ORIGINAL ARTICLE

Specific haplotypes of the *RET* proto-oncogene are over-represented in patients with sporadic papillary thyroid carcinoma

F Lesueur, M Corbex, J D McKay, J Lima, P Soares, P Griseri, J Burgess, I Ceccherini, S Landolfi, M Papotti, A Amorim, D E Goldgar, G Romeo

J Med Genet 2002;**39**:260–265

See end of article for authors' affiliations

Correspondence to:
Dr G Romeo, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France; romeo@iarc.frRevised version received 17 January 2002
Accepted for publication 23 January 2002**Background:** Papillary thyroid carcinoma (PTC), which may be sporadic (95%) or familial (5%), has a prevalence adjusted for age in the general population of 1:100 000. Somatic rearrangements of the *RET* proto-oncogene are present in up to 66% of sporadic tumours, while they are rarely found in familial cases.**Purpose:** In order to determine if some variants of this gene, or a combination of them, might predispose to PTC, we looked for an association of *RET* haplotype(s) in PTC cases and in controls from four countries matched for sex, age, and population.**Methods:** Four single nucleotide polymorphisms (SNPs) across the *RET* coding sequence were typed and haplotype frequencies were estimated. Genotype and haplotype distributions were compared among these cases and controls.**Results:** Ten haplotypes were observed, the seven most frequent of which have been previously described in sporadic Hirschsprung patients and controls. The single locus analyses suggested association of exon 2 and exon 13 SNPs with sporadic PTC. The haplotype analysis showed over-representation of one haplotype in French and Italian sporadic PTC, whereas a different haplotype was significantly under-represented in French familial PTC.**Conclusions:** Our data suggest that some variants of *RET* and some specific haplotypes may act as low penetrance alleles in the predisposition to PTC.

The *RET* proto-oncogene (MIM 164761) is normally expressed in cells of neural crest origin. Its mutations are associated with a wide spectrum of disease. In Hirschsprung disease (HSCR), a congenital disorder of the enteric nervous system (MIM 142623), point mutations, insertions, or heterozygous deletions of the whole gene lead to a loss of function of the Ret protein.^{1–5} In multiple endocrine neoplasia type 2A and 2B (MEN 2A, MEN 2B, MIM 171400) and in medullary thyroid carcinoma (MTC, MIM 155240), point mutations in the coding sequence of *RET* result in a constitutive activation of the tyrosine kinase domain of the Ret receptor.^{5–14} Finally, in papillary thyroid carcinoma (PTC, MIM 188550), which is the most common form of thyroid malignancy, occurring sporadically in 95% of cases,¹⁵ the disease is very often associated with somatic rearrangements of the *RET* proto-oncogene, resulting in constitutive activation of the *RET* tyrosine kinase domain.^{16–18}

HSCR is characterised by genetic heterogeneity. When inherited, long segment HSCR is usually transmitted as an autosomal dominant trait with incomplete penetrance and variable expressivity, whereas short segment HSCR is transmitted in a complex way.¹⁹ The same definition of a complex genetic disorder can be applied to familial PTC because a variety of genes are involved in its predisposition^{20–24} and the mode of inheritance of familial cases is non-Mendelian. Familial PTC, which accounts for only 5% of the total PTC cases, is reported to be characterised by more aggressive behaviour than sporadic PTC.²⁵ We have previously shown that *RET* was not linked with disease in the majority of PTC families²⁶ and we recently mapped a major gene predisposing to this tumour phenotype in 42% of the families with recurrence of PTC.²⁴ While a somatic rearrangement of *RET* (*RET*/*PTC1*) was found only once in the tumour material of a familial PTC case,²⁷ *RET* rearrangements are observed in 2.5–35% of sporadic PTC

tumours.^{16 28 29} No germline mutations of *RET* have ever been observed to cosegregate with PTC.

In complex disorders, common polymorphic variants can be associated with the disease phenotype, thus modifying the risk of occurrence. Over the three last years, several groups have used *RET* SNPs to determine whether polymorphic variants of *RET* might represent low penetrance alleles predisposing to *RET* associated disorders.^{30–34} It has thus been shown that four silent SNPs in the coding sequence of *RET* are associated with HSCR. A45A (G135A in exon 2) and L769L (T2307G in exon 13) are over-represented whereas G691S (C2071A in exon 11) and S904S (C2712G in exon 15) are under-represented in Spanish and German patients affected with HSCR.^{31 32}

In the same manner, the rarer allele (T) of the SNP S836S (C2508T in exon 14) is over-represented among sporadic cases of MTC from Germany and USA and is apparently associated with the somatic mutation M918T in the tumoral DNA from the same patients.³³ Over-representation of the T polymorphism of S836S was confirmed in Italian MTC patients,³⁴ although in this last study the difference did not achieve statistical significance. This same study also shows that the T allele of the SNP S836S (C2508T) is clearly under-represented in Italian HSCR patients.³⁴ These association studies therefore suggest an opposite role played by some *RET* polymorphisms in the aetiology of sporadic HSCR and MTC.

