)	3 (0.6)
K603E					1 (0.2)
C609F=R=G=S=Y	6 (2.5)	1 (0.5)	1 (3.9)		5 (1.0)
C611R=G=F=S=W=Y	1 (0.4)	6 (3)			12 (2.5)
C618R=G=F=S=Y	15 (6.1)	11 (5)	1 (3.85)	4 (6.9)	29 (6)
C620R=G=F=S=W=Y	9 (3.7)	14 (7)	1 (3.85)	5 (8.6)	31 (6.4)
C630R=F=S=Y	4 (1.6)	1 (0.5)			1 (0.2)
C634R=G=F=S=W=Y	85 (34.8)	73 (36)	17 (65.4)	19 (32.8)	144 (29.9)
S649L					2 (0.4)
K666E					1 (0.2)
E768D	9 (3.7)	2 (1)	1 (3.9)	1 (1.7)	14 (2.9)
L790F	8 (3.3)	26 (13)			43 (8.9)
Y791F *	2 (0.8)	14 (7)			7 (1.5)
V804L	3 (1.2)	19 (9)		3 (5.2)	12 (2.5)

V804M	49 (20.2)				83 (17.2)
A883F					2 (0.4)
S891A	23 (9.4)	6 (3)	1 (3.85)		20 (4.1)
R912P					1 (0.2)
M918T	17 (7.0)	32 (16)	3(11.5)	5 (8.6)	30 (6.0)
non-SNP RET variants, non classified in ATA	12 (4.9)				36 (7.5)

* Erlic et al (6), Toledo et al (5), and ARUP MEN 2 data base classified Y791F as a rare non-pathogenic variant whereas ATA MTC guidelines in 2009 and 2015 considered this variant as pathogenic variant.