Hirschsprung disease in MEN 2A: increased spectrum of RET exon 10 genotypes and strong genotype–phenotype correlation

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The RET proto-oncogene encodes a transmembrane receptor with tyrosine kinase activity. Germline mutations in RET are responsible for a number of inherited diseases. These include the dominantly inherited cancer syndromes multiple endocrine neoplasia types 2A and 2B (MEN 2A and MEN 2B) and familial medullary thyroid carcinoma (FMTC), as well as some cases of familial Hirschsprung disease (HSCR1). RET mutations in HSCR1 have been shown to cause a loss of RET function, while the cancer syndromes result in RET oncogenic activation. Occasionally MEN 2A or FMTC occurs in association with HSCR1, albeit with low penetrance. An initial report linked HSCR1 in MEN 2A solely to the C618R and C620R RET mutations. In this study we have analyzed 44 families with MEN 2A. HSCR1 co-segregated with MEN 2A in seven (16%) of the 44 families. The predisposing RET mutation in all seven families had been previously reported in MEN 2A or FMTC and occurred in exon 10 at codons 609, 618 or 620, resulting in C609Y, C618S, C620R or C620W substitution. MEN 2A families with RET exon 10 Cys mutations had a substantially greater risk of developing HSCR1 than those with the more common RET exon 11 Cys634 or exon 14 c804 mutations (P = 0.0005). These findings suggest that expression of HSCR1 in MEN 2A may be peculiar to RET exon 10 Cys mutations. However, HSCR1 in MEN 2A is not exclusive to C618R or C620R RET mutations and can occur with other exon 10 Cys amino acid substitutions. The strong correlation between disease phenotype and position of the MEN 2A RET mutation suggests that oncogenic activation of RET alone is insufficient to account for co-expression of the diseases.

INTRODUCTION

Germline mutations of the RET proto-oncogene, a transmembrane tyrosine kinase receptor mapping to chromosome 10q11.2, have been found to predispose to the development of two autosomal dominantly inherited diseases: (i) the medullary thyroid cancer syndromes multiple endocrine neoplasia type 2 (MEN 2), subclassified into types 2A and 2B, and familial medullary thyroid carcinoma (FMTC) (1–5); (ii) familial Hirschsprung disease (HSCR1) (6–8). MEN 2A is characterized by the occurrence of medullary thyroid carcinoma (MTC) with variable expression of pheochromocytoma and hyperparathyroidism. In the FMTC variant MTC occurs in isolation, while MEN 2B gives rise to a more aggressive form of MTC, pheochromocytoma and a hyperganglionosis of the gut without parathyroid disease. Most frequently germline mutations predisposing to MEN 2A and FMTC involve one of six highly conserved Cys residues within the extracellular domain of RET exon 10 at codons 609, 611, 618 and 620 or exon 11 at codons 630 and 634 (1–5,9). Less commonly missense sequence changes affecting the tyrosine kinase domain occur in exon 13 (c768) and exon 14 (c804) occur in patients with FMTC (10–12). Finally, germline mutations predisposing to MEN 2B are located solely in exon 16 at codon 918 (3–5). RET mutations in the cancer syndromes appear to result in oncogenic activation (13,14).

Hirschsprung disease (HSCR) is a congenital disorder affecting ~1 in every 5000 live births (15). The disease is defined histologically by the absence of intrinsic ganglion cells of the gastrointestinal tract, producing a functional bowel obstruction in infancy. A number of deletions, frameshift, missense and nonsense sequence alterations, resulting in insufficient RET protein product or its impaired transport, have been described in HSCR1 (6–8,16). Other genes which contribute to HSCR susceptibility include the endothelin-B receptor (EDNRB) and endothelin 3 (EDN3) genes (17–19). Therefore, germline RET alterations predispose to two distinct syndromes, MEN 2 and HSCR1, seemingly through disparate effects on RET activity. Yet most intriguing is that HSCR1 has been found to co-segregate with MEN 2A in rare families, albeit with low penetrance (20–22).