No study of an association between *RET* SNPs and PTC has been reported so far. In PTC, as in MTC, variation in the DNA

Abbreviations: PTC, papillary thyroid carcinoma; SNP, single nucleotide polymorphism; HSCR, Hirschsprung disease; MTC, medullary thyroid carcinoma; MEN, multiple endocrine neoplasia

Table 1 Primers, PCR conditions, and restriction enzymes used for the genotyping

Exon	Primers sequence (5' > 3')	Annealing temp (°C)	Mg conc (mmol/l)	Product size (bp)	Restriction enzyme	Size of fragments
2	Fwd: TCACCTACATCCCTACTCC Rv: CTTATGCGGACACTGAGC	60	1.2	263	EagI	207+87
13	Fwd: CTCTCTGTCTGAACTGGGC Rv: TCACCTGCGAGCTGGCCTTA	65	1.2	238	TaqI	139+99
14	Fwd: AAGACCCAAGCTGCCTGA Rv: GTGGTGGGTCAAGGTGTGG	66	1.5	328	–	–
15	Fwd: GACTCGTGCTATTTTCCTC Rv: TATCTTCTAGGCTTCCCAA	59	1.5	235	RsaI	106+129

sequence of *RET* might modify expression of the Ret protein and/or lead to activation of the gene. Therefore, we looked for an association of *RET* haplotype(s) in patients from four different countries. For this, we selected four SNPs (located in exons 2, 13, 14, and 15) on the basis of published data^{30–33} from the seven known SNPs encompassing the coding sequence of *RET*. We analysed 247 sporadic PTC cases together with 319 controls.

Since thyroid is the cancer site with the highest familial relative risk among primary cancer sites (FRR=8.60³⁵), we also studied *RET* haplotypes in a sample of 80 French familial PTC cases.

PATIENTS, MATERIALS, AND METHODS

Sporadic PTC and controls

We studied 247 subjects affected with sporadic PTC who were recruited from four countries: Italy (n=100), Portugal (n=95), France (n=26), and Australia (n=26). Healthy blood donors from Portugal (n=62), France (n=102), and Australia (n=55) served as controls. For Italy, controls (n=100) were recruited from patients attending the same hospital as PTC patients for reasons other than cancer or thyroid disease. Controls were matched for age, sex, and geographical origin and were unrelated to the PTC subjects.

DNA from PTC patients was extracted from normal thyroid tissue surrounding the tumour or from peripheral blood. Genomic DNA of controls was obtained from peripheral blood leucocyte samples. DNA was extracted according to a standard phenol-chloroform protocol.

Familial PTC cases

Familial PTC patients came from the families collected at IARC through the International Consortium for the Genetics of Non-Medullary Thyroid Carcinoma. Eighty unrelated French patients with a family history of PTC (at least one relative with PTC) were selected. DNA samples were extracted from blood using the Puregene Kit (Gentra Systems, Minneapolis, MN).

Genotyping

Exons 2, 13, 14, and 15 of *RET* were amplified from genomic DNA using the primers shown in table 1. The PCR reactions were carried out in a volume of 25 µl and included 1 × buffer PCR, 200 mmol/l of each deoxy-NTP, 50 ng DNA, MgCl₂ as indicated in table 1, 0.2 U *Taq* Platinum polymerase (Gibco BRL, Life Technologies), and 10 pmol of each primer. The reactions were carried out in a 9600 GeneAmp PCR System with the following thermal profile: denaturation at 96°C for five minutes; 30 cycles of 94°C for 30 seconds, annealing temperature as indicated in table 1 for 45 seconds, and 72°C for one minute; with a final extension at 72°C for five minutes.

The SNPs G135A (A45A in exon 2), T2307G (L769L in exon 13), C2508T (S836S in exon 14), and C2712G (S904S in exon

15) were genotyped by differential restriction digestion with the appropriate enzymes, as previously described for exons 2, 13, and 15^{31–33–36} according to the manufacturer's recommendations (MBI fermentas, Biolabs). The restriction digestion products were fractionated by electrophoresis through 3% agarose gels containing ethidium bromide and visualised under UV transillumination. For exon 14, direct sequencing of the PCR product was carried out.

Statistical analysis

Allelic frequencies for the four *RET* SNPs were calculated from the genotype frequencies and compared among countries by the chi-square test. Hardy-Weinberg equilibrium was tested in controls from each country. The extent of linkage disequilibrium between SNPs was expressed in terms of D' = D/Dmax or D/Dmin.³⁷ Pairwise linkage disequilibrium coefficients were calculated by using Transposer macros.³⁸

Association with the disease was tested by using chi-square tests when computationally feasible or Fisher's exact test for tables with small expected cell counts.

For controls and sporadic PTC patients, haplotype frequencies were estimated by the maximum likelihood method from genotype data through the use of the E-M algorithm under the assumption of Hardy-Weinberg equilibrium (Arlequin software³⁹). In familial PTC, haplotypes frequencies were estimated using the same method because genotypic information from parents was incomplete.

