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Linkage analysis has been performed in four pedigrees with multiple endocrine neoplasia type 2A (MEN 2A) or familial medullary thyroid carcinoma (MTC) using pericentromeric chromosome 10 probes. Important information regarding carrier status has been provided in 10 individuals, many of whom would not have been identified by pentagastrin stimulation testing. We have also used pulsed field gel electrophoresis (PFGE) to link the probes H4.IRBP and pMCK2 to a 150 kb fragment. Using PFGE, no evidence was found in DNA from lymphocytes of a major DNA rearrangement in two individuals affected with MEN 2A and an individual with MEN 2B compared with normals. Metastatic MTC from one patient has been used to generate a cDNA library which will be used to screen for candidate MEN 2A and MEN 2B gene(s). (Henry Ford Hosp Med J 1992;40:220-3)

The identification of carriers of the gene(s) responsible for the development of the multiple endocrine neoplasia type 2A (MEN 2A) syndrome has traditionally relied on provocative pentagastrin-stimulated calcitonin testing. Difficulties are frequently encountered in the interpretation of this test. These include dependence upon age at time of test performance and nonparallelism of diluted samples to the assay standard curve. In "at risk" individuals the test should ideally be repeated annually in an attempt to identify the premalignant stage of C-cell hyperplasia.

The availability of closely linked flanking markers for the MEN 2A (1,2) locus has made it possible to accurately predict gene carrier status on a single blood sample when linkage has been established in the family.

We have performed linkage analysis in four of six Australian families with either MEN 2A or familial medullary thyroid carcinoma (MTC) and have been able to provide important carrier status information to 10 individuals.

In addition, we have performed pulsed field gel electrophoresis (PFGE) to improve the genetic map in the 10q11.2 region and have generated a cDNA library from MTC which will be helpful in the isolation of candidate MEN 2A gene(s).

Materials and Methods

Linkage analysis

Genomic DNA used for linkage analysis was extracted from peripheral blood leukocytes by a method described previously (3). Restriction enzyme digested DNA was electrophoresed in 0.8% agarose gels and transferred to nylon filters (Genescreen Plus) by Southern blotting (4).

Probes

Eight probes (H4.IRBP [RBP3], pMCK2 [D10S15], TB10.163 [D10S22], and pGEM-32 [FNRB]) were used to study linkage relationships in four families. Gel isolated fragments of the cosmid probes MEN203 and cIRBP.9 (RBP3) were also used in linkage analysis. A 9 kb DNA fragment isolated by a NotI, Bg1II double digestion of the cosmid MEN203 detected the Bg1ll polymorphism but still contained some repetitive sequences. A subsequent MspI digestion of the above fragment isolated a unique 2.2 kb subfragment which also detected the Bg1II polymorphism. A unique 3 kb fragment isolated by a NotI, TaqI double digestion of the cosmid probe cIRBP.9 detected the TaqI polymorphism. The probes H4.IRBP and pMCK2 were also used in pulsed field gel analysis.

Insert DNA was isolated from the probes and labeled with random primers to a specific activity of 1×10^9 cpm/μg. Filters were hybridized overnight. Final washes were in 2 × SSC, 1% SDS at 42 °C, or 0.1 × SSPE, 0.1% SDS at 65 °C. Filters were stripped in 0.2N NaOH and checked for removal of probe prior to reuse.

Pulse field gel electrophoresis

PFGE was performed on DNA from our control individuals. In two controls, DNA was obtained from independent lymphoblastoid cell lines and in the other two controls DNA was obtained from lymphocytes isolated from fresh blood. The DNA from two independent MEN 2A individuals and the MEN 2B individual was isolated from lymphocytes of fresh blood. DNA was isolated from the lymphoblastoid cell line CY6. This cell line contains a single 10:16 translocation in a mouse, A9 background. The chromosome 10 content was 10pter-10q26 (this cell line was provided by D. F. Callen).

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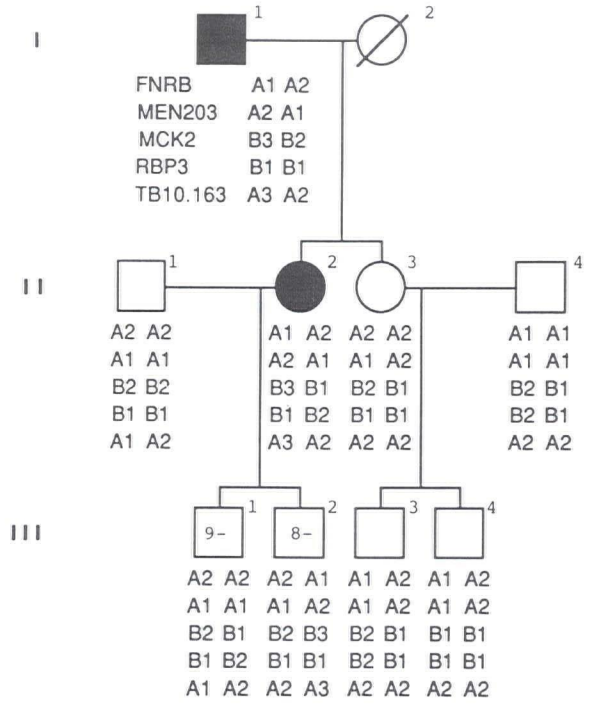

Fig 1—DNA typing of family 1 with closely linked markers. Solid circle/solid square = individuals with proven MEN 2A. Numbers in symbols indicate age in years at last pentagastrin stimulation test. + or − = positive or negative test result.

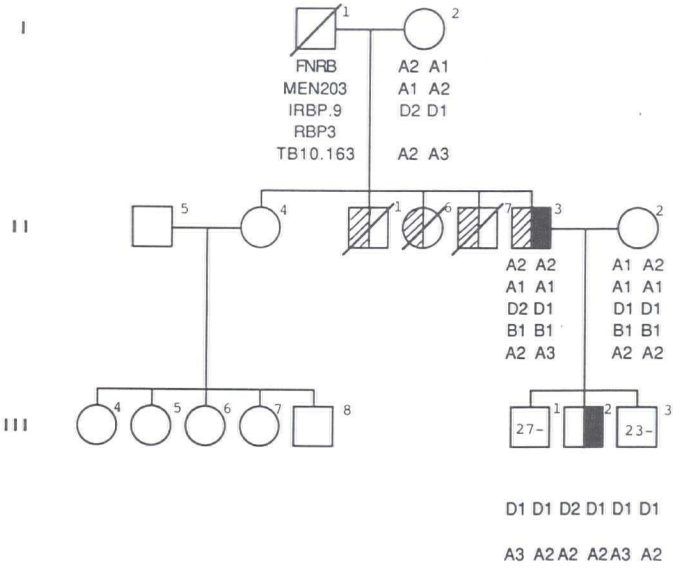

Fig 2—DNA typing of family 3 with closely linked markers. Solid circle/solid square = individuals with proven MEN 2A. Half-filled circles/half-filled squares = individuals with familial MTC. Half-shaded circles/half-shaded squares = individuals with Hirchsprung's disease. Numbers in symbols indicate age in years at last pentagastrin stimulation test. + or − = positive or negative test result.

A Pharmacia PFGE apparatus with a hexagonal electrode was used. The buffer was 0.5 × TBE and the gel was 1% agarose (Bethesda Research Laboratories). DNA in 0.7% low melting point agarose (Pharmacia) was digested with restriction endonucleases Not1, Nru1, and Mlu1 (New England Biolabs). Samples were electrophoresed at 150 mA constant current, 5 °C, with a switching of 70s for 30 hours. To resolve the 1,600 kb Mlu1 fragment, the buffer was reduced to 0.25 × TBE and the samples electrophoresed at 80 mA constant current, 5 °C, with a switching of 1,200s for five days, and then with a switching of 70s for 20 hours.

cDNA library

Total RNA was prepared from metastatic MTC in a cervical lymph node by standard GTC/CsC1 procedures (5). From 70 µg total RNA, 5.92 µg poly A^+ RNA was collected by column chromatography using oligo dT cellulose columns (Pharmacia).

The cDNA library was constructed using the lambda ZAP-cDNA synthesis kit (Stratagene).

Results

Linkage analysis

We have collected data on six families with either MEN 2A or familial MTC and have performed linkage analysis in four of these families. The results of linkage analysis are shown in Figs 1 and 2.

Several observations can be made from our families. Individual III-2 in pedigree 1 (Fig 1) has a high probability of being a gene carrier. In pedigree 2 a recombination event has been identified in one individual between the RBP3 and D10S22 (TB10.163) loci. In pedigree 3 (Fig 2) the index case II-3 has both Hirschprung's disease and MTC. Several of this individual's siblings died in infancy or childhood with complications of Hirschprung's disease. Hirschprung's disease and MTC do not appear to be linked in this family as individual III-2 has MTC but normal bowel function. Southern analysis of DNA extracted from stored paraffin blocks of tissue from individual II-1 in pedigree 3 was not possible as the DNA was degraded. Linkage analysis has been particularly helpful in individual III-1 in this pedigree as the result of an initial pentagastrin stimulation test revealed nonparallelism of diluted samples to the assay standard curve. Pedigree 4 has been uninformative when analyzed with six different linked probes. Blood samples are currently being collected from individuals in pedigree 5, which is also of interest in that two individuals have recently developed positive pentagastrin stimulation tests. DNA samples from pedigree 6 are being obtained.

Linkage analysis in our families has been particularly helpful in establishing that one individual, aged 8 years, is a gene carrier and that nine individuals between the ages of 8 and 35 years have been reasonably excluded as gene carriers. Flanking markers have been informative only in pedigree 3.

Pulsed field gel mapping

Results are shown in Tables 1 and 2 and Fig 3 (A and B). H4.IRBP and pMCK2 hybridize to Not1 restriction fragments

Table 1
Hybridization with H.4IRBP

Cell Line	Restriction Endonuclease Not1	Nru1	Mlu1
Controls 1 and 2	100 kb	800 kb	150, 250, 600, 750, and 1,600 kb
Controls 3 and 4*	100 kb	800, 900 kb	150, 250, 600, 750, and 1,600 kb
CY6	ND	800 kb	250 and 1,600 kb
MEN2A 1 and 2/ MEN2B 1	ND	800 kb	150, 250, 600, 750, and 1,600 kb

*No *Mlu1* hybridization fragments were detected.
ND = not determined.

Table 2
Hybridization with pMCK2

Cell Line	Restriction Endonuclease Not1	Nru1	Mlu1
Controls 1 and 2	400 kb	800 kb	150, 250, 600, 750, and 1,600 kb
Controls 3 and 4*	400 kb	800, 900 kb	150, 250, 600, 750, and 1,600 kb
CY6	ND	800 kb	250 kb
MEN2A 1 and 2/ MEN2B 1	ND	800 kb	150, 250, 600, 750, and 1,600 kb

*No *Mlu1* hybridization fragments were detected.
ND = not determined.

of 100 kb and 400 kb, respectively. Both probes hybridized to an 800 kb Nru1 restriction fragment for controls 1 and 2, and to 800 and 900 kb Nru1 restriction fragments for controls 3 and 4. In addition, both probes hybridized to Mlu1 restriction fragments of 150, 250, 600, 750, and 1,600 kb for three of the four controls. DNA from control 4 that hybridized to the two probes was unresolved on the gels. The pattern of hybridization to the Mlu1 restriction fragments indicates partial restriction of DNA at the Mlu1 restriction sites adjacent to or in the RBP3 and D10S15 loci. For CY6, H4.IRBP hybridized to Mlu1 fragments of 250 and 1,600 kb and pMCK2 hybridized to only a 250 kb Mlu1 restriction fragment. This indicates that the genomic sequence for RBP3, on the 10:16 translocation, contains an Mlu1 restriction site and the 250 and 1,600 kb restriction fragments are adjacent on the chromosome.

No change in the pattern of hybridization was observed for the two MEN 2A or MEN 2B individuals. No detectable rearrangement of the RBP3 or D10S15 loci was observed in these individuals in DNA from lymphocytes.

cDNA Library

The cDNA library which was generated from metastatic MTC is currently being screened for candidate genes. 10^6 clones are being screened initially using a flow-sorted human chromo-

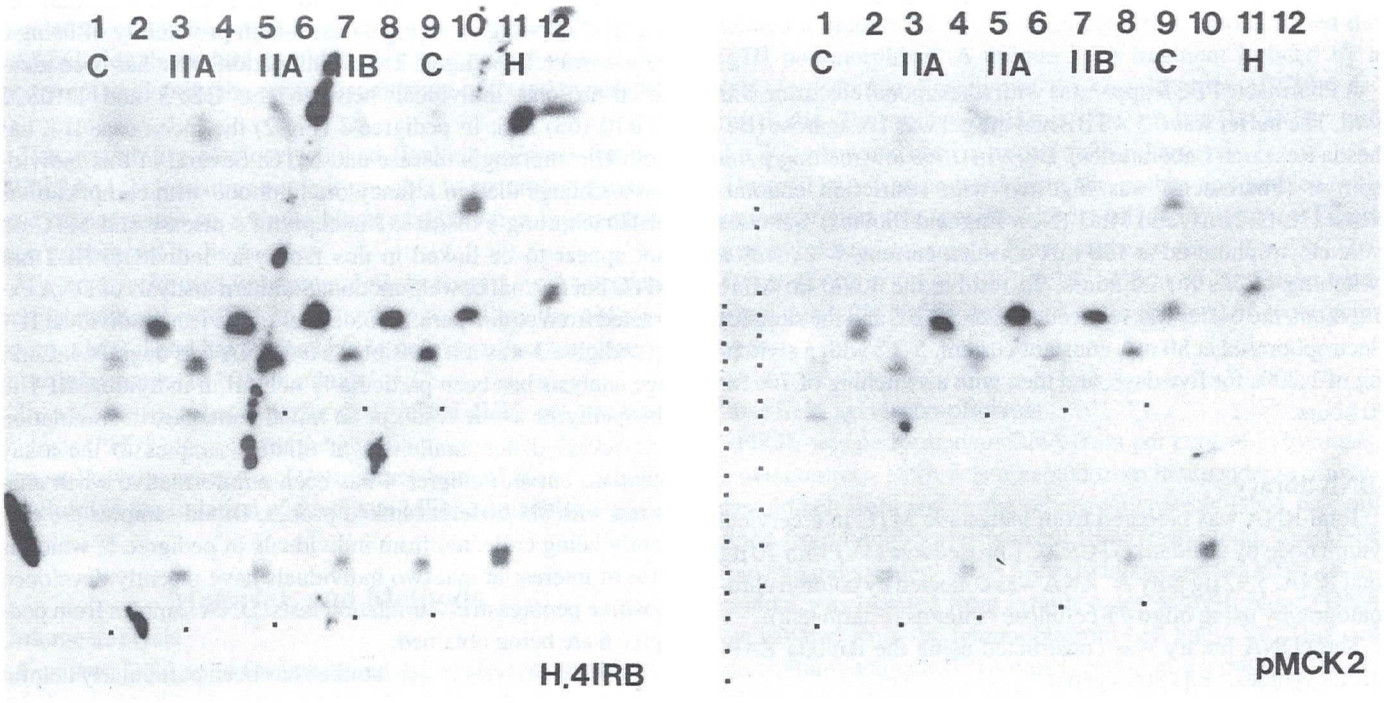

Fig 3—A) autoradiograph (left) of filter hybridized with H4.IRBP, and B) autoradiograph (right) of the same filter hybridized with pMCK2. Lane 1, control 1 MluI; lane 2, control 1 NruI; lane 3, MEN2A-1 MluI; lane 4, MEN2A-1 NruI; lane 5, MEN2A-2 Mlu1; lane 6, MEN2A-2 Nru1; lane 7, MEN2B-1 Mlu1; lane 8, MEN2B-2 NruI; lane 9, control 3 MluI; lane 10, control 3 NruI; lane 11, CY6 Mlu1; lane 12, CY6 NruI. DNA standards are shown to the left of lane 1 in photograph B; the L DNA standards are concatamers of Lambda cI857 (New England Biolabs); and the Y DNA standards are yeast chromosome markers of Saccharomyces cerevisae strain YPH80 (New England Biolabs). Faint signals are highlighted with a solid square.

some 10 library (ATCC 57736). The insert sizes of six random recombinant clones ranged from 0.5 to 2 kb.

A panel of hybrid cell lines will then be used to identify those clones which localize to the centromeric region of chromosome 10.

Discussion

Accurate identification of MEN 2A gene carriers is now possible using probes closely linked to the gene for MEN 2A (MEN2A). This enables intensive follow-up of identified carriers with annual pentagastrin stimulation testing and enables early identification of premalignant C-cell hyperplasia. Thyroidectomy in patients with only C-cell hyperplasia has been shown (6) to significantly reduce morbidity and mortality due to MTC. In contrast, those individuals in whom the gene(s) is not present do not require regular pentagastrin testing. It is unlikely that the gene carrier we have identified would have been identified by pentagastrin testing because of the low sensitivity of this test at ages less than 12 years (7).

MEN2A has been linked to the 10q11.2 region (8), and since recombination fractions in the pericentromeric regions of chromosomes may underestimate the physical distance between the locus and linked markers, we have begun constructing a physical map of the 10q11.2 region. Our data show that two markers, pMCK2 and H4.IRBP, are linked by a 150 kb MluI fragment. Orientation of other markers and the use of hybrid cell lines in PFGE will enable further expansion of this map.

Since our PFGE data do not indicate major DNA rearrangement in DNA of lymphocytes from patients with MEN 2A, we have commenced screening of an MTC cDNA library based on the hypothesis that pathogenic mRNA molecules should be expressed in MTC. Those cDNAs which are mapped to 10q11.2 will be examined closely as potential MEN2A candidate genes.

Acknowledgments

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