Preliminary data by others (21) exclusively linked co-expression of HSCR1 and MEN 2A to a Cys→Arg substitution at RET codons 618 and 620 in exon 10. However, in an initial study (22) we reported two families with the combined phenotype harboring a
Figure 1. Pedigrees of seven families with MEN 2A and HSCR1. Arrows indicate patients with histological evidence of HSCR1. Pedigrees IIIa, IIIb, IVa and IVb represent relevant segments from two large and possibly related MEN 2a kindreds (~102 and 53 affected individuals respectively) which have been previously reported (see 22).

C618S mutation. Therefore, further analysis was needed to better clarify the genetic profile of HSCR1 in MEN 2A.

In this study we have investigated our families with MEN 2A and FMTC for the presence of HSCR1 and their predisposing RET mutation. Our results suggest that: (i) the number of MEN 2A RET genotypes associated with co-expression of HSCR1 is greater than initially suspected; (ii) the HSCR1 phenotype in MEN 2A appears uniquely linked to RET exon 10 Cys missense mutations.

RESULTS

The predisposing MEN 2A mutation in all seven families (Fig. 1) expressing HSCR1 involved missense changes at one of three highly conserved Cys residues in RET exon 10 at codons 609, 618 and 620 (Fig. 2). The identical exon 10 mutation was detected in each family member affected with MEN 2A, irrespective of the presence of HSCR1. No sequence changes were observed in RET exons 11 and 13–16.

One of the seven families possessed the C620R substitution previously reported by others (30). The remaining six families had a c609 TAC, c618 AGC, c618 TCC or c620 TGG Cys mutation, resulting in Cys→Tyr, Cys→Ser or Cys→Trp amino acid changes (Fig. 2 and Table 1).
The HSCR1 phenotype involved 10 males and six females and occurred within sibships in four of the seven MEN 2A families. All patients with HSCR1 who did not succumb to complications of their disease in early life underwent thyroidectomy for a C-cell abnormality (see Materials and Methods). HSCR1 was not observed in isolation without other stigmata of MEN 2A. Thirteen of the 16 patients had retrievable medical records and histological evidence of HSCR1. Of these 13 patients seven had long segment and six had short segment disease. Identical histological evidence of HSCR1. Of these 13 patients seven had long segment and six had short segment disease. Identical histological evidence of HSCR1. Of these 13 patients seven had long segment and six had short segment disease. Identical histological evidence of HSCR1. Of these 13 patients seven had long segment and six had short segment disease. Identical histological evidence of HSCR1. Of these 13 patients seven had long segment and six had short segment disease. Identical histological evidence of HSCR1. Of these 13 patients seven had long segment and six had short segment disease. Identical histological evidence of HSCR1. Of these 13 patients seven had long segment and six had short segment disease. Identical histological evidence of HSCR1. Of these 13 patients seven had long segment and six had short segment disease. Identical histological evidence of HSCR1. Of these 13 patients seven had long segment and six had short segment disease. Identical histological evidence of HSCR1. Of these 13 patients seven had long segment and six had short segment disease. Identical histological evidence of HSCR1. Of these 13 patients seven had long segment and six had short segment disease.

Haplotype analyses using highly polymorphic microsatellites flanking RET suggested that families III and IV could share a single founder chromosome. An extensive pedigree analysis of the origin of the two families did not disclose a common ancestor, however, both families reside in geographic proximity to each other.