Haplotypes frequencies were compared using Monte Carlo simulations to estimate the significance level of the test statistic (CLUMP software⁴⁰). After combining haplotypes with a frequency lower than 0.05 to avoid small expected cell counts, test T1 (chi-square on the whole table) was carried out. Comparisons of each haplotype versus the others were also performed by chi-square test.

The significance level of each statistical test was taken to be 0.05; this threshold may seem quite high considering the number of tests performed, but is adequate considering the exploratory character of this study conducted in limited samples.

RESULTS

Comparison of allelic frequencies and linkage disequilibrium between populations

The four polymorphisms studied, their position, nucleotide changes, and allele frequencies are shown in table 2. All the four SNPs are in the coding sequence of *RET* but represent silent mutations. Allele frequencies did not significantly differ among the four populations (Italian, French, Portuguese, and Australian) (table 2). No significant deviation from Hardy-Weinberg equilibrium was observed for any polymorphism. We also checked the pairwise linkage disequilibrium coefficients D' in the four populations separately and did not observe any significant variation between these populations

Table 2 Description of *RET* polymorphisms and allele frequencies in the four control populations

Exon	Amino acid change	Nucleotide substitution	Less frequent allele frequencies			
			Italy	Portugal	France	Australia
Exon 2	A45A	G135A	0.30	0.33	0.29	0.24
Exon 13	L769L	T2307G	0.24	0.19	0.27	0.25
Exon 14	S836S	C2508T	0.06	0.05	0.07	0.05
Exon 15	S904S	C2712G	0.21	0.25	0.26	0.18

Table 3 Allele frequencies and pairwise linkage disequilibrium coefficients (D' above diagonal) between the *RET* SNPs in pooled control subjects from Italy, Portugal, France, and Australia. P values for the test $D'=0$ are provided below the diagonal

	Allele frequencies*	D'			
		Exon 2	Exon 13	Exon 14	Exon 15
Exon 2	0.29		0.42	-0.69	-0.58
Exon 13	0.24	$p<0.001$		0.95	-1
Exon 14	0.06	$p<0.01$	$p<0.001$		-1
Exon 15	0.19	$p<0.001$	$p<0.001$	$p<0.001$	

*Frequency of the rarer allele is given.

Bold indicates significant results at the $\alpha=0.05$ level.

(data not shown). These results allowed us to analyse the data of the four populations pooled together. The pairwise linkage disequilibrium coefficients D' in the pooled populations are given in table 3.

Association of individual polymorphic loci with PTC (single locus analyses)

In order to test the impact that polymorphisms of the *RET* proto-oncogene might have on the aetiology of PTC, we investigated the genotype distribution of the four SNPs in exons 2, 13, 14, and 15 in patients with sporadic PTC. Table 4 presents the results of single locus analyses in the four populations

considered independently and in the pooled populations. Of the four loci, only the two SNPs in exons 2 and 13 showed significant association with PTC; homozygotes for G G of exon 2 were over-represented in Italian PTC patients. The same trend was observed in the Portuguese and French populations, but not in the Australian population; homozygotes for G G of exon 13 were over-represented in the PTC patients when the four populations were pooled together. This trend was observed in each population taken independently, except in the Australian population.

Allele G of exon 2 is in weak association with allele A of exon 13 ($D'=0.42$, table 2), thus the effect observed for exon

Table 4 Single locus analysis for each of the four SNPs of *RET* in the different populations studied. The studied populations comprised 247 sporadic PTC patients from Italy (n=100), Portugal (n=95), France (n=26), and Australia (n=26); 319 controls were matched for age, sex, and geographical origin (100 for Italy, 62 for Portugal, 102 for France, and 55 for Australia)

SNP	Frequency†		Odds ratio‡					
	PTC	Controls	Model	All†	Italy	Portugal	Australia	France
Exon 2	0.26	0.29	Codom	0.83	0.70	0.78	1.31	0.72
			Dom	0.73	0.54*	0.68	1.19	0.71
			Rec	0.94	0.85	1.10	0.44	2.13
Exon 13	0.22	0.24	Codom	1.17	1.26	1.48	0.92	1.20
			Dom	1.04	1.08	1.32	0.95	0.96
			Rec	2.22*	3.57	5.56	0.71	2.44
Exon 14	0.05	0.06	Codom	0.89	0.90	0.86	1.29	1.13
			Dom	0.85	0.90	0.74	1.30	1.14
			Rec	—	—	—	—	—
Exon 15	0.19	0.19	Codom	1.01	0.91	0.75	0.48	1.84
			Dom	1.03	0.96	0.76	0.45	1.83
			Rec	1.07	1.53	2.11	—	—

*Significant p value (less than 0.05).

†Frequency of the rare allele in the pooled population.

‡Odds ratio is given for each model: codominant (comparison of allelic frequencies), dominant (heterozygotes and rare homozygotes have the same risk), and recessive (heterozygotes and frequent homozygotes have the same risk), respectively.

