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Predictive DNA Testing and Prophylactic Thyroidectomy in Patients at Risk for Multiple Endocrine Neoplasia Type 2A

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Background

Missense germ-line mutations in the RET protooncogene are associated with multiple endocrine neoplasia type 2A (MEN 2A). Detection of these mutant alleles in kindred members predicts disease inheritance and provides the basis for preventative thyroidectomy.

Methods

A polymerase chain reaction (PCR)-based genetic test for the 19 known RET mutations was designed to study 132 members of 7 kindreds with MEN 2A. Haplotypes also were constructed using genetic markers flanking the MEN 2A locus. Plasma calcitonin (CT) concentrations were determined before and after provocative testing.

Results

Direct DNA testing and haplotype analysis showed that 21 of 58 kindred members at risk for disease had inherited a mutation in the RET protooncogene associated with MEN 2A. Plasma CT concentrations were elevated in 9 of the 21 family members, but were normal in 12. After genetic counseling, 13 of the 21 kindred members (6 with normal and seven with elevated plasma CT levels), consented to immediate thyroidectomy. In each patient, the resected thyroid gland showed C-cell hyperplasia with or without medullary thyroid carcinoma. There were no metastases to regional lymph nodes, and postoperative stimulated plasma CT levels were normal.

Conclusion

The PCR-based direct DNA test for RET mutations is accurate, rapid, and reproducible. For all 132 individuals evaluated, the results of direct DNA analysis were consistent with haplotype studies. The direct test for mutations in the RET protooncogene is the preferred method for screening MEN 2A kindreds. In family members who have inherited a RET mutation, total thyroidectomy is indicated, regardless of the plasma CT values.

Multiple endocrine neoplasia type 2A (MEN 2A) is characterized by the occurrence of medullary thyroid carcinoma (MTC), pheochromocytoma, and hyperparathyroidism. The disease has an autosomal dominant inheritance pattern with near complete penetrance but variable expressivity. Virtually all affected patients develop MTC whereas half or less develop pheochromocytomas or hyperparathyroidism.[1] Medullary thyroid carcinoma is the only endocrinopathy that is uniformly malignant, and it is the most common cause of death in patients with MEN 2A.

The C cells, from which the MTC is derived, secrete calcitonin (CT), and increased plasma levels of this hormone indicate the presence of a C-cell disorder. A sensitive diagnostic test consisting of the intravenous infusion of the potent CT secretagogues, calcium and pentagastrin, has been used to screen families with MEN 2A. Our practice has been to test family members beginning at 5 years of age and to continue annually thereafter until 45 years of age (if the intervening stimulated plasma CT levels are normal). This provocative regimen is associated with bothersome side effects, and some family members have refused repetitive testing.

In 1987, genetic linkage analysis mapped the MEN 2A locus to the pericentromeric region of chromosome 10.[2,3] Confirmatory studies further refined the position of this locus and demonstrated genetic homogeneity in a large number of families throughout the world.[4-9] With the subsequent development of highly informative polymorphic DNA markers closely linked to and flanking the MEN 2A locus, presymptomatic prediction of disease susceptibility became feasible.[10-13] The practical use of this indirect test is critically dependent on the availability of DNA from at least two affected family members and on an appropriate pedigree structure for linkage analysis.

Recently, missense mutations in the RET protooncogene were found to be associated with the inheritance of the MEN 2A phenotype.[14,15] This finding provided the basis for direct predictive testing and presented novel management alternatives in kindreds with MEN 2A. Family members who had inherited an associated RET mutation would be candidates for thyroidectomy, regardless of the stimulated plasma CT values. Conversely, family members who had not inherited a RET mutation could be assured that neither they nor their descendants would develop the disease. Because therapeutic decisions would be based on genetic testing, which in principle only need be performed once in a kindred member's lifetime, it was critical that the method be highly accurate and reproducible.

In the current study, members of seven MEN 2A kindreds were evaluated by haplotype studies, direct mutation analysis, and biochemical testing. A genetic counselor, a nurse, and a surgeon reviewed all test results with appropriate family members and explained the clinical implications. A management plan then was defined for each family member who was at risk for inheriting MEN 2A.

METHODS

Subjects for Study

One hundred thirty-two individuals from seven kindreds with MEN 2A (K2, K3, K5, K17, K22, K64, and K80) were studied. Initial screening included a history, a physical examination, and laboratory tests to detect the presence of hyperparathyroidism and pheochromocytoma. In patients at 50% risk for disease (having an affected parent or sibling), CT levels were determined by radioimmunoassay (Nichols Institute, San Juan Capistrano, CA) on plasma samples collected before and 1, 2, 3, and 5 minutes after the intravenous infusion of calcium gluconate (2 mg/kg/min) and pentagastrin (Peptavlon, Wyeth-Ayerst Laboratories, Philadelphia PA [0.5 μg/kg/5 sec]).

Genetic Counseling

Genetic counselors met with family members, whether or not they were at risk for disease, to inform them of the pattern of disease inheritance and the basis for the genetic tests. The counselors also reviewed the results of the genetic tests with family members and discussed the therapy available. A surgeon and a nurse then explained the benefits and complications of the specific therapy. All participants gave informed consent in accord with procedures established by the Washington University Human Studies Committee.

Genetic Testing

DNA Preparation

The DNA was purified from peripheral blood lymphocytes by automated extraction (Applied Biosystems, Inc., Foster City, CA) or by a manual method.[16] Also, DNA used in genetic analyses performed previously (the results of which also were considered in the current study) was purified from lymphocyte cell lines established previously from the pedigree members.

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Haplotype Analysis

The following six microsatellite markers were used in this study: D10S176 (sTCL1);[13] D10S1100 (sSSD3), D10S1099 (sSSD2), and D10S1098 (sKMC2)[17]: D10S141;[18] and RET (sTCL2).[19] Each forward primer was 5′ end-labeled with [Γ-^{32}P]dATP,[20] and the reactions were performed in a volume of 5μl containing 50 ng of genomic DNA essentially as described elsewhere.[13,17-19] Haplotypes were constructed manually from inspection of family relationships.