RET missense changes at Cys codon 609, 618 or 620 in exon 10 occurred in 10 of the 44 MEN 2A families without evidence of HSCR1. The HSCR1 phenotype was not present in any of the 23 MEN 2A families with the Cys codon 634 mutation (exon 11) or four FMTC families harboring codon 804 (GTG→ATG, GTG→CTG and GTG→TTG) exon 14 mutations. Mutations involving RET codons 611 (exon 10), 768 (exon 13) and 804 (exon 14) predispose to the development of MTC alone consistently without pheochromocytoma or hyperparathyroidism (24). The results of our study suggest yet another intriguing genotype–phenotype correlation. We propose that Cys mutations within exon 10 may uniquely predispose to HSCR1 and MEN 2A/FMTC, whereas those at RET codon 634 do not. Several lines of evidence are consistent with our findings. First, Ito et al. (25) recently analyzed the transforming capacity of c-RET transfected into NIH 3T3 cells which harbored Cys mutations at codons 609, 611, 618 and 620 in exon 10 and codon 634 in exon 11. Results of the study showed that exon 10 Cys mutations had a 3- to 5-fold lower transforming activity than that of the Cys c634 mutant. Furthermore, expression of RET on the cell surface directly paralleled the transforming activity of each mutant protein. Therefore, expression of codon 609, 611, 618 or 620 RET mutants was diminished in comparison with those harboring the RET codon 634 mutation. A similar decrease in RET expression has been previously reported in protein mutants harboring the RET missense changes described in sporadic and familial HSCR (16,26).

**DISCUSSION**

Several mutation-specific variations in disease phenotype have been described in MEN 2A. Allele-specific variation in predisposition to pheochromocytoma and parathyroid disease has been associated with missense mutations at RET codon 634 and the C634R mutation respectively (23,24). Furthermore, several non-cysteine missense changes in the catalytic domain of RET at codons 768 (exon 13) and 804 (exon 14) predispose to the development of MTC alone consistently without pheochromocytoma or hyperparathyroidism (24). The results of our study suggest yet another intriguing genotype–phenotype correlation. We propose that Cys mutations within exon 10 may uniquely predispose to HSCR1 and MEN 2A/FMTC, whereas those at RET codon 634 do not. Several lines of evidence are consistent with our findings. First, Ito et al. (25) recently analyzed the transforming capacity of c-RET transfected into NIH 3T3 cells which harbored Cys mutations at codons 609, 611, 618 and 620 in exon 10 and codon 634 in exon 11. Results of the study showed that exon 10 Cys mutations had a 3- to 5-fold lower transforming activity than that of the Cys c634 mutant. Furthermore, expression of RET on the cell surface directly paralleled the transforming activity of each mutant protein. Therefore, expression of codon 609, 611, 618 or 620 RET mutants was diminished in comparison with those harboring the RET codon 634 mutation. A similar decrease in RET expression has been previously reported in protein mutants harboring the RET missense changes described in sporadic and familial HSCR (16,26).

Second, the results of Ito’s investigation indicated that different amino acid substitutions at any given Cys mutation affecting RET...
Sixteen patients from these seven MEN 2A families, with the exception of the C620W substitution, were represented in the transfectant analysis. The increased spectrum of MEN 2A amino acid substitutions linked to the HSCR1 phenotype in our study is consistent with Ito’s findings. Therefore, it appears that exon 10 Cys missense changes equally predispose to HSCR1, the risk not being restricted to a Cys→Arg substitution as previously reported.

The absence of a RET codon 634 mutation in our families co-expressing MEN 2A and HSCR1 is conspicuous, since codon 634 is the most common site mutated in MEN 2A (12,23). Twenty two (52%) of the 44 families in this study carried the codon 634 mutation, while 39% had an exon 10 mutation and the remaining 9% harbored the codon 804 missense change seen in FMTC. However, our findings concur with isolated reports of HSCR1 associated with MEN 2A. To date six confirmed additional families co-expressing MEN 2A and HSCR1 have been described by others (21,27–29). In all cases the predisposing RET mutation occurred in exon 10 at Cys codon 618 or 620 and produced a Cys→Arg amino acid change. Several families with C609W, C609Y or C620R RET mutations expressing solely HSCR1 without MEN 2A have also been reported, although the rigor with which occult MTC was pursued by biochemical testing in these patients is unknown (21,30,31).