Table 5 Comparison of haplotype frequencies between sporadic PTC and controls

Haplotypes	Frequency†		Odds ratio‡				
	PTC	Controls	All†	Italy	Portugal	Australia	France
G T C C	0.42	0.43	0.96	1.06	1.43	1.17	0.6
G T C G	0.18	0.17	1.09	0.97	0.80	0.39	2.33*
A G C C	0.14	0.14	1.00	0.92	2.08	0.94	1.02
A T C C	0.12	0.14	0.81	0.62	0.56	1.88	0.70
G G T C	0.05	0.05	0.94	0.81	2.64	0.87	1.18
G G C C	0.08	0.05	1.68*	2.82*	0.77	0.71	2.41
A T C G	<0.01	0.02	0.26	–	0.25	–	–
G T T C	0.02	<0.01	–	–	–	–	–
A G T G	0.01	<0.01	–	–	–	–	–
G G C G	<0.01	0.01	–	–	–	–	–
p value for T1 test§			0.30	0.16	0.14	0.38	0.40

*Significant p value (less than 0.05).

†Haplotype frequencies when the four populations are pooled.

‡Odds ratio provided for each haplotype corresponds to the comparison of the haplotype with all the others.

§After combining haplotypes with a frequency lower than 5%, the T1 test performs a chi-square test on the whole table.

13 G is not the result of linkage disequilibrium with exon 2 G (and vice versa). The results suggest that each of the G alleles has a separate effect on risk, or that the G G haplotype carries a functional at risk allele.

RET haplotype analysis in PTC and unrelated controls

A total of 10 haplotypes was observed in the case-control groups (table 5). The six most frequent haplotypes exist in the four populations. The additional rare haplotypes were pooled in one class to allow comparison between PTC and controls.

Although a chi-square test performed on the whole table (test T1, see Patients, materials, and methods) was not significant, some of the haplotypes displayed different frequencies between cases and controls, indicating putative associations with the disease.

In the French population, the G T C G haplotype was significantly over-represented in PTC. This was not observed in the three other populations. This association was the result of the fact that in our French control group, the frequency of this haplotype was lower than in the three other control groups. This indicated that the association was probably not a genuine one.

In the Italian population, the G G C C haplotype was over-represented in PTC patients. This was consistent with the result obtained with the single locus analysis (over-representation of the homozygous G G in exon 2, table 4). This haplotype was also over-represented in French PTC, although the difference between cases and controls was not statistically significant ($p=0.12$). When the four populations were pooled (table 5), the G G C C haplotype was significantly over-represented ($p=0.03$). In the Portuguese and Australian populations, the frequencies of this haplotype were not different in cases and controls. The G G C C haplotype may therefore reflect the presence of a low penetrance predisposing allele for PTC in the Italian and French populations. However, owing to the small sample set and the number of tests performed, this result should be confirmed on larger samples.

RET genotype and haplotype analyses in familial PTC

When comparing the four SNP allele frequencies in the 80 French familial PTC and the 102 French controls (table 6), we observed an under-representation of the rare T allele of the SNP in exon 14 (S836S) which did not exist in sporadic PTC.

In familial PTC, we observed the seven haplotypes already observed in the French population (table 6) but one of them was under-represented in familial PTC (the G G T C haplotype). This under-representation of G G T C was not observed in the sporadic PTC. This haplotype was the only one in the French population comprising the rare T allele of S836S in exon 14. This result was consistent with the single locus analysis.

Table 6 Single locus analysis for each of the four SNPs of RET, and comparison of haplotype frequencies between French familial PTC (n=80) and controls (n=102)

SNP	Model	Odds ratio†
Exon 2	Codom	0.97
	Dom	1.01
	Rec	1.28
Exon 13	Codom	0.93
	Dom	0.97
	Rec	1.40
Exon 14	Codom	0.26*
	Dom	0.24*
	Rec	–
Exon 15	Codom	1.58
	Dom	1.49
	Rec	–
Haplotypes	Odds ratio‡	
G T C C	0.84	
G T C G	1.78	
A G C C	1.18	
A T C C	0.86	
G G T C	0.26*	
G G C C	1.94	
p value for T1 test§	0.33	

*Significant p value (less than 0.05).

†Odds ratio is given for each model: codominant, dominant, and recessive, respectively.

‡Odds ratio provided for each haplotype corresponds to the comparison of the haplotype with all the others.

§After combining haplotypes with a frequency lower than 5%, the T1 test performs a chi square test on the whole table.

G T C G and G G C C haplotypes were over-represented in the familial PTC, as they were in sporadic PTC, although these results were not statistically significant.

