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Address for correspondence and reprints: Dr. Francesc Palau, Unitat de Genètica, Hospital Universitari La Fe, Avenue Campanar 21, 46009 Valencia, Spain. E-mail: palau@evalgb.geneti.uv.es

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Prevalence and Parental Origin of De Novo *RET* Mutations in Multiple Endocrine Neoplasia Type 2A and Familial Medullary Thyroid Carcinoma

To the Editor:

Multiple endocrine neoplasia type 2A (MEN 2A), familial medullary thyroid carcinoma (FMTC), and multiple endocrine neoplasia type 2B (MEN 2B) are three dominantly inherited disorders of neural crest development. MTC, a malignant tumor of the thyroid C-cells, is a component of the three syndromes. In MEN 2A, MTC is associated with pheochromocytoma in ~50% of the gene carriers and with hyperplasia and/or adenoma of the parathyroid glands in 20%–30% of predisposed individuals. In MEN 2B, MTC is associated with pheochromocytoma and developmental abnormalities such as marfanoid habitus; overgrowth of neuronal tissue in the lips, tongue, and conjunctivae; and hyperplasia of the autonomic cells of the gut. "FMTC" refers to families in which at least two individuals are affected with MTC but without objective evidence of adrenal and parathyroid involvement (Ponder 1995).

All these disorders are caused by allelic germ-line mutations of the *RET* proto-oncogene, which codes for a transmembrane receptor tyrosine kinase protein whose ligand is a neurotrophin called "GDNF" (Jing et al. 1996; Trupp et al. 1996). The majority of mutations occur in one of two regions of the *RET* protein. A unique point mutation at codon 918 in the tyrosine kinase domain has been identified in >98% of MEN 2B patients and is pathognomonic of the syndrome (Carlson et al. 1994*b;* Eng et al. 1994, and in press; Hofstra et al. 1994; Rossel et al. 1995). Missense mutations of one of five cysteine codons (609, 611, 618, 620, or 634) in the extracellular domain have been identified in 98% of MEN 2A cases and in 70% of patients affected with FMTC (Mulligan et al. 1993, 1994*b;* Schuffenecker et al. 1994; Eng et al., in press). Finally, a missense mutation at either codon 768 or codon 804 in the tyrosine kinase domain has been shown to be responsible for 5%–10% of the FMTC cases (Bolino et al. 1995; Eng et al. 1995*b,* and in press).

Whereas a high proportion of MEN 2B cases are the result of de novo germ-line mutations (Carlson et al. 1994*a;* Ponder 1995), MEN 2A (and, by definition, FMTC) are considered to be almost always associated with familial inheritance of the disease. To date, only three de novo mutations have been reported in MEN 2A cases (Mulligan et al. 1994*a;* Wohllk et al. 1996).

Taking advantage of the large series of families in the French MTC register, we now report that de novo mutations of the *RET* proto-oncogene contribute significantly to MEN 2A syndrome. When origin of the de novo mutations could be traced in families, it was exclusively of paternal origin. In addition, a sex-ratio distortion was observed among the founders.

Approximately 200 families diagnosed with MEN 2 have been registered since 1984, in the GETC (Groupe d'Etude des Tumeurs à Calcitonine) register. Among these families, 87 are of the MEN 2A phenotype, defined as families with at least one patient with pheochromocytoma and/or hyperparathyroidism associated with MTC; 56 families are affected with FMTC, characterized by at least two individuals with MTC and without