Given the strong genotype–phenotype correlation, albeit with incomplete penetrance, and differing severity of HSCR1 in patients with identical RET mutations, it appears that a RET exon 10 Cys mutation is necessary but insufficient to explain development of HSCR1 in MEN 2A. Allelic variation at yet another locus, such as GDNF, GDNFR-α, NTN or NTNR-α, may modify HSCR1 susceptibility. To date a second RET sequence variation such as those found in sporadic or familial HSCR1 has not been observed in MEN 2A patients (21).

We observed HSCR1 in 16% of our MEN 2A families. This is higher with respect to other series, however, it may reflect due diligence given to systematically question our MEN 2A patients regarding its occurrence. Case ascertainment resulted from nationwide referrals and was based on risk of hereditary MTC alone. To avoid bias patients referred for RET analysis with sporadic or familial HSCR were intentionally excluded from the study.

In summary, this study represents the largest single series of MEN 2A families expressing HSCR1 and the broadest spectrum of MEN 2A genotypes reported in these unusual families. Our findings suggest that a tight genotype–phenotype correlation exists between MEN 2A/FMTC RET exon 10 Cys mutations and the development of HSCR1. Additional loci that may alter susceptibility to HSCR1 are yet to be identified.

MATERIALS AND METHODS

Patients and families

Patients in the study were ascertained from nationwide referrals and represented members at risk from clinically established MEN 2A kindred or who had a diagnosis of sporadic MTC or pheochromocytoma which prompted the genetic investigation. The HSCR1 phenotype was found to co-segregate with MEN 2A in seven of the 44 separate families with multi-generational MEN 2A or FMTC. Sixteen patients from these seven MEN 2A families were affected with HSCR. In 13 of the 16 patients the diagnosis of HSCR1 was confirmed histologically by review of relevant operative and pathology reports which showed absence of submucosal and myenteric neuronal ganglia and increased acetylcholinesterase staining of nerve fibers in the aganglionic bowel. In three patients from family VII the diagnosis of HSCR1 was suspected by history and review of death certificates which dated prior to 1940. The level of aganglionosis in the 13 patients ranged from mid-rectum to duodenum.

Six of the 16 patients died in infancy or early childhood of complications related to HSCR1. The remaining 10 patients underwent thyroidectomy for C cell disease at a mean age of 15.6 years (range 2–47 years). Of those undergoing thyroid surgery five patients had histological evidence of MTC and the remaining five had C cell hyperplasia. A single patient with MTC and short segment HSCR1 developed a pheochromocytoma at age 29 years.

PCR amplification

Peripheral blood samples were collected in acid/citrate/dextrose and genomic DNA extracted by standard techniques. Total reaction volume was 50 µl.

Exon 10. Amplification of RET exon 10 was performed using 1 µg genomic DNA template, 2.2 µmol/l each primer, 200 µM each dNTP, 1.25 U Taq DNA polymerase (Gibco-BRL, Gaithersburg, MD), 10 mM Tris, pH 8.3, 50 mM KCl, 2.0 mM MgCl2. Thermal cycling (MJ Research, Watertown, MA) conditions were 40 cycles at 95°C, 1 min and 72°C, 1 min, followed by a final 10 min denaturing step to facilitate heteroduplex formation.

Exons 11 and 13–16. Reaction conditions were identical to those for exon 10 except for the use of 1.5 mM MgCl2 in amplification of exon 14. Thermocycling conditions consisted of 40 cycles at 95°C, 35 s and 66°C, 35 s, followed by a final denaturing step (10 min).

Mutational analysis

Mutational analysis was carried out using a denaturing gradient gel electrophoresis (DGGE) approach as previously described (32), confirmed by direct DNA sequencing.