DISCUSSION

It is now well established that polymorphisms may have a relatively strong effect on disease susceptibility. This has been illustrated by a number of reported examples, the last one in chronological order being represented by variants in the *NOD2* gene associated with susceptibility to Crohn's disease.^{41–42}

In the present study, we observed that some variants within RET could represent low penetrant alleles for the PTC phenotype. Of the four SNPs studied, the strongest association with

PTC was found for polymorphism A45A (G135A) in exon 2 and L769L (T2307G) in exon 13. They were also the ones found to be associated with sporadic HSCR.³⁰⁻³² However, the A allele of exon 2 was found to be at risk for HSCR whereas it was found to be a protective allele for PTC in our study. This observation is consistent with the fact that mutations of *RET* occurring in HSCR disease and in thyroid cancer have opposite effects. In HSCR, known mutations lead to a loss of function of the RET protein, whereas in thyroid cancer, known mutations lead to a constitutive activation of the receptor (germinal point mutations in MTC and somatic *RET*/PTC rearrangements in sporadic PTC).

With the four SNPs studied, a total of 10 haplotypes of *RET* were observed when samples from the four countries were pooled. The seven most frequent haplotypes have been described by Borrego *et al*³⁰ in controls of Spanish and German origin. Distribution of some of these haplotypes differed between cases and controls. The G G C C haplotype is over-represented in both Italian and French sporadic PTC and this over-representation is significant when all four populations are pooled (OR 1.68, 95% CI 1.04-2.71). The G G C C haplotype includes the G allele of exon 2 and the G allele of exon 13, the two alleles that are associated with an increasing risk in the single locus analysis.

There is only one other haplotype that includes the two Gs, G G T C, but it is very rare owing to the very low frequency of the exon 14 T allele (6.0%, table 3). To confirm the association and to ascertain if the increased risk is the result of independent effects of exon 2 and exon 13 or to some exon 2-exon 13 containing haplotypes, larger studies are required.

Another interesting finding raised by this study is the differential association with *RET* in sporadic and familial PTC. The haplotype G G T C is found in only 1.9% of the French familial PTC compared to 6.9% in controls, and might therefore protect against the development of PTC in families that segregate the PTC phenotype (OR 0.26, 95% CI 0.08-0.87). This under-representation is not observed in sporadic PTC. Interestingly, an under-representation of the exon 14 T allele is observed in the French familial PTC while an over-representation of the T allele has been reported in MTC patients.³³⁻³⁴ This T allele is even more represented in MTC patients with the somatic mutation M918T of *RET*.³³ These results suggest an opposite role played by the exon 14 T allele in the aetiology of thyroid carcinoma of follicular and parafollicular cell origin.

Recently, we mapped a major predisposing gene on chromosome 2q21 involved in 42% of 80 families with recurrence of PTC.²⁴ The influence of modifier genes in these families is suggested by the intrafamilial heterogeneity of the phenotype (different variants of PTC and presence of both benign and malignant tumours in the same family). Using the French subset of the PTC families used for mapping of locus 2q21, we showed in the present study that the G G C C haplotype is also more represented in the French familial PTC (OR 1.94, 95% CI 0.83-4.56) and could therefore act as a modifier or low predisposing allele.

In a previous study,²⁶ we tested by linkage the involvement of *RET* as a major predisposing gene in a subset of the 80 PTC families used to map a new gene on chromosome 2. The proportion of families linked to *RET* could not be estimated, owing to the small number of families and to the high genetic heterogeneity between these families. The aim of the present study was to evaluate if common alleles of *RET* could represent a low risk for PTC. The magnitude of the effect between the *RET* SNPs/haplotypes and PTC is quite modest at best, that is, ORs of around 2 for a haplotype with a frequency of 5%. By itself, this locus would not be expected to contribute much to familial clustering with a predicted familial risk of 1.04 (about 2% of the overall familial effect). Thus, it is not surprising that there would be no evidence of linkage between *RET* and PTC in a family based linkage analysis.

The mechanism by which the silent polymorphisms may act in the development of PTC or other *RET* related diseases is unknown. Assuming a direct effect of one of the four *RET* polymorphisms considered, a series of possible effects should be taken into account, including transcript stability, RNA splicing, and DNA protein binding and protein folding. Hypotheses have been made regarding the possible mechanisms. For instance, it has been proposed that the silent sequence variant at codon 45 in exon 2 could lead to aberrantly spliced products, resulting in a protein with a 21 amino acid deletion in the extracellular domain, altering a part of the extracellular signal peptide sequence.⁴³ The C>T transition of polymorphism S836S in exon 14 generates a putative binding site (ACTCaAGT) for a thyroid specific factor not yet identified.³⁴ As an attempt to explain a possible role of the T allele of S836S over-represented in MTC, Griseri *et al*³⁴ carried out gel retardation assays and did not find any difference between normal (C) and variant (T) oligonucleotide binding profiles. A possible interference of this SNP with correct *RET* mRNA processing was also experimentally excluded.³⁴

To test the possible interference of the other three SNPs independently and of some combinations of all of them (corresponding to the haplotypes showing different breakdown between cases and controls, G G C C, G T C G, G G T C) with correct splicing, we have used appropriate gene predictions that are included in the NIX software (see Data access). The exon prediction analyses performed could not detect any abnormal alternative splicing site. Alternatively, the over-represented haplotypes might be in linkage disequilibrium with another nucleotide variant, of either the *RET* gene or flanking regions, displaying a low penetrant causative effect by itself. If our results are confirmed in other populations, a search for mutations should be undertaken in subjects sharing the specific haplotypes (G G C C in Italian PTC patients and G T C G in French PTC patients) and functional analyses will also be needed to elucidate the precise mechanism of the haplotypes associated with the PTC phenotype.