clinical and biochemical evidence for adrenal and parathyroid disease. Thirteen families (11 MEN 2A and 2 FMTC) of these 143 (87+56, above) had clinical histories suggestive of either de novo disease or nonpenetrance of a disease allele present in earlier generations. In each family, there was no history of MTC or pheochromocytoma in the parents and/or brothers and sisters of the proband. In addition, in most of the families, biochemical documentation regarding C-cell hyperplasia was available and reinforced the indication of de novo or nonpenetrant disease. In eight families (see fig. 1), DNA samples from the probands' parents were available to test whether a de novo *RET* mutation had occurred. Genomic DNA samples of affected probands and their parents (and other relatives, when available) were amplified by PCR, and exons 10 and 11 were sequenced as described elsewhere (Schuffenecker et al. 1994). A missense mutation of codon 620, changing a cysteine to an arginine, was identified in family F RG with FMTC. In the remaining seven MEN 2A families, we identified missense mutations of codon 634. Of these seven families, five had a C634R mutation, one had a C634Y mutation, and the other had a C634G mutation (fig. 1). All these mutations already have been described. Preferential localization of mutations at codon 634, with predominance of the C634R mutation in MEN 2A patients, is in accordance with mutation data already published (Mulligan et al. 1994*b;* Schuffenecker et al. 1994; Eng et al., in press). In each of these eight families, analysis of DNA from the probands' parents demonstrated that the disease was due to de novo mutation of the *RET* gene (fig. 1). We excluded the alternative of nonpaternity or incorrect sampling, by the analysis of four highly polymorphic loci on chromosome 10—D10S141, *RET*-INT5, s-TCL2, and ZNF22 (Lairmore et al. 1993; Love et al. 1993*a,* 1993*b;* Pasini et al. 1995)—and one polymorphic locus on chromosome 2, YNH24 (Nakamura et al. 1987). The molecular analysis confirmed clinical and biochemical data, demonstrating de novo mutation in 8/143 (5.6%) families in the French register. It is highly likely that, in the five families for which we could not genotype the probands' parents, the disease is also due to a new mutation in the *RET* proto-oncogene. Accordingly, as many as 9% of MEN 2 families (excluding MEN 2B) could be associated with de novo mutations of the *RET* proto-oncogene.

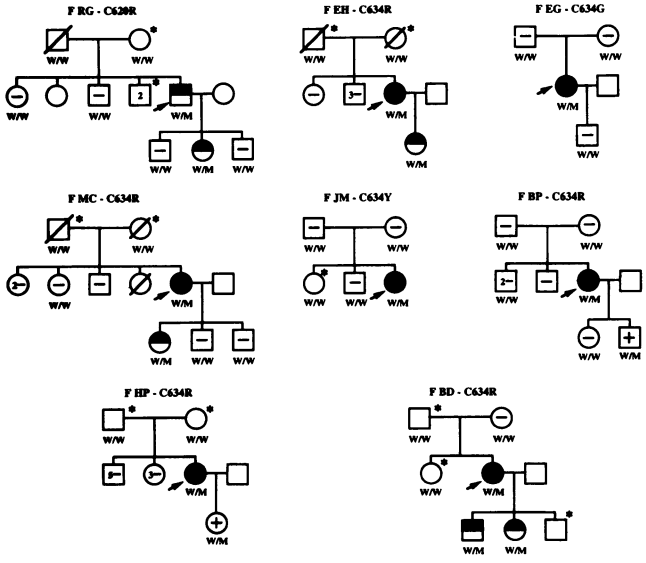

Figure 1 Pedigrees of eight MEN 2 families with de novo *RET* mutations. We analyzed the sequence of *RET* exons 10 and 11 in one FMTC family and seven MEN 2A families in which results of clinical and biochemical investigation of C-cell status were compatible with de novo disease. In F RG we identified a mutation of codon 620, whereas in others we identified missense mutations of codon 634. These mutations are indicated on the upper line next to the identification codes of the families. W = wild-type sequence; and M = mutant sequence. Completely blackened circles denote MEN 2A–affected females; half-blackened circles and squares denote MTC-affected individuals; unblackened circles and squares with an asterisk to the upper right denote individuals with normal basal calcitonin values; circles and squares containing a minus sign denote individuals with a normal pentagastrin-stimulation test; and circles and squares containing a plus sign denote individuals with a positive pentagastrin-stimulation test.