DGGE analysis of exons 10 and 11. Amplified PCR products were electrophoresed at 65°C and 14 mA/gel for 18 h (Hofer Scientific Instruments, San Francisco, CA) through a 1 mm thick 9% acrylamide gel (acrylamide: bis-acrylamide 20:1) with a 50–80% linear gradient of denaturants [100% = 7 M urea, 40% (v/v) formamide] in 1× TA buffer (40 mM Tris–acetate, 20 mM sodium acetate, pH 7.8).

DGGE analysis of exons 13 and 15. Amplified PCR products were electrophoresed at 65°C and 12 mA/gel for 17 h through a 9% acrylamide gel (acrylamide: bis-acrylamide 18:1) with a 45–75% linear gradient of denaturants in 1× TA buffer.

DGGE analysis of exon 14. Amplified PCR products were electrophoresed at 65°C and 14–15 mA/gel for 18 h through a 7% acrylamide gel (acrylamide: bis-acrylamide 16:1) with a 50–80% linear gradient of denaturants in 1× TA buffer.

DGGE analysis of exon 16. PCR products were electrophoresed at 65°C and 14–15 mA/gel for 18 h through a 9% acrylamide gel
(acrylamide: bis-acrylamide 16:1) with a 30–60% linear gradient of denaturants in 1× TA buffer.

Electrophoresis was carried out with continuous recirculation of buffer from the lower to upper electrode chamber. Gels were stained for 10 min prior to visualization. As per laboratory protocol, results were confirmed by a second independent blood sample and identical analysis.

Products of PCR were electrophoretically separated on a 2% low melting agarose gel, eluted using the Wizard PCR prep system (Promega, Madison, WI) and sequenced with an automated DNA sequencer (ABI 373A; Applied Biosystems, Foster City, CA) and ABI dideoxy terminator cycle sequencing kit (Perkin Elmer, Foster City, CA).

**Primer sequences**

Primers were selected following MEL T87 analysis (33) and primer sequences

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**Primer sequences**

Primers were selected following MEL T87 analysis (33) and encompassed RET codons 609, 611, 618 and 620 (exon 10), 630 and 634 (exon 11), 768 (exon 13), 804 (exon 14), 883 (exon 15) and 918 (exon 16). Primers were as follows. Exon 10: 5′-primer, 5′-GCGCCGCCCGCCCCGCCGCCCCGCCGCCCCCGC-

3′-primer, 5′-GCGGGGGCGGGCGGGGCGGGGCGGGCAG-

CGGCCGACCTGGTTCTCCA TGGAGTC-3′. Exon 11: 5′-primer, 5′-CCTCACACCACCCCCAC-

3′-primer, 5′-GCGCCCCGCCCGCCCCGCCGC-

GGAGGCCGCTGACTGATGTGTC-3′; 3′-primer, 5′-GCGTCGGG-

TGCAGTGGCTGCCC-3′. Exon 14: 5′-primer, 5′-CCCAAGGCC-

CCCTCTCCCGC-3′; 3′-primer, 5′-GCGGGGGGCGGGC-

GGCGGGCGGCGGCGGGCGGTGTCAGGTGTCGGT-3′. Exon 15: 5′-primer, 5′-GCGCCCCGCCCGCCCCGCCG-

CGCGGACTGCTGATGTGTCGTATTTTTCC-3′; 3′-primer, 5′-

GTCGGGCTGCGCGCGCGCGCGCGGGTCAA-

TGTCTTATTCC-3′; 3′-primer, 5′-GTCGGGCTGCGCGCGCGCGCGCGGGTCAA-

**Haplotype analysis**

PCR amplification and detection of microsatellite polymorphisms at the D10S183, D10S469 and TCL2 loci flanking RET were performed as previously described (34–36).

**Statistical methods**

Significance of the RET genotype–phenotype correlations was determined by two-tailed Fisher’s exact test using a standard 2 × 2 contingency table.

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