ACKNOWLEDGEMENTS

We are grateful to Suzanne Pauly and Cyril Lafaye for their technical assistance. Fabienne Lesueur is recipient of a Special Training Award from the International Agency for Research on Cancer. Data access. URLs for data in this report are as follows: Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/omim> (for Hirschsprung disease (MIM 142623), for multiple endocrine neoplasia type 2 (171400), for medullary thyroid carcinoma (155240), for papillary thyroid carcinoma (MIM 188550), for *RET* proto-oncogene (MIM 164761). NIX software is available on <http://www.hgmp.mrc.ac.uk/NIX/>

Authors' affiliations

F Lesueur, M Corbex, J D McKay, D E Goldgar, G Romeo, International Agency for Research on Cancer, Lyon, France
J Lima, P Soares, A Amorim, IPATIMUP, Porto, Portugal
P Griseri, I Ceccherini, Laboratorio di Genetica Molecolare, Istituto Giannina Gaslini, Genova, Italy
J D McKay, J Burgess, Department of Endocrinology, Royal Hobart Hospital, Hobart, Tasmania, Australia
S Landolfi, M Papotti, Dipartimento di Anatomia Patologica, University of Turin, Italy
P Soares, Faculty of Medicine, University of Porto, Portugal
A Amorim, Faculty of Sciences, University of Porto, Portugal
G Romeo, Laboratorio di Genetica Medica, Dipartimento di Medicina Interna, Cardioangiologia, Epatologia, University of Bologna Medical School, Italy

REFERENCES

- 1 Edery P, Lyonnet S, Mulligan LM, Pelet A, Dow E, Abel L, Holder S, Nihoul-Fekete C, Ponder BA, Munnich A. Mutations of the RET proto-oncogene in Hirschsprung's disease. *Nature* 1994;**367**:378-80.
- 2 Romeo G, Ronchetto P, Luo Y, Barone V, Seri M, Ceccherini I, Pasini B, Boccardi R, Lerone M, Kääriäinen H, Martucciello G. Point mutations

- affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. *Nature* 1994;**367**:377-8.
- 3 **Attie T**, Pelet A, Edery P, Eng C, Mulligan LM, Amiel J, Boutrand L, Beldjord C, Nihoul-Fekete C, Munnich A. Diversity of RET proto-oncogene mutations in familial and sporadic Hirschsprung disease. *Hum Mol Genet* 1995;**4**:1381-6.
 - 4 **Pasini B**, Borrello MG, Greco A, Bongarzone I, Luo Y, Mondellini P, Alberti L, Miranda C, Arighi E, Bocciardi R, Seri M, Barone V, Radice MT, Romeo G, Pierotti MA. Loss of function effect of RET mutations causing Hirschsprung disease. *Nat Genet* 1995;**10**:35-40.
 - 5 **Eng C**, Mulligan LM. Mutations of the RET proto-oncogene in the multiple endocrine neoplasia type 2 syndromes, related sporadic tumours, and Hirschsprung disease. *Hum Mutat* 1997;**9**:97-109.
 - 6 **Schuffenecker I**, Billaud M, Calender A, Chambe B, Ginet N, Calmettes C, Modigliani E, Lenoir GM. RET proto-oncogene mutations in French MEN 2A and FMTC families. *Hum Mol Genet* 1994;**3**:1939-43.
 - 7 **Bolino A**, Schuffenecker I, Luo Y, Seri M, Silengo M, Tocco T, Chabrier G, Houdent C, Murat A, Schlumberger M. RET mutations in exons 13 and 14 of FMTC patients. *Oncogene* 1995;**10**:2415-19.
 - 8 **Eng C**, Smith DP, Mulligan LM, Healey CS, Zvelebil MJ, Stonehouse TJ, Ponder MA, Jackson CE, Waterfield MD, Ponder BA. A novel point mutation in the tyrosine kinase domain of the RET proto-oncogene in sporadic medullary thyroid carcinoma and in a family with FMTC. *Oncogene* 1995;**10**:509-13.
 - 9 **Rossel M**, Schuffenecker I, Schlumberger M, Bonnardel C, Modigliani E, Gardet P, Navarro J, Luo Y, Romeo G, Lenoir G, Billaud M. Detection of a germline mutation at codon 918 of the RET proto-oncogene in French MEN 2B families. *Hum Genet* 1995;**95**:403-6.
 - 10 **Boccia LM**, Green JS, Joyce C, Eng C, Taylor SA, Mulligan LM. Mutation of RET codon 768 is associated with the FMTC phenotype. *Clin Genet* 1997;**51**:81-5.
 - 11 **Gimm O**, Marsh DJ, Andrew SD, Frilling A, Dahia PL, Mulligan LM, Zajac JD, Robinson BG, Eng C. Germline dinucleotide mutation in codon 883 of the RET proto-oncogene in multiple endocrine neoplasia type 2B without codon 918 mutation. *J Clin Endocrinol Metab* 1997;**82**:3902-4.
 - 12 **Hofstra RM**, Fattoruso O, Quadro L, Wu Y, Libroia A, Verga U, Colantuoni V, Buys CH. A novel point mutation in the intracellular domain of the ret protooncogene in a family with medullary thyroid carcinoma. *J Clin Endocrinol Metab* 1997;**82**:4176-8.
 - 13 **Berndt I**, Reuter M, Saller B, Frank-Raue K, Groh P, Grussendorf M, Raue F, Ritter MM, Hopner W. A new hot spot for mutations in the ret protooncogene causing familial medullary thyroid carcinoma and multiple endocrine neoplasia type 2A. *J Clin Endocrinol Metab* 1998;**83**:770-4.
 - 14 **Pigny P**, Batters C, Wemeau JL, Houcke ML, Crepin M, Caron P, Giraud S, Calender A, Buisine MP, Kerckaert JP, Porchet N. A novel 9-base pair duplication in RET exon 8 in familial medullary thyroid carcinoma. *J Clin Endocrinol Metab* 1999;**84**:1700-4.
 - 15 **Alsanee O**, Clark O. Familial thyroid cancer. *Curr Opin Oncol* 2001;**13**:44-51.
 - 16 **Grieco M**, Santoro M, Berlingieri MT, Melillo RM, Donghi R, Bongarzone I, Pierotti MA, Della Porta G, Fusco A, Vecchio G. PTC is a novel rearranged form of the ret proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinomas. *Cell* 1990;**60**:557-563.
 - 17 **Bongarzone I**, Monzini N, Borrello MG, Carcano C, Ferraresi G, Arighi E, Mondellini P, Della Porta G, Pierotti MA. Molecular characterization of a thyroid tumor-specific transforming sequence formed by the fusion of ret tyrosine kinase and the regulatory subunit RI alpha of cyclic AMP-dependent protein kinase A. *Mol Cell Biol* 1993;**13**:358-66.
 - 18 **Bounacer A**, Wicker R, Caillou B, Cailleux AF, Sarasin A, Schlumberger M, Suarez HG. High prevalence of activating ret proto-oncogene rearrangements, in thyroid tumors from patients who had received external radiation. *Oncogene* 1997;**15**:1263-73.
 - 19 **Pasini B**, Ceccherini I, Romeo G. RET mutations in human disease. *Trends Genet* 1996;**12**:138-44.
 - 20 **Bignell GR**, Canzian F, Shayeghi M, Stark M, Shugart YY, Biggs P, Mangion J, Hamoudi R, Rosenblatt J, Buu P, Sun S, Stoffer SS, Goldgar DE, Romeo G, Houlston RS, Narod SA, Stratton MR, Foulkes WD. Familial nontoxic multinodular thyroid goiter locus maps to chromosome 14q but does not account for familial nonmedullary thyroid cancer. *Am J Hum Genet* 1997;**61**:1123-30.
 - 21 **Canzian F**, Amati P, Harach HR, Kraimps JL, Lesueur F, Barbier J, Levillain P, Romeo G, Bonneau D. A gene predisposing to familial thyroid tumors with cell oxyphilia maps to chromosome 19p13.2. *Am J Hum Genet* 1998;**63**:1743-8.
 - 22 **Gemmell RM**, West JD, Boldog F, Tanaka N, Robinson LJ, Smith DI, Li F, Drabkin HA. The hereditary renal cell carcinoma 3;8 translocation fuses FHIT to a patched-related gene, TRC8. *Proc Natl Acad Sci USA* 1998;**95**:572-7.