In order to establish the parental origin of the de novo *RET* mutation, we genotyped all available members of seven families, at *RET*-INT5 intragenic CA repeat and at three extragenic flanking microsatellite polymorphisms—that is, D10S141 in the centromeric position and s-TCL2 and ZNF22 in the telomeric position. PCR amplification and analysis of these polymorphic loci were performed and analyzed as described elsewhere (Lairmore et al. 1993; Love et al. 1993*a,* 1993*b;* Pasini et al. 1995). In the remaining family, F JM, since the proband was the only mutation carrier and had no progeny, it was impossible to determine whether the mutated *RET* allele that she carried was of paternal or maternal origin; as a consequence, we did not perform haplotype analysis in this family. In the seven families that we analyzed, we found that de novo *RET* mutation had arisen on the paternally derived chromosome. Results of haplotype analysis are shown for one family (F BP), in figure 2. These data, together with results reported for three MEN 2A patients (Mulligan et al. 1994*a;* Wohllk et al. 1995), indicate that all MEN 2A de novo mutations reported to date (10 out of 10) occur exclusively on the paternal allele. Preferential susceptibility of paternally derived DNA already has been described in MEN 2B (Carlson et al. 1994*a;* Kitamura et al. 1995) and in a number of neoplastic diseases, such as neurofibromatosis type 1, bilateral retinoblastoma, osteosarcoma, and Wilms tumor (reviewed in Sapienza and Hall 1994). In MEN 2B and a number of these tumor disorders, de novo mutations have been associated with increased paternal age (Carlson et al. 1994*a;* Sapienza and

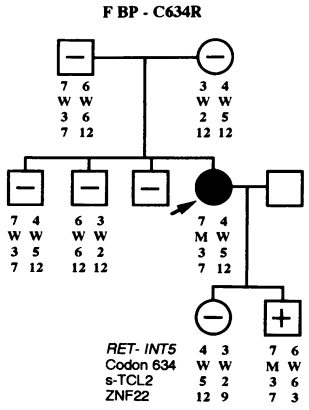

Figure 2 Paternal origin of de novo *RET* mutation in F BP. The haplotype on which the de novo mutation arose is clearly derived from the grandfather. The proband, II-4, transmitted it to her son, III-2, but not to her affected daughter, III-1. Informative flanking microsatellites shown are *RET*-INT5 on the centromeric side of the C634R mutation and s-TCL2 and ZNF22 on the telomeric side.

Hall 1994). To test this possibility in our series of patients, we compared the ages of parents of de novo MEN 2A patients to an average maternal age of 27.6 years and an average paternal age of 30.6 years (parental ages for the French population during 1940–85, given by the Institut National des Etudes Démographiques). We determined a mean maternal age of 34.8 years (range 25–54 years) and a mean paternal age of 39.3 years (range 29–57 years). A one-tailed *t*-test demonstrated that both paternal age and maternal age were significantly greater than the average national age ($P = .012$ for paternal age; and $P = .034$ for maternal age). The present data suggest that preferential susceptibility of paternal DNA to de novo mutations is associated with advanced paternal age in the MEN 2 syndromes and is not restricted to the rare MEN 2B clinical form.

In MEN 2B, Carlson et al. also had reported a sex-ratio distortion. This sex-ratio distortion was observed in those individuals in whom the M918T allele was paternally derived (Carlson et al. 1994*a*). Therefore, we examined in our set of families whether there was a sex-ratio distortion among founders and next-generation offspring. Of 8 patients with de novo disease, 7 were female (of 19 females in this generation) and 1 was male (of 17 males in this generation). In the probands' offspring, among gene carriers having either a histologically proved MTC or a positive pentagastrin-stimulation test, five were female (of six females in this generation) and two were male (of seven males in this generation) (fig. 1). When compared with the expected ratio of 100 females and 106 males, the gender distribution was significantly different among probands ($P = .029$, exact one-sided binomial test) but not in the next-generation ($P = .20$). The overall sex-ratio was significantly different ($P = .013$). Since the proportion of males and females is similar in the first and second generations, we can determine that the excess of affected females is associated with the disease and is not the reflection of an excess of females in these particular families. It may be simply a chance finding (type I error); however, it also could reflect genomic imprinting effects. The small number of affected males observed when the *RET* mutation is paternally derived could reflect a reduced fitness of the Y chromosome–bearing germ cells with the *MEN2A* mutation. Alternatively, there might be an adverse effect of *MEN2A* alleles on male embryos, as has been discussed with regard to *MEN2B RET* mutations (Carlson et al. 1994*a*; Sapienza 1994). However, examination of 27 large French MEN 2A families shows that the number of male gene carriers is only slightly lower than the number of affected females (data not shown).

