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Germline mutations in the *PTEN/MMAC1* gene in patients with Cowden disease

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Cowden disease, also known as multiple hamartoma syndrome, is an autosomal dominant cancer syndrome with a high risk of breast and thyroid cancer. The gene involved has been localized to chromosome 10q22–23. Recently, the tumour suppressor gene *PTEN/MMAC1*, encoding a putative protein tyrosine or dual-specificity phosphatase, was cloned from that region and three mutations were detected in patients with Cowden disease. We confirmed that the *PTEN/MMAC1* gene is indeed the gene for Cowden disease by a refined localization of the gene to the interval between *D10S1761* and *D10S541*, which contains the *PTEN/MMAC1* gene and by mutation analysis in eight unrelated familial and 11 sporadic patients with Cowden disease. Eight different mutations were detected in various regions of the *PTEN/MMAC1* gene. One mutation was detected twice. All detected changes in the gene can be predicted to have a very deleterious effect on the putative protein. Five of the nine patients have a mutation in exon 5 coding for the putative active site and flanking amino acids. Evaluation of the clinical data of the patients in which a mutation could be detected gives no clear indications for a correlation between the genotype and phenotype. In 10 patients no mutation could be detected so far. In support of the linkage data, no evidence has emerged from the phenotype of these

patients suggestive for genetic heterogeneity.

INTRODUCTION

Cowden disease (CD) (MIM 158350), also known as multiple hamartoma syndrome, is a rare familial cancer disease named after the first patient described in 1963 (1). The disease is inherited in an autosomal dominant pattern. Characteristic for CD are oral and facial papules together with hamartomatous features of the thyroid, breast and digestive tract (1,2). Multiple trichilemmomas are considered to be the pathological hallmark of the disease (3). CD patients have a predisposition to develop both benign and malignant neoplasms. Female patients have a high risk of developing fibrocystic disease and carcinomas of the breast; also goiter, adenomas and follicular cell carcinomas of the thyroid gland and polyps of the digestive tract are part of the disease (4–7).

Apart from the CD manifestations described above a variety of neurological symptoms can be found. Lhermitte–Duclos disease (LDD), or dysplastic gangliocytoma of the cerebellum is, together with megalencephaly, the most important central nervous system manifestation (8). Other neurological signs range from tremor and ataxia to epilepsy and mental retardation (8–10, reviewed in 11).

Recently, in an extensive linkage study in 12 CD families, we localized the gene for CD to 10q22–23 between the markers *D10S215* and *D10S564* (12). There were no indications for genetic heterogeneity. The recently cloned tumour suppressor gene *PTEN/MMAC1*, encoding a putative tyrosine or dual-specificity phosphatase (13,14), was suggested to be involved in CD (15). We confirmed that the gene is involved in CD by refinement of the linkage data and mutation analysis in the *PTEN/MMAC1* gene in eight familial and 11 sporadic CD patients. We identified eight different mutations in nine patients.

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Table 1. Summary of the clinical data of the patients and description of the mutations

Patient	Sex	Age	Skin	Thyroid	Breast	Intestine	Urogenital	LDD	Other neurological signs	Head	Mutation and predicted effect
n331	f	43	-	goitre	carcinoma <i>in situ</i> benign lesions	polyps	ovarian cysts uterine fibromas	+	-	nm	Insertion of TTAC (exon 2, nucleotide 1192/1193; frameshift, aa insertion, premature stop in codon 63)
N4 (F)	3m/2f	30-62	+(5)	goitre (3)	adenocarcinoma (2) benign tumors (2)	n.d.	ovarian cysts uterus myomatosis	+(1)	+(5)	mac (2) meg (3)	GT to TT (intron 4, splice site mutation)
n40	m	57	+	goitre	n.r.	n.d.	n.d.	-	+	mac	CAC to CGC : His ₁₂₃ Arg (exon 5)
n130	f	42	+	adenoma	-	-	n.d.	-	-	mac	TGT to CGT : Cys ₁₂₄ Arg (exon 5)
N2 (F)	2m/7f	29-65	+(9)	goitre (7)	adenofibromas (3) adenocarcinomas (1)	polyps (3)	uterus myomatosis (1)	-	+	mac (8) nm (1)	CGA to TGA : Arg ₁₃₀ Stop (exon 5)
n264	f	38	+	goitre	fibrocystic disease fibroadenomas	n.d.	n.d.	-	+	meg	CGA to TGA : Arg ₁₃₀ Stop (exon 5)
n275	m	46	+	-	n.r.	polyps	n.d.	+	+	mac	GAA to TAA : Glu ₁₅₇ Stop (exon 5)
n342	m	47	+	goitre	n.r.	polyps	kidney cysts	-	+	mac	Insertion of A in codon 183 (exon 6) frameshift, premature stop in codon 189
n269	m	33	+	-	n.r.	n.d.	n.d.	+	+	mac	Deletion of GA in N ₂₆₂ (exon 7) (frameshift, premature stop)