 - 23 **Malchoff CD**, Sarfarazi M, Tendler B, Forouhar F, Whalen G, Joshi V, Arnold A, Malchoff DM. Papillary thyroid carcinoma associated with papillary renal neoplasia: genetic linkage analysis of a distinct heritable tumor syndrome. *J Clin Endocrinol Metab* 2000;**85**:1758-64.
 - 24 **McKay JD**, Lesueur F, Jonard L, Pastors A, Williamson J, Hoffman L, Burgess J, Duffield A, Papotti M, Stark M, Sobol H, Maes B, Murat A, Kaariainen H, Bertholon-Gregoire M, Zini M, Rossing MA, Toubert ME, Bonichon F, Cavarec M, Bernard AM, Boneu A, Lepret F, Haas O, Lassot C, Schlumberger M, Canzian F, Goldgar DE, Romeo G. Localization of a susceptibility gene for familial nonmedullary thyroid carcinoma to chromosome 2q21. *Am J Hum Genet* 2001;**69**:440-6.
 - 25 **Grossman RF**, Tu SH, Duh QY, Siperstein AE, Novosolov F, Clark OH. Familial nonmedullary thyroid cancer. An emerging entity that warrants aggressive treatment. *Arch Surg* 1995;**130**:892-7.
 - 26 **Lesueur F**, Stark M, Tocco T, Ayadi H, Deslisle MJ, Goldgar DE, Schlumberger M, Romeo G, Canzian F for the NMTC Consortium. Genetic heterogeneity in familial nonmedullary thyroid carcinoma: exclusion of linkage to RET, MNG1, and TCO in 56 families. *J Clin Endocrinol Metab* 1999;**84**:2157-62.
 - 27 **Corvi R**, Lesueur F, Martinez-Alfaro M, Zini M, Decaussin M, Murat A, Romeo G. RET rearrangements in familial papillary thyroid carcinomas. *Cancer Lett* 2001;**170**:191-8.
 - 28 **Bongarzone I**, Butti MG, Coronelli S, Borrello MG, Santoro M, Mondellini P, Pilotti S, Fusco A, Della Porta G, Pierotti MA. Frequent activation of ret protooncogene by fusion with a new activating gene in papillary thyroid carcinomas. *Cancer Res* 1994;**54**:2979-85.
 - 29 **Cinti R**, Yin L, Ilc K, Berger N, Basolo F, Cuccato S, Giannini R, Torre G, Miccoli P, Amati P, Romeo G, Corvi R. RET rearrangements in papillary thyroid carcinomas and adenomas detected by interphase FISH. *Cytogenet Cell Genet* 2000;**88**:56-61.
 - 30 **Borrego S**, Ruiz A, Saez ME, Gimm O, Gao X, Lopez-Alonso M, Hernandez A, Wright FA, Antiñolo G, Eng C. RET genotypes comprising specific haplotypes of polymorphic variants predispose to isolated Hirschsprung disease. *J Med Genet* 2000;**37**:572-8.
 - 31 **Borrego S**, Saez ME, Ruiz A, Gimm O, Lopez-Alonso M, Antiñolo G, Eng C. Specific polymorphisms in the RET proto-oncogene are over-represented in patients with Hirschsprung disease and may represent loci modifying phenotypic expression. *J Med Genet* 1999;**36**:771-4.
 - 32 **Fitze G**, Schreiber M, Kuhlisch E, Schackert HK, Roesner D. Association of RET protooncogene codon 45 polymorphism with Hirschsprung disease. *Am J Hum Genet* 1999;**65**:1469-73.
 - 33 **Gimm O**, Neuberg DS, Marsh DJ, Dahia PL, Hoang-Vu C, Raue F, Hinze R, Dralle H, Eng C. Over-representation of a germline RET sequence variant in patients with sporadic medullary thyroid carcinoma and somatic RET codon 918 mutation. *Oncogene* 1999;**18**:1369-73.
 - 34 **Griseri P**, Sancandi M, Patrone G, Bocciardi R, Hofstra R, Ravazzolo R, Devoto M, Romeo G, Ceccherini I. A single-nucleotide polymorphic variant of the RET proto-oncogene is underrepresented in sporadic Hirschsprung disease. *Eur J Hum Genet* 2000;**8**:721-4.
 - 35 **Goldgar DE**, Easton DF, Cannon-Albright LA, Skolnick MH. Systematic population-based assessment of cancer risk in first-degree relatives of cancer probands. *J Natl Cancer Inst* 1994;**86**:1600-8.
 - 36 **Ceccherini I**, Hofstra RM, Luo Y, Stulp RP, Barone V, Stelwagen T, Bocciardi R, Nijveen H, Bolino A, Seri M, Ronchetto P, Pasini B, Bozzano M, Buys C, Romeo G. DNA polymorphisms and conditions for SSCP analysis of the 20 exons of the ret proto-oncogene. *Oncogene* 1994;**9**:3025-9.
 - 37 **Thompson EA**, Deeb S, Walker D, Motulsky AG. The detection of linkage disequilibrium between closely linked markers: RFLPs at the ALCIII apolipoprotein genes. *Am J Hum Genet* 1988;**42**:113-24.
 - 38 **Cox D**, Canzian F. Genotype transposer: automated genotype manipulation for linkage disequilibrium analysis. *Bioinformatics* 2001;**17**:738-9.
 - 39 **Excoffier L**, Slakin M. Maximum-likelihood estimation of molecular haplotype frequencies in diploid population. *Mol Biol Evol* 1995;**12**:921-7.
 - 40 **Sham PC**, Curtis D. Monte Carlo tests for associations between disease and alleles at highly polymorphic loci. *Ann Hum Genet* 1995;**59**:97-105.
 - 41 **Hugot JP**, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001;**411**:599-603.
 - 42 **Ogura Y**, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, Cho JH. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001;**411**:603-6.
 - 43 **Borrego S**, Eng C, Sanchez B, Saez ME, Navarro E, Antinolo G. Molecular analysis of the ret and GDNF genes in a family with multiple endocrine neoplasia type 2A and Hirschsprung disease. *J Clin Endocrinol Metab* 1998;**83**:3361-4.