In summary, we have demonstrated that as many as 9% of all MEN 2A and FMTC cases are de novo and that, thus far, the new mutation occurs exclusively on the paternal allele. This systematic study provides the first population register–based estimate of the prevalence of de novo *RET* mutations in the MEN 2A/FMTC syndromes. This has some clinical implications for newly diagnosed, apparently sporadic cases of MTC. Our data indicate that 5.6%–9% of MEN 2A/FMTC cases are de novo. Of these, a high proportion will show other stigmata of MEN 2A synchronously and can be recognized as hereditary MTC cases. However, retrospective data on 274 MEN 2A cases registered by the EuroMen study group have shown that pheochromocytoma occurred 2–11 years subsequent to MTC in 40.2% of these patients (Modigliani et al. 1995). Therefore, we can surmise that 2.2%–3.6% (40.2% × [5.6%–9%]) of newly diagnosed apparently sporadic MTC cases could have occult de novo MEN 2A/FMTC. These patients are at risk for being classified as sporadic cases and not being given further surveillance or counseling. The 2.2%–3.6% figure is within the range found in studies that systematically performed *RET* mutation analysis in series of apparently sporadic cases of MTC (defined as patients with no sign or symptom referable to MEN 2 and with no family history). Among these series, 1.5%–5.9% of cases were found to have germline *RET* mutations (either de novo mutations or mutations transmitted from a nonpenetrant parent) in exon 10 or exon 11 (Eng et al. 1995*a*; Wohllk et al. 1996; I. Schuffenecker and F. Eustache, unpublished data). From a clinical point of view, our data suggest that all apparently sporadic MTC patients should be examined for de novo *RET* mutations. However, from a statistical and public-policy standpoint, it could be argued that the yield of such a systematic testing would be low. Nonetheless, given the efficacy of early or prophylactic thyroidectomy in the treatment or prevention of MTC, it

also could be maintained that it is worth the effort and the cost to identify all occult de novo MEN 2 cases.

ISABELLE SCHUFFENECKER,[1,3] NICOLE GINET,[1] DAVID GOLDGAR,[4] CHARIS ENG,[5] BÉATRICE CHAMBE,[1] ANDRÉE BONEU,[6] CHANTAL HOUDENT,[7] DANIÈLE PALLO,[8] MARTIN SCHLUMBERGER,[9] CHARLES THIVOLET,[2] GILBERT M. LENOIR[1,3] AND LE GROUPE D'ÉTUDE DES TUMEURS À CALCITONINE

[1]*Laboratoire de Génétique and* [2]*Service d'Endocrinologie, Hôpital Edouard Herriot,* [3]*CNRS UMR 5641, Université Claude Bernard, Faculté de Médecine, and* [4]*Unité d'Epidémiologie Génétique, Centre de Recherche sur le Cancer, Lyon;* [5]*Division of Cancer Epidemiology and Control, Dana-Farber Cancer Institute, Harvard Medical School, Boston;* [6]*Service de Médecine Nucléaire, Centre Claudius Régaud, Toulouse;* [7]*Service d'Endocrinologie, Hôpital de Boisguillaume, Rouen;* [8]*Service d'Endocrinologie, Centre Hospitalier Universitaire de Grenoble, Grenoble; and* [9]*Service de Médecine Nucléaire, Institut Gustave Roussy, Paris*

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Address for correspondence and reprints: Dr. Isabelle Schuffenecker or Dr. Gilbert M. Lenoir, Laboratoire de Génétique, Hôpital Edouard Herriot, 1 Place d'Arsonval, 69437 Lyon cedex 03, France.