Direct Mutation Analysis

Genomic DNA was amplified using PCR and oligonucleotide primers for exons 10, (10F $^{5'}$GCGCCCCAGGAGGCTGAGTG$^{3'}$ and 10R $^{5'}$CGTGGTGGTCCCGGCCGCC$^{3'}$), and exon 11, (11AF$^{5'}$CCTCTGCGGTGCCAAGCCTC$^{3'}$ and 11AR $^{5'}$CACCGGAAGAGGAGTAGCTG$^{3'}$). Exons 10 and 11 were amplified using 50 ng of genomic DNA and 2 μM of each set of primers in a PCR volume of 5 μl containing 10 mM of Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 5 mM NH_4Cl, 200 μM each dNTP, 0.5 units Taq polymerase (Amplitaq, Perkin Elmer-Cetus, Norwalk, CT). The reactions were carried out in an automatic thermocycler (Biometra TRIO-Thermoblock, Emerston Instruments, Richmond Hill, Ontario, Canada) using 25 cycles (exon 10) or 30 cycles (exon 11) of denaturation at 94 C for 30 seconds, annealing at 65 C (exon 10) or 68 C (exon 11) for 30 seconds, with extension at 72 C for 1 minute.

Individual aliquots of PCR products from exon 10 subsequently were incubated with one of six restriction enzymes (Nla IV, Mbo II, BstU I [New England Biolabs, Beverly, MA], Cfo I, Rsa I, and Taq I [Boehringer Mannheim, Indianapolis, IN]) according to conditions specified by the manufacturers. Similarly, aliquots from exon 11 reaction mixtures were incubated with one of four restriction enzymes Cfo I, Rsa I, Hae III, and Dde I (Boehringer Mannheim). Reaction products were separated by electrophoresis through 10% nondenaturing polyacrylamide gels and detected by ultraviolet visualization after ethidium bromide (EtBr) staining. The DNA sequencing of PCR amplified products for exons 10 and 11 was carried out as described previously.[14]

Operative Technique

A total thyroidectomy was performed, and lymph nodes in the central zone of the neck (from the hyoid bone to the thoracic inlet and laterally to the great vessels) were resected. The parathyroid glands were removed and autografted into the brachioradialis muscle of the nondominant forearm. After thyroidectomy and parathyroid autotransplantation, the patient was placed on L-thyroxin (100 μg/day), 1,25 dihydroxyvitamin D_3 (1 μg/day) and calcium gluconate (1000 mg/day). Approximately 8 weeks after the operation, the oral calcium and vitamin D were stopped. The serum calcium concentration and the serum parathyroid hormone concentration in each antecubital vein were determined 2 weeks thereafter.

Pathology

The thyroid glands from all 13 patients were evaluated grossly, sectioned vertically and then horizontally from the superior to the inferior pole. The central vertical portion of each lobe was then fixed in formalin and embedded in paraffin. Hematoxylin/eosin-stained sections were evaluated for the presence of C-cell hyperplasia and MTC using the criteria of Wolfe[21] and Mendelsohn.[22] Immunohistochemical stains for CT using a polyclonal antibody (1:4000 dilution; DAKO, Carpinteria, CA) and carcinoembryonic antigen using a monoclonal antibody (1:4000 dilution; Boehringer-Mannheim) were performed on the formalin-fixed, paraffin-embedded tissue according to a standard avidin-biotin peroxidase technique.[23] Stock human neoplasms, known to contain the determinants, served as positive controls.

Tissue Calcitonin Measurement

The concentration of calcitonin was measured in the medial and lateral vertical portions of each thyroid lobe.

Calcitonin was extracted from human thyroid tissues using a modification of the method of Tashjian and Voelkel.[24] Briefly, the frozen tissue was weighed and minced in the cold room (4 C) and transferred to a 17 × 100 mm round bottom polypropylene tube. Ten to 20 vol (mL:g) of extraction buffer (0.1 N acetic acid, 0.1% Triton X-100) was added to the tube and the contents were homogenized at low speed (setting of 2–3) for 10 to 15 seconds with a Polytron Homogenizer fitted with the ST-10 generator (Brinkmann Instruments, Westbury, NY), and then at high speed (setting of 8–9) for 3 seconds. The homogenate was transferred to a microcentrifuge tube and spun at 14,000 RPM for 15 minutes in an Eppendorf microcentrifuge in the cold room. The clear supernate was transferred to a clean tube and stored at −60 C until assayed. At the time of assay, the extract was neutralized with 0.1 N NaOH and 0.1 M Tris pH 7.5. Calcitonin in tissue extracts was measured with a modification of our previously described radioimmunoassay.[25] Tissue CT values greater than 30 ng/g wet weight were considered abnormal.

RESULTS

Patient Evaluation

Of the 132 members from the 7 MEN 2A kindreds (Fig. 1), 48 had an established diagnosis of MEN 2A (by

clinicopathologic data review), and 58 were at 50% risk for inheriting the disease, but had no clinical evidence of endocrine neoplasia. Twenty-six unaffected spouses of MEN 2A kindred members served as controls.

Evaluation of the PCR-Based Direct DNA Predictive Test

We evaluated the prototype for the direct test by mutation analysis of 132 individuals from 7 kindreds segregating MEN 2A. A PCR-based direct DNA test was developed for the 19 known RET mutations associated with MEN 2A and familial non-MEN medullary thyroid carcinoma (Table 1). The test primarily relies on the detection of restriction enzyme sites that either are created or eliminated because of point mutations within exons 10 and 11 of germ-line DNA. A panel of nine restriction endonucleases was chosen to detect the mutations and produce fragments that could be resolved easily using polyacrylamide gel electrophoresis and EtBr staining.

The laboratory personnel performing these studies were blinded with respect to the following: kindred name, kindred-specific mutation data, birth order within a sibship, individual's birth date, gender, and MEN 2A phenotype. One coded DNA sample from an affected family member from each kindred was prepared as a positive control in the assay panel. If a mutation-specific restriction endonuclease pattern was not found with the control sample, the PCR products were sequenced directly.

A haplotype study was included to independently monitor the direct DNA test result. The coded samples were analyzed using a set of six polymorphic microsatellite genetic markers that flank the MEN 2A locus. Haplotypes were constructed based on pedigree structure but without knowledge of MEN 2A disease status for family members and without information from direct mutation analysis. Because the pedigrees had been made into haplotypes previously, the study also served to evaluate the consistency of microsatellite genotype analysis. Once the direct DNA mutation analysis, haplotype construction, and CT testing were complete, the code was removed and results were compared. Haplotypes also were reevaluated with knowledge of the disease status of the pedigree members.

PCR-Based Direct DNA Predictive Test

Detection of RET Mutations by Restriction Endonuclease Digestion

For 15 of the 19 RET protooncogene mutations, restriction enzyme cleavage was used to detect mutations. For 13 mutations, new restriction enzyme cleavage sites were created, and in two cases, different point mutations

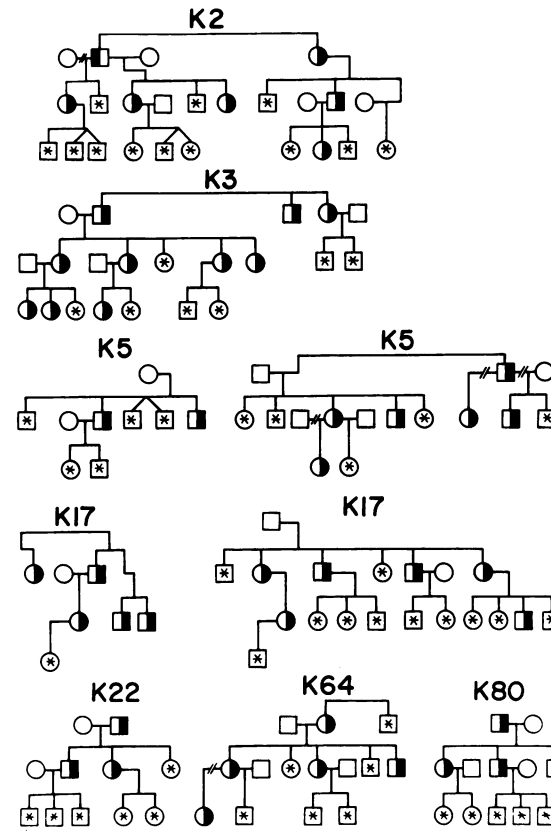


Figure 1. Structure of the family segments selected from 7 kindreds (K2, K3, K5, K17, K22, K64, and K80) with MEN 2A. Each square represents a male and each circle represents a female. The half darkened symbols represent kindred members with the proven clinical diagnosis of MEN 2A. * A kindred member who is at risk for inheriting MEN 2A.

within cysteine codon 634 eliminated Fnu4H I cleavage sites, thus giving rise to larger fragments (Table 1, Fig. 2a). For seven mutations (Cys611, TGC→TGG; Cys618, TGC→AGC; Cys618, TGC→TTC; Cys634, TGC→GGC; Cys634, TGC→AGC; Cys634, TGC→TCC; Cys634, TGC→TTC), the restriction enzyme used to detect the mutation also cleaved the normal allele, providing an internal positive control for endonuclease digestion. Reference DNA samples were processed in parallel to serve as positive controls for the other eight mutations.

Detection of RET Mutations by PCR Sequencing

Mutations that are not revealed by restriction endonuclease analysis (4 total, Table 1) can be detected by direct sequencing of exon 10 and 11 PCR products. The normal and mutant sequences are present in the genomic DNA substrate used for sequencing but, as Figure 2B shows for Kindred 2, they can be distinguished easily. Resolution of mutant alleles using single strand conformational polymorphism analysis (SSCP) was evaluated as an alternative detection method. However, for these

Table 1. RESTRICTION ENZYME PANEL AND RET PROTOONCOGENE MUTATIONS ASSOCIATED WITH MEN 2A

RET Mutation (ref)	Exon	Base Pair Change	Preferred Restriction Enzyme	Fragment Sizes (bp)			Kindred
				UC	N	M	
Cys611 → Trp[14]	10	TGC → TGG	Nla IV	187	78 72 28 9	65 + 13 72 28 9	K22*
Cys618 → Tyr[14]	10	TGC → TAC	Rsa I	187	187	131 + 56	K34
Cys618 → Arg[14]	10	TGC → CGC	Cfo I	187	184 3	129 + 55 3	K88
Cys618 → Phe†	10	TGC → TTC	Mbo II	187	158 29	124 + 34 29	K80*
Cys618 → Ser[14]	10	TGC → AGC	Mbo II	187	158 29	139 + 19 29	K10
Cys620 → Arg[14]	10	TGC → CGC	BstU I	187	187	138 + 49	K17*, K64*
Cys620 → Phe†	10	TGC → TTC	Taq I	187	187	138 + 49	K19
Cys634 → Arg[14]	11	TGC → CGC	Cfo I	234	234	173 + 61	K3*
Glu-Leu-Cys634 → Asp-Val-Arg[15]	11	GAC-CTG-TGC → GAC-GTC-CGC	Cfo I	234	234	173 + 61	—
Cys634 → Tyr[15]	11	TGC → TAC	Rsa I	234	234	174 + 60	K5
Cys634 → Gly[15]	11	TGC → GGC	Hae III	234	194 30 10	133 + 61 30 10	K41
Cys634 → Trp†	11	TGC → TGG	Cfo I	234	234	170 + 64	K73
Cys634 → Ser†	11	TGC → AGC	Dde I	234	170 59 5	113+ 57 59 5	PJGK
Cys634 → Ser[15]	11	TGC → TCC	Fnu4H I	234	123 62 + 14 32 3	123 76 32 3	—
Cys634 → Phe[15]	11	TGC → TTC	Fnu4H I	234	123 62 + 14 32 3	123 76 32 3	—
Mutations without Restriction Enzyme Site Alterations							
Cys609 → Tyr†	10	TGC → TAC	NA	NA	NA	NA	—
Cys611 → Arg†	10	TGC → CGC	NA	NA	NA	NA	—
Cys618 → Gly[15]	10	TGC → GGC	NA	NA	NA	NA	—
Cys620 → Tyr[14]	10	TGC → TAC	NA	NA	NA	NA	K2

UC = uncut; N = normal alleles, cut; M = mutant alleles, cut; NA = not applicable; PJGK = Paul J. Goodfellow Kindred.
* Kindreds included in the current study.
† Unpublished data.

four mutations, we found that SSCP gels either did not have sufficient resolving power or that band shifts were not routinely reproducible (data not shown).

Genetic Testing Results for Seven MEN 2A Kindreds

Direct DNA Mutation Analysis

Restriction endonuclease analyses of the mutation-positive control samples from six coded kindreds revealed five different point mutations (K22, K80, K17, K64, K3, K5; Table 1, Fig. 2A). For one coded sample, a restriction site alteration was not detected (K2). The PCR products from exons 10 and 11 were sequenced, and a mutation in exon 10 was identified (Table 1, Fig. 2B) as expected from prior analysis of this kindred.[14] Based on these results, each family member was tested with the appropriate restriction enzyme/exon combination or by DNA sequence analysis.

Direct mutation analysis of genomic DNA from the

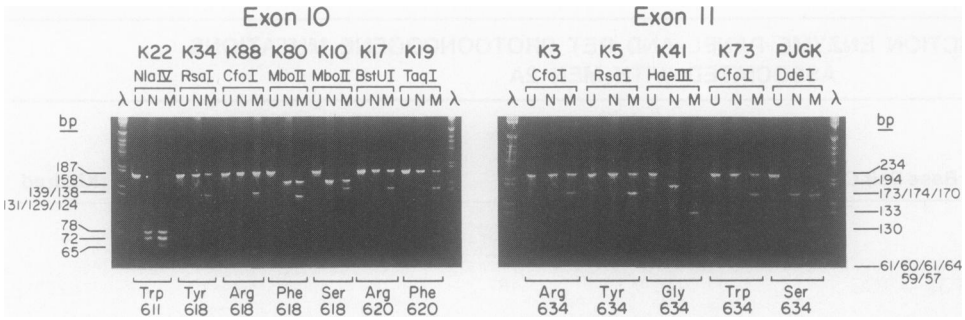


Figure 2A. Restriction endonuclease detection of RET protooncogene mutations associated with MEN 2A. Genomic DNA from 12 kindreds (K or PJG) was amplified by PCR using oligonucleotide primers for exons 10 or 11 of the RET protooncogene. The products were cleaved with restriction enzymes as indicated, size-fractionated by electrophoresis through 10% polyacrylamide gels, and stained with EtBr. U = uncut, PCR amplification product with no endonuclease treatment (187 bp for exon 10, 234 bp for exon 11); N = normal, genomic DNA from individual unaffected with MEN 2A; M = mutant, PCR amplification of genomic DNA from MEN 2A-affected individual. Both the normal and 13 mutant alleles are evident in these restriction digests. Codons and amino acid substitutions produced by missense mutation in cysteine residues are indicated at the bottom of the figure. λ = DNA size standard (1 Kb DNA ladder, GibcoBRL, Grand Island, NY). bp = fragment size in base pairs. Fragments close in size are listed together, e.g., 131/129/124. Fragments smaller than 50 bp are not resolved on these gels. PJGK = Paul J. Goodfellow kindred.

58 individuals at 50% risk for MEN 2A identified 21 individuals who had an inherited RET mutation associated with disease. Thirty-seven family members had two normal RET alleles. Only normal RET alleles were found in the 26 unaffected control individuals. Three individuals from kindred 3, who had been diagnosed and treated for MTC at another health care facility, had normal RET alleles. This suggested that either the DNA test produced three false-negative results or the medical diagnoses were incorrect. The mutation associated with MEN 2A in this kindred, Cys634-Arg (TGC→CGC), has been documented[14] and verified in a large number of family members using a combination of SSCP, DNA sequencing, and Cfo I restriction enzyme cleavage analysis of exon 11 PCR products (data not shown). We performed direct PCR sequencing of the exon 11 PCR products for these three individuals and found only normal RET alleles (data not shown). The histopathology slides from the thyroidectomy specimens were reviewed in each of the three cases. There was no evidence of MTC in the material from the three patients. One patient had abundant C cells; however, they were not in a nodular pattern. Considering the molecular genetic test results and the histopathology, we concluded that these patients did not have MEN 2A.

The PCR-based technique used in these studies was highly reliable and reproducible. Polymerase chain reaction amplification of exons 10 or 11 failed on the first trial in only six instances (<5%) within the 132 samples. Furthermore, we did not detect any failure of restriction endonucleases to cleave substrate DNA.

Haplotype Analysis

Six microsatellite genetic markers closely linked within an interval of less than 3 centiMorgan (sex-average) and flanking the MEN 2A locus were used to independently determine inheritance of MEN 2A alleles. The order of the markers and interval spacing is as follows: *10ptel*—D10S176

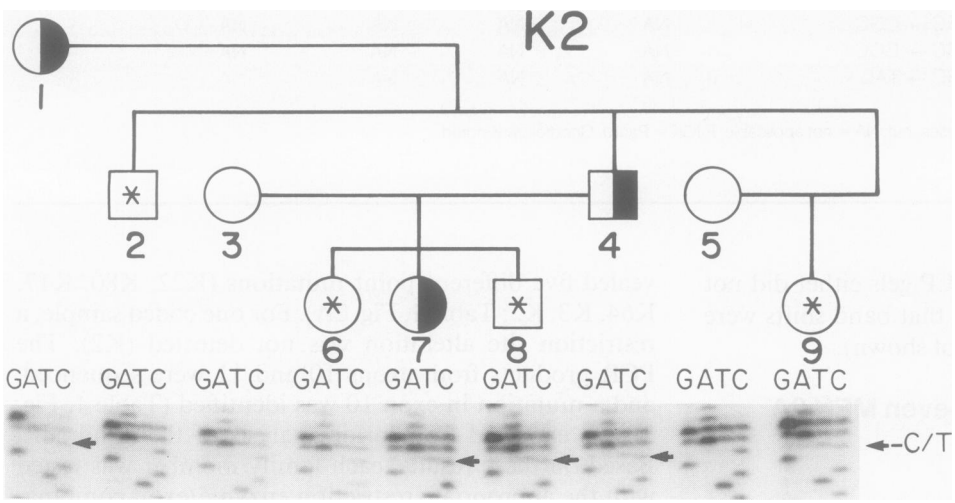
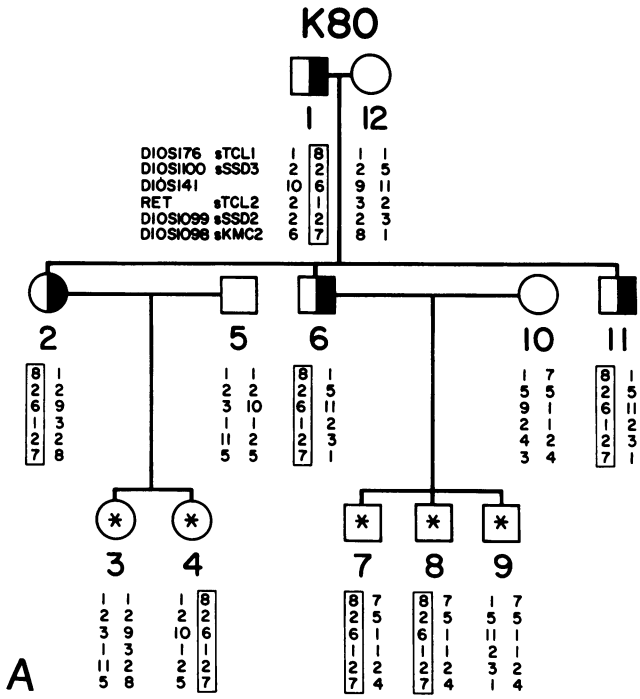


Figure 2B. RET protooncogene exon 10 DNA sequence analysis from genomic DNA of MEN 2A. Family kindred 2 members affected with MEN 2A(1,4,7) are indicated with a half-filled circle (female) or square (male), and those at-risk (2,6,8,9) with an asterisk (*) in the pedigree drawing. A mutation in RET protooncogene Cys620→Tyr (TGC→TAC) is revealed by DNA sequencing of PCR amplification products from exon 10. The antisense strand sequences for nine members of Kindred 2 are shown. The nucleotide (T) mutation is evident in the sequence ladder, as is the nucleotide (C) present in the normal sequence (arrows). Of the four individuals at-risk for MEN 2A, two (8 and 9) have the mutation characteristic for their family, and two (2 and 6) have only normal RET alleles. Compression of the sequencing ladder is evident immediately above the region containing the position of mutation.



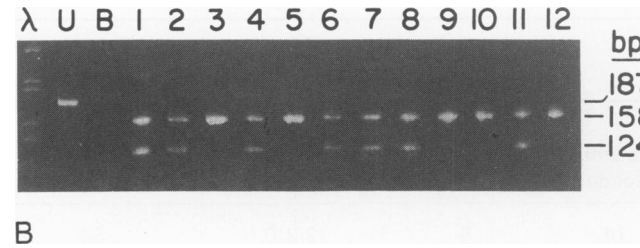


Figure 3. Haplotype analysis and inheritance of a RET protooncogene mutation associated with MEN 2A in Kindred 80. (A) The haplotypes for individuals from Kindred 80 are shown. Those affected with MEN 2A are indicated by half-filled circles (female) or squares (male) in the pedigree drawing and those at-risk are indicated with an asterisk (*). Six microsatellite genetic markers were genotyped by PCR amplification of genomic DNA from the 12 individuals. The pericentromeric markers flanking the RET protooncogene are shown in order along chromosome 10. The haplotype segregating with MEN 2A is enclosed with a rectangle. The same three individuals at risk for MEN 2A (4,7,8) have inherited the MEN 2A allele as indicated from direct mutation analysis, whereas the remaining two (3,9) have inherited the normal allele from their affected parent. (B) Restriction digest patterns are shown for 12 family members. The normal allele contains one Mbo II restriction site producing fragments of 158 and 29 bp, whereas the 158 bp fragment is cleaved into fragments of 124 bp and 34 bp in addition to the 29 bp fragment in the mutant allele. bp = fragment size in base pairs. U = uncut, PCR-amplified DNA from exon 10 without endonuclease digestion (187 bp). λ = DNA size standard (1 Kb DNA ladder, GibcoBRL). B = blank, negative control. Two cleavage products (158 bp normal, and 124 bp mutant) are resolved in the polyacrylamide gel shown. Of the five individuals at-risk for MEN 2A (3,4,7,8) three (4,7,8) had inherited the MEN 2A mutation common to their family, whereas the remaining two (3 and 9) inherited only the normal RET alleles.

(sTCL-1)-*Cen*—D10S1100 (sSSD3)—100 Kb—D10S141—300 Kb—RET(sTCL2)—1350 Kb—D10S1099 (sSSD2)—550 Kb—D10S1098 (sKMC2)—*10qtel.* The physical distance between D10S176 and the nearest locus, D10S1100, is not known. The genetic distance between D10S176 and D10S1100 is 0.9 cM (unpublished data). In 128 cases of 132 individuals tested, haplotypes could be constructed or inferred from family relationships. When information about previously diagnosed family members with MEN 2A was included, haplotypes could be constructed and predictions could be made as to which at-risk individuals had inherited an MEN 2A allele. For example, haplotypes constructed for kindred 80 indicated that three of the five at-risk individuals were found to have inherited the haplotype associated with MEN 2A (Fig. 3A), a result consistent with direct DNA mutation analysis (Fig. 3B).

No discordance was observed between the haplotype results and direct DNA mutation analysis except with respect to one marker, D10S141. In seven cases, we observed an apparent lack of transmission of a parental allele that was evident in normal individuals and those with MEN 2A alleles. This inconsistency also was observed in genomic DNA from the Centre d'Etude du Polymorphisme Humain pedigree collection, and therefore, is likely to occur because of inhibition of PCR amplification unrelated to the presence of MEN 2A in these families.

Operative Management

Haplotype analysis and direct DNA testing identified 21 individuals who had inherited mutations in the RET protooncogene. The patients ranged in age from 6 to 21 years (mean 13.2 years). In 12 of the 21 patients (mean age 12 years, range 6–21 years), the stimulated plasma CT levels were within normal limits. In the remaining 9 patients (mean age 15.1 years, range 8–20 years), the stimulated plasma CT levels were elevated.

Of the 12 individuals with normal stimulated plasma CT levels, 6 (or their parents) decided not to have an immediate thyroidectomy. Four chose to delay the operation until spring or summer recess from school. In two other children, the parents wished to delay thyroidectomies until the plasma CT levels became elevated. Of the nine family members with elevated stimulated plasma CT levels, two wished to delay thyroidectomies for several months for personal reasons. For the remaining 13 individuals (6 with normal plasma CT levels and 7 with elevated levels), total thyroidectomy, total parathyroidectomy, lymph node dissection, and heterotrophic parathyroid autotransplantation were performed (Tables 2 and 3). A total of 176 lymph nodes were resected from the central zone of the neck (13.5 per patient), and on histologic examination, none contained metastases. In each of the 13 patients, the stimulated plasma CT levels were normal after total thyroidectomy.

Table 2. PATIENTS AT RISK FOR MEN 2A WHO HAD INHERITED A RET MUTATION AND WERE FOUND TO HAVE AN ELEVATED STIMULATED PLASMA CT CONCENTRATION

Patient/ Gender	Age (yrs)	Plasma CT (pg/mL) basal/peak		Histology	Lymph Nodes +/Total	Ca (mg/dL)	PTH G/NG
		Preoperative	Postoperative				
1/F	8	12/210	5/3	MMTC/CCH	0/18	9.4	1.4
2/F	8	17/193	9/12	MMTC/CCH	0/5	10.1	25.3
3/F	14	25/340	8/7	GMTC/CCH	0/13	9.1	3.8
4/F	16	110/344	17/17	MMTC/CCH	0/27	9.1	3.8
5/M	18	28/830	4/2	GMTC/CCH	0/12	9.2	5.1
6/M	18	39/356	14/18	MMTC/CCH	0/10	9.4	11.6
7/M	20	28/886	7/5	GMTC/CCH	0/16	9.4	18.2

pg/mL = picogram/mL; peak = peak plasma CT level after intravenous administration of calcium and pentagastrin; preoperative = before thyroidectomy; postoperative = following thyroidectomy; MMTC = microscopic medullary thyroid carcinoma; GMTC = macroscopic; CCH = C-cell hyperplasia; PTH = serum parathyroid hormone concentration in the antecubital vein above the parathyroid graft in the grafted (G) arm compared to the nongrafted (NG) arm.
The PTH concentrations on the 7 patients were measured by different radioimmunoassay techniques in various commercial laboratories. Data are expressed as a ratio of PTH concentrations in the two arms of a patient where the same immunoassay technique was used.

Two weeks after the oral calcium and vitamin D replacement was stopped, the serum calcium concentration was within the normal range in each patient. In each patient there was a higher concentration of serum PTH in the antecubital vein of the parathyroid grafted arm compared with the nongrafted arm. There were no complications of the surgery except for transient minor arm pain at the site of the parathyroid autograft in one patient.

Pathology

Of the seven patients whose preoperative plasma CT levels were elevated (Table 2), each had microscopic evidence of MTC on histologic examination. Two of the seven had macroscopic disease. Of the six patients with normal preoperative plasma CT levels (Table 3), all had C-cell hyperplasia, one had macroscopic disease, and two had microscopic disease. C-cell hyperplasia and MTC were identified in sections taken from the upper and middle portions of the thyroid lobes. C-cell hyperplasia was characterized histologically by a combination of well circumscribed, round clusters of nodules of C cells without intervening stroma of a desmoplastic type and increased individual C cells arranged around and within the thyroid follicles (Figs. 4A and 4B).

Medullary thyroid carcinoma displayed a combination of distinctive cytologic, architectural, and stromal features. The transition from C-cell hyperplasia to carcinoma was typified by cytologic changes in the nucleus and cytoplasm, including increased nuclear size, variable nuclear shape, coarse chromatin, and pleomorphism

Table 3. PATIENTS AT RISK FOR MEN 2A WHO HAD INHERITED A RET MUTATION AND WERE FOUND TO HAVE A NORMAL STIMULATED PLASMA CT CONCENTRATION

Patient/ Gender	Age (yrs)	Plasma CT (pg/mL) basal/peak		Histology	Lymph Nodes +/Total	Ca (mg/dL)	PTH G/NG
		Preoperative	Postoperative				
1/M	6	10/39	2/4	CCH	0/16	9.9	15.4
2/M	6	11/223	8/11	MMTC/CCH	0/9	9.5	3.8
3/M	9	20/280	6/5	CCH	0/9	8.4	14.6
4/M	13	13/192	8/7	GMTC/CCH	0/18	10.0	1.8
5/M	14	9/57	5/5	MMTC/CCH	0/26	10.0	11.0
6/M	14	20/78	7/8	CCH	0/6	9.1	1.7

pg/mL = picogram/mL; peak = peak plasma CT level after intravenous administration of calcium and pentagastrin; preoperative = before thyroidectomy; postoperative = following thyroidectomy; MMTC = microscopic medullary thyroid carcinoma; GMTC = macroscopic; CCH = C-cell hyperplasia; PTH = serum parathyroid hormone concentration in the antecubital vein above the parathyroid graft in the grafted (G) arm compared to the nongrafted (NG) arm.
The PTH concentrations in the 6 patients were measured by different radioimmunoassay techniques in various commercial laboratories. Data are expressed as a ratio of PTH concentrations in the two arms of a patient where the same immunoassay technique was used.

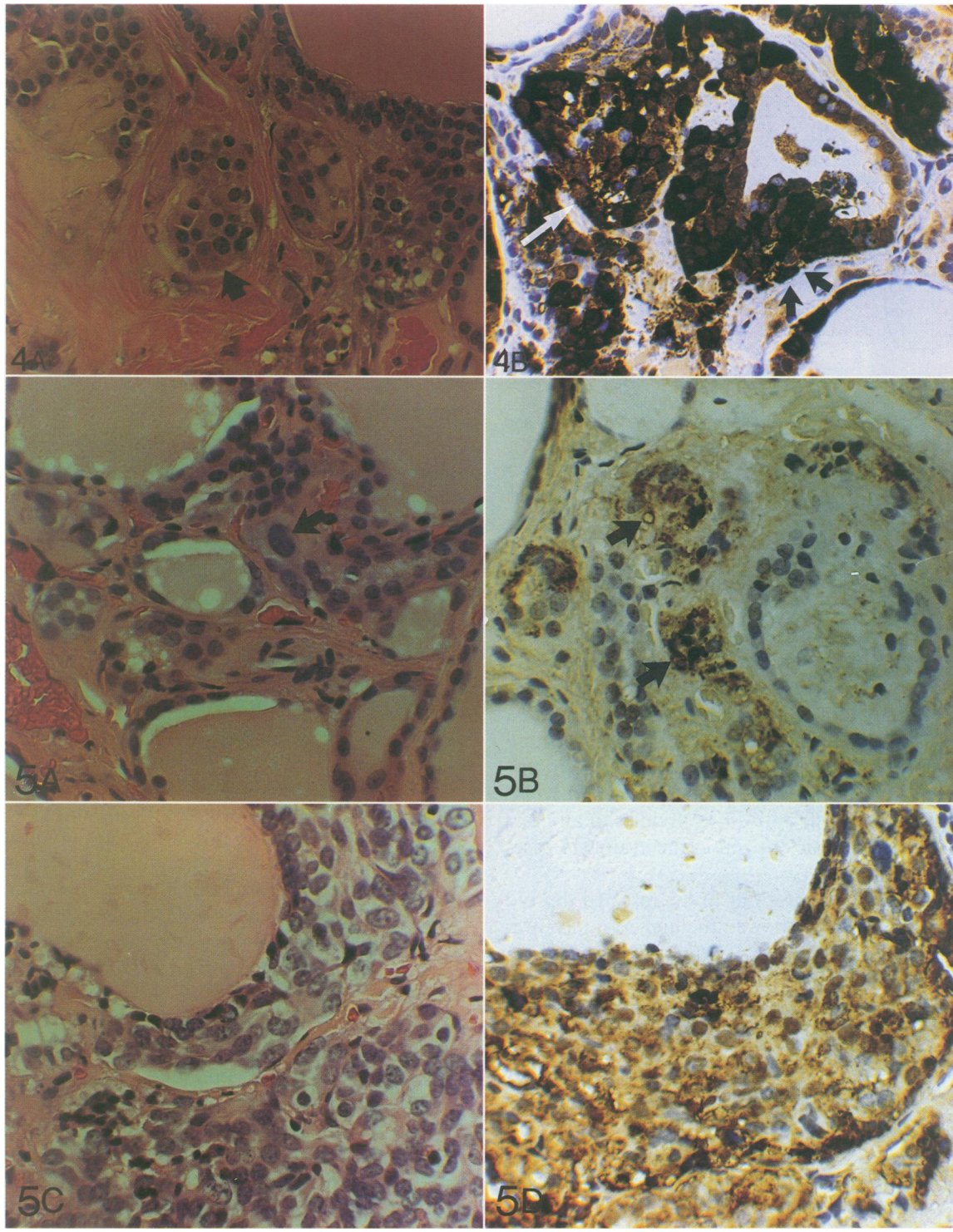


Figure 4. Histology from the resected thyroid gland of Patient 4 (Table 2). (A) One of several microscopic foci of MTC showing invasive nests of tumor in a desmoplastic stroma (arrow). A congo red stain was negative for amyloid by polarization microscopy. (B) Immunoperoxidase staining for CT shows aggregates of CT-positive cells budding from a follicle (small arrows) and an adjacent nest of medullary carcinoma (white arrow). (H&E, ×400; peroxidase-antiperoxidase, ×400).

Figure 5. Histology from the resected gland of Patient 4 (Table 3). (A) An individual C cell with nuclear enlargement (arrow). Small aggregates of C cells with minimal nuclear abnormalities are present in the interstitium. (B) Immunoperoxidase staining for CT shows CT-positive hyperplastic C cells (arrows). (C) A transitional focus of atypical C-cell hyperplasia merges with an adjacent medullary carcinoma. (D) Immunoperoxidase staining for CT shows positive C-cells in an area of transition from hyperplasia to medullary carcinoma. (H&E, ×400, peroxidase-antiperoxidase, ×400).

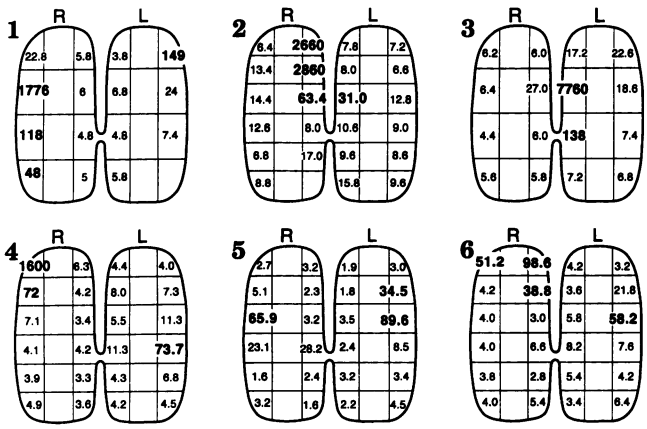


Figure 6. Tissue calcitonin concentrations in the resected thyroid glands of the six patients with normal preoperative plasma CT determinations (Table 3). The thyroid glands were blocked as explained in the text and the concentration of calcitonin in tissue is expressed as ng/g wet weight. Note the higher concentrations of tissue CT in the middle portion of the gland and the upper pole regions (bold). The central vertical strip of each thyroid gland was used for histological evaluation.

(Figs. 5A and 5B). Giant multilobulated tumor-cell nuclei were present in areas of carcinoma, but were not observed in cases in which only C-cell hyperplasia was present. Architecturally, a loss of cellular cohesion, with clear spaces between cells, a desmoplastic stroma, and an infiltrative pattern heralded the development of carcinoma (Figs. 4A, 5C, and 5D). C-cell nodules became less round and more polygonal or angulated in areas of the transition to carcinoma. Both C-cell hyperplasia and MTC were immunoreactive to anti-CT antibodies in all cases and to carcinoembryonic antigen in the evaluated cases (Figs. 4B and 5D).

The calcitonin concentrations in the resected thyroid glands of the 13 patients showed the highest concentrations in the upper pole regions, compatible with the increased density of C-cells there. The distribution of tissue CT concentrations in the thyroid glands of the six patients with normal preoperative stimulated plasma CT levels is shown in Figure 6.

DISCUSSION

Before predictive genetic testing, the early diagnosis of MEN 2A was based on the results of biochemical tests to detect the presence of MTC, pheochromocytomas, and hyperparathyroidism. The various screening procedures are expensive and time consuming. Furthermore, although the provocative test for the detection of MTC is reliable, the associated side effects become more bothersome to patients with years of repetitive testing.

The discovery of the chromosomal locus for MEN 2A led to the development of an indirect test based on linkage analysis This test, although highly accurate in identifying family members who had inherited MEN 2A, is laborious and depends on the availability of a suitable pedigree, including a certain number of family members with disease. The recent finding that mutations in the RET protooncogene are associated with MEN 2A provided the opportunity to develop a direct DNA test for MEN 2A.

In this study, we evaluated 132 members of 7 large kindreds with MEN 2A by haplotype analysis and direct DNA testing. The study was performed in a blinded fashion so that laboratory investigators performing the tests were not aware of the disease status of various kindred members. Biochemical screening with measurement of plasma CT levels also was performed on kindred members at risk for inheriting disease. When the code was removed, there was complete concordance between direct DNA testing and haplotype analysis. We evaluated 58 family members who had a 50% risk of developing MEN 2A and identified 21 who had inherited a mutation in the RET protooncogene. We did not detect a failure of restriction enzyme cleavage; however, because surgical decisions will be based on these findings, it is imperative that adequate positive controls be included with each analysis so that the highest accuracy is maintained. Duplicate digestions, inclusion of internal control DNAs with cleavage sites for the relevant enzyme, and confirmation by sequence analysis or by microsatellite haplotype analysis are among the options available.

Genetic counselors had an important role in this study because they were responsible for informing kindred members of the theoretical and practical aspects of the genetic tests. Also, they primarily were involved in explaining the results of the tests and the therapeutic options available. Only after this interaction did nurses and surgeons discuss the therapeutic options with the family members. Kindred members who were at risk for MEN 2A but who had not inherited a mutation in the RET protooncogene were informed that they had not inherited the disease and that they and their descendants would no longer need to be evaluated for disease. Kindred members who had inherited a RET mutation were offered total thyroidectomy, even though 12 of the 21 patients had normal plasma CT levels after provocative testing. All but 2 of the 21 patients agreed to thyroidectomy either immediately or at a more convenient time in the near future. Of the 13 patients who chose immediate thyroidectomy, only 4 had macroscopic evidence of MTC, and the remaining 9 had either microscopic MTC, C-cell hyperplasia, or both. In each of the 13 patients, the C-cell disorder was confined to the thyroid gland. The C-cell abnormality was confirmed by documenting increased tissue CT concentrations in the resected thyroid glands. There was no spread of the malignancy to regional lymph nodes, and postoperative stimulated plasma CT values were normal. There was minimal mor-

bidity associated with the operation, which is important because many of the children having thyroidectomies were younger than 10 years of age. After termination of postoperative calcium and Vitamin D replacement therapy, each patient had a normal serum calcium concentration.

It can be argued that a patient, regardless of age, becomes a candidate for thyroidectomy as soon as a RET mutation associated with MEN 2A is identified. Based on our previous experience and the current report, we recommended thyroidectomies for children as young as 5 years of age with disease-specific mutations. From all evidence, it appears that this approach will be curative or preventative of MTC.

For the patients in this study, we plan to repeat provocative testing with plasma CT determination at 2 and 5 years after thyroidectomy. If plasma CT levels remain normal, continued provocative testing would appear unnecessary. These patients must, however, continue to be screened for the development of pheochromocytomas and hyperparathyroidism.

The discovery that mutations in the RET protooncogene are associated with MEN 2A has simplified the management of kindreds with this disease. The role of direct genetic testing in MEN 2A has established the place of preventative operative therapy in this familial cancer syndrome. In the future, such preventive operative therapy may have a place in the management of patients with the more common malignancies, such as carcinoma of the breast and carcinoma of the large bowel.

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Discussion

DR. MURRAY F. BRENNAN (New York, New York): The Program Committee is to be congratulated for opening this