For the families the number of patients with a specific symptom are given between brackets. f, female; F, familial; LDD, Lhermitte-Duclos disease; m, male; mac, macrocephaly; meg, megalencephaly diagnosed by MRI scanning; n.d., no signs but not examined; nm, normal; n.r., not relevant; +, present; -, absent; Skin abnormalities include trichilemmomas, lichenoid and verrucous papules on the face, oral mucosal papillomatosis, hyperkeratotic lesion on the distal extremities, and punctate keratosis on the palms and soles. Neurological signs include tremor, clumsiness, incoordination, hearing defects, epilepsy and dizziness.

RESULTS

Fine mapping

Nineteen CA-repeat markers spanning the 10q22-23 region were tested in the families N1-N5 (12) and revealed that the Cowden critical region is between *D10S1761* and *D10S541* (Fig. 1). In the primary linkage study individual N4-II-10, determining the proximal border of the critical region, was considered to be affected (12) although his clinical status was disputed. He was known to have hyperkeratotic papules, macrocephaly and mild ataxia. Recently, clinical re-examination was carried out by an independent clinician leading to the conclusion that he is unaffected. Apart from macrocephaly and mild unsteadiness, he has two hyperkeratotic papules on his forearm and a naevus naevocellularis on his scalp. His skin abnormalities only do not fulfil the criteria for diagnosis of CD (12) and macrocephaly in itself does not justify the diagnosis of CD. Surgical treatment for cervical disc disease and a period of alcohol abuse might explain his mild unsteadiness. The haplotype in combination with the diagnosis of this individual are in accordance of *PTEN/MMAC1* being the Cowden gene (Fig. 1).

Mutation analysis of the *PTEN/MMAC1* gene

Mutation analysis of the *PTEN/MMAC1* gene was performed using lymphocyte DNA of eight familial and 11 sporadic CD patients. The nine described exons of the gene were amplified and sequenced. In two families (N2 and N4) and seven sporadic

patients a heterozygous mutation was detected. The mutations are listed in Table 1. We identified missense, nonsense, frameshift and splice site mutations.

There are three different mutations in the active site sequence motif HCxxGxxRS/T of protein tyrosine phosphatases and dual specificity phosphatases (16,17). Two of these mutations are missense mutations, His₁₂₃Arg and Cys₁₂₄Arg, and one is a nonsense mutation Arg₁₃₀Stop, which occurred twice (Fig. 2). All three can be predicted to lead to a complete or severe loss of phosphatase activity (17). By sequencing the A-lane with the reversed primer, the Arg₁₃₀Stop mutation was shown to cosegregate with CD in family N2 (Fig. 3). A second nonsense mutation Glu₁₅₇Stop was detected in the sequences flanking the active site and therefore can be expected to reduce the phosphatase activity.

In family N4 the first nucleotide of intron 4 was changed from G into T causing a mutation in the nearly invariant splice site sequence GU to UU. This will lead either to exon skipping or to insertion of intron 4 into the transcript. The mutation was shown to completely cosegregate with CD in the family by sequencing of the T-lane.

The three remaining mutations lead to a frameshift and premature termination of the protein at codons 63, 189 and 297 which are the eighth, sixth and 36th codon after the frameshift mutation, respectively. Also these mutations can be predicted to be very deleterious to the protein.

Mutations were confirmed in independently amplified PCR fragments. To exclude common polymorphisms, 50 controls were tested in whom none of the mutations were found.

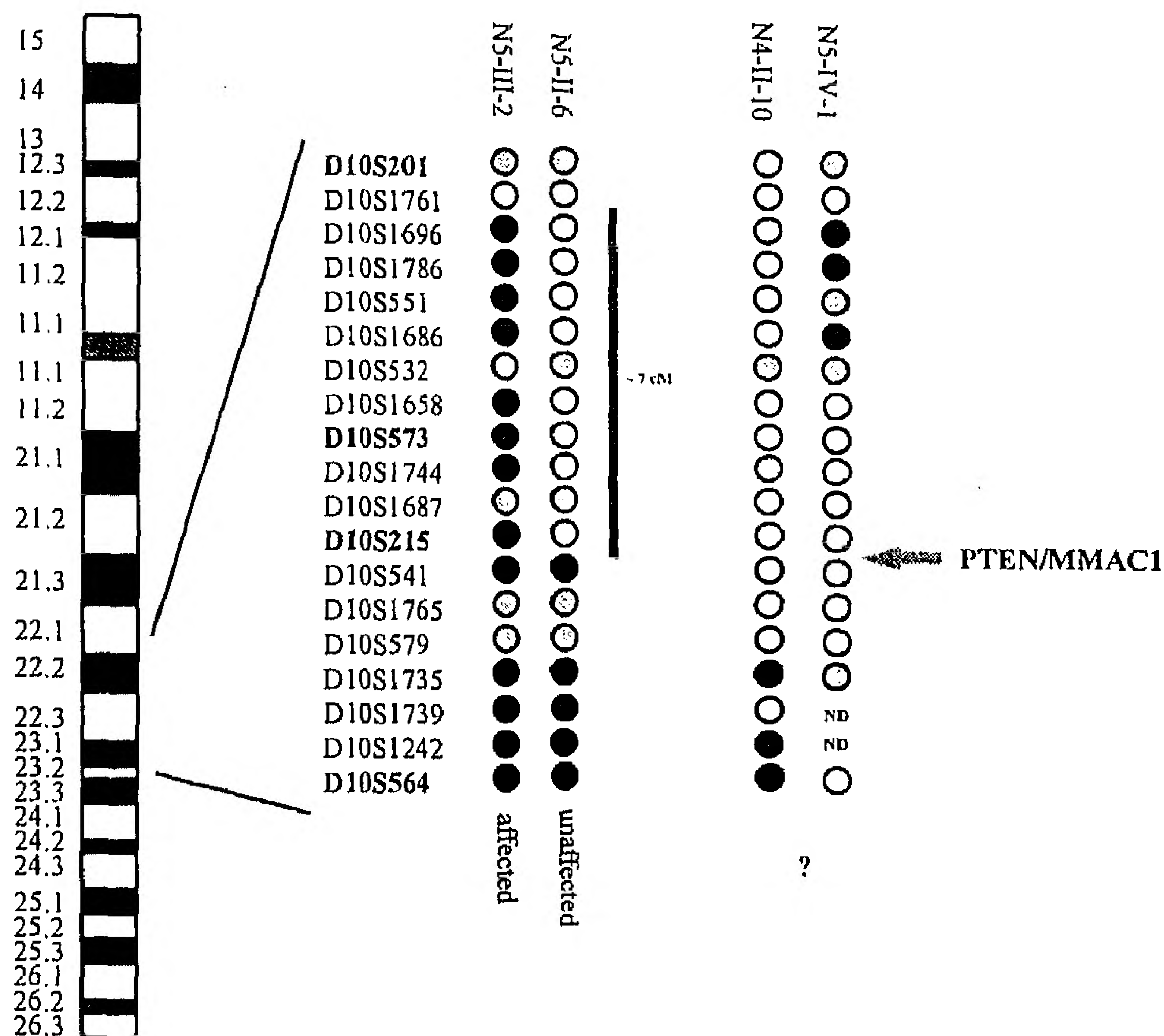


Figure 1. Refinement of the localization of the gene for CD; key recombinations. Black circles indicate the affected haplotype, open circles the unaffected haplotype and grey circles uninformativity. The Cowden critical region is depicted as a black bar. Markers which were used in the linkage analysis described in (12) are given in bold. The location of the *PTEN/MMAC1* gene is marked by an arrow. The haplotypes of two healthy sibs from the CD families N4 and N5, who both have macrocephaly, are depicted on the right.

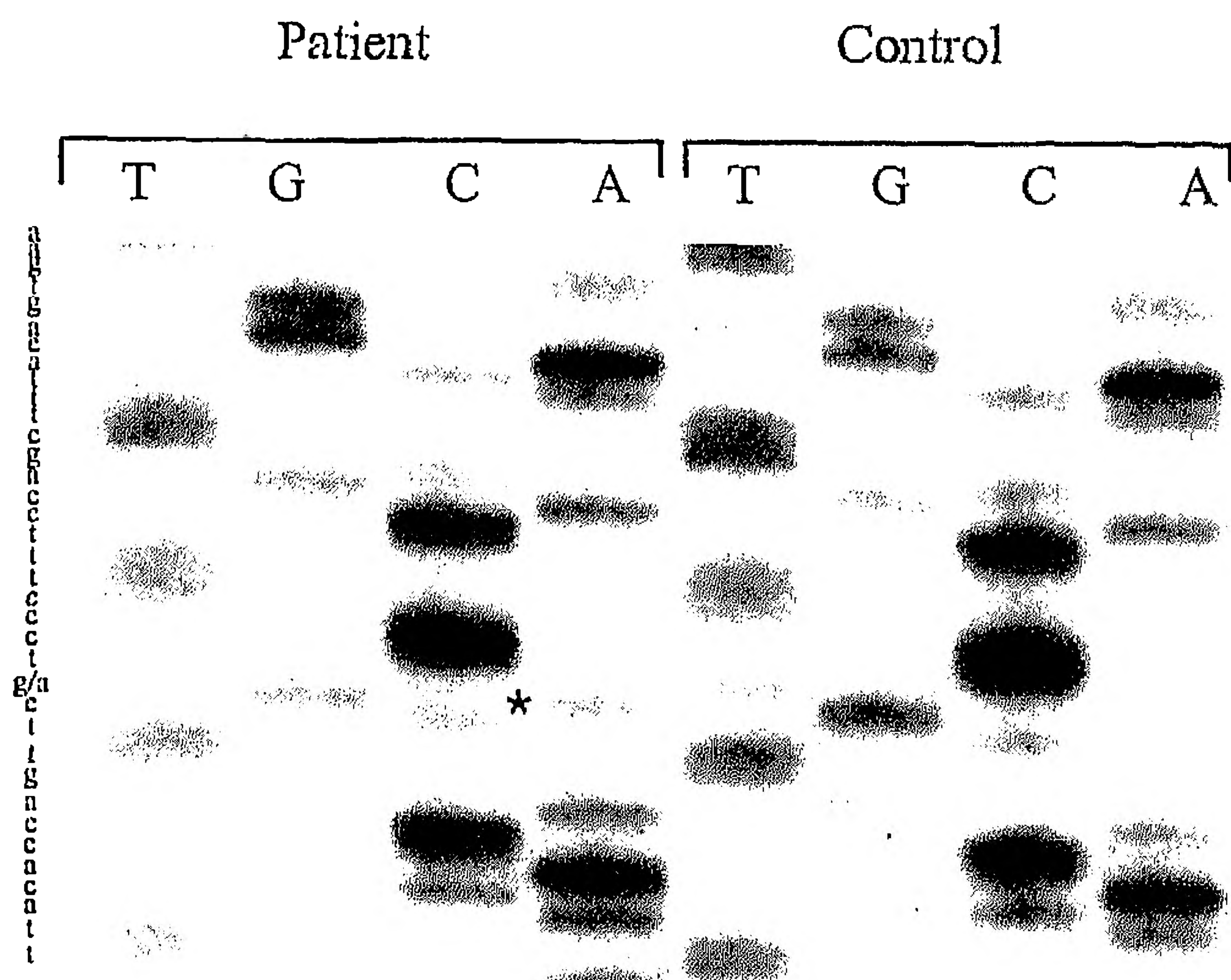


Figure 2. Excerpt of the sequence of exon 5 using the radioactively labeled reversed primer. The heterozygous transversion of G to A in a patient of family N2 is marked by an asterisk. The substitution is predicted to cause a premature stop of the protein at codon 130.

DISCUSSION

We have refined the localization of the CD gene and performed mutation analysis in the *PTEN/MMAC1* gene thereby confirming that this gene is causative of CD (15). A mutation was detected in nine patients, two of whom are familial (N2 and N4). Five of these mutations are in exon 5 in addition to three of four described by Liaw *et al.* (15). This suggests that exon 5 (amino acids

86–165), coding for the active site and flanking amino acids, is a ‘hotspot’ for mutations in patients with CD. So far, missense mutations are only found in the active site. The Arg130Stop mutation was detected twice in our patient group and once in a glioma (14) and is caused by a mutation in a CG dinucleotide. Since these are known to have a relatively high mutation frequency, the CG in codon 130 might be prone to become mutated. In 10 patients no mutation was found. Three of these patients are from families linked to 10q22–23 (12). CD in these individuals might be due to a change in the promoter sequences, deletion of an entire exon which remains undetected with the method used, or a mutation in an intron leading to aberrant splicing. In support of the linkage data, no evidence has emerged from the phenotype of these 10 patients suggestive for genetic heterogeneity.

Since different types of mutations were detected in several regions of the gene, we made an attempt to correlate symptoms and specific mutations. Skin abnormalities including trichilemmomas were detected in patients with all types of mutations but not in patient n331 although trichilemmomas are pathognomonic for CD (3). Patient n331 testifies to the observation in families with CD that the skin abnormalities are not fully penetrant (4). The combination of oncological features including LDD justifies in our opinion the diagnosis of CD. At the same time it raises the question which and how many non-dermatological criteria allow the clinical diagnosis. The detection of a mutation in this patient indicates that the *PTEN/MMAC1* gene is a good candidate for familial cancer disorders with (a combination of) neoplasms associated with CD, for example familial breast cancer and/or thyroid cancer, in the absence of skin abnormalities.

Involvement of the thyroid was found in the majority of patients with the exception of patient n275 with the Glu157Stop mutation