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Expression of *DAZ*, an *Azoospermia Factor* Candidate, in Human Spermatogonia

To the Editor:

Three percent to 4% of men have severe defects in sperm production that result in infertility (van Zyl et al. 1975; Hull et al. 1985). Most of these men are otherwise healthy, and the cause of their spermatogenic failure is rarely identified with certainty. Little is known about possible contributions from genetic factors, but some cases may be due to mutations that disrupt male germ-cell development without affecting the soma. Such "pure male sterile" genes have been identified in invertebrates (Ellis and Kimble 1994; Castrillan et al. 1993). In humans, no pure sterile factor—male or female—has been unequivocally defined at the biochemical level, although some critical genetic loci have been mapped.

The *azoospermia factor* (*AZF*), found on the long arm of the Y chromosome, may be the most thoroughly studied pure male sterile locus in humans. In 1976, Tiepolo and Zuffardi reported microscopic deletions of Yq in six men with azoospermia (no sperm in semen). On the basis of these findings, they proposed that Yq carries an *AZF* gene or gene complex required for spermatogenesis (Tiepolo and Zuffardi 1976). Recently, Tiepolo and Zuffardi's hypothesis has received strong experimental support (Ma et al. 1992; Kobayashi et al. 1994; Najmabadi et al. 1996; Vogt et al. 1996). In particular, 13% of men with nonobstructive azoospermia were found to have deletions of a consistent, specific portion of Yq—an "*AZF* region" (Reijo et al. 1995). The deletions were not present in the affected individuals' fathers but had arisen de novo, establishing that the deletions were the cause of azoospermia rather than an incidental finding.

De novo deletions of this *AZF* region have been found to result in a wide range of spermatogenic defects. In some *AZF*-deleted individuals, testis biopsies revealed the complete absence of germ cells ("Sertoli cell–only syndrome"). In other individuals, early spermatogenic cells were observed ("testicular maturation arrest"). In two of the latter individuals, spermatogenesis had sometimes progressed through meiosis to the stage of condensed spermatids (Reijo et al. 1995). Indeed, we have shown recently that deletion of *AZF* is compatible with completion of spermatogenesis, albeit at greatly reduced output: two men with severe oligozoospermia (markedly reduced but nonzero semen sperm counts) were found to have de novo, *AZF*-region deletions similar or identical to those found in unrelated azoospermic men (Reijo et al. 1996*a*). Thus, spermatogenesis can, in some instances, proceed without *AZF*. However, spermatogenic output is severely diminished in the absence of *AZF*, and in some cases germ cells (even spermatogonial stem cells) are found to be completely lacking. As we and our colleagues have suggested, this wide array of *AZF* phenotypes might be explained most simply by a defect (with variable expressivity) in spermatogonia, the stem cells that are the source of the spermatogenic lineages in the adult testis (Reijo et al. 1995, 1996*a*).

The molecular identity of *AZF* is not yet known with certainty, but much experimental evidence has accumulated in favor of *DAZ* (*deleted in azoospermia*), a multicopy gene cluster located in this *AZF* region (Reijo et al. 1995; Saxena et al. 1996). As revealed by northern blotting of human tissue RNAs, *DAZ* is transcribed specifically in testis. On the basis of sequence comparisons, *DAZ* appears to encode an RNA-binding protein. Although no human *DAZ* point mutants have been reported, recent genetic findings in *Drosophila* provide strong support for the hypothesis that *DAZ* is *AZF*. Eberhart and colleagues have characterized a *Drosophila* gene, *boule,* whose product shows striking amino acid similarity to the human DAZ protein, especially in the RNA-binding domain. Just as with human *AZF*, loss-of-function mutations in *Drosophila boule* disrupt spermatogenesis and result in azoospermia but spare the soma (Eberhart et al. 1996). Thus, the *Drosophila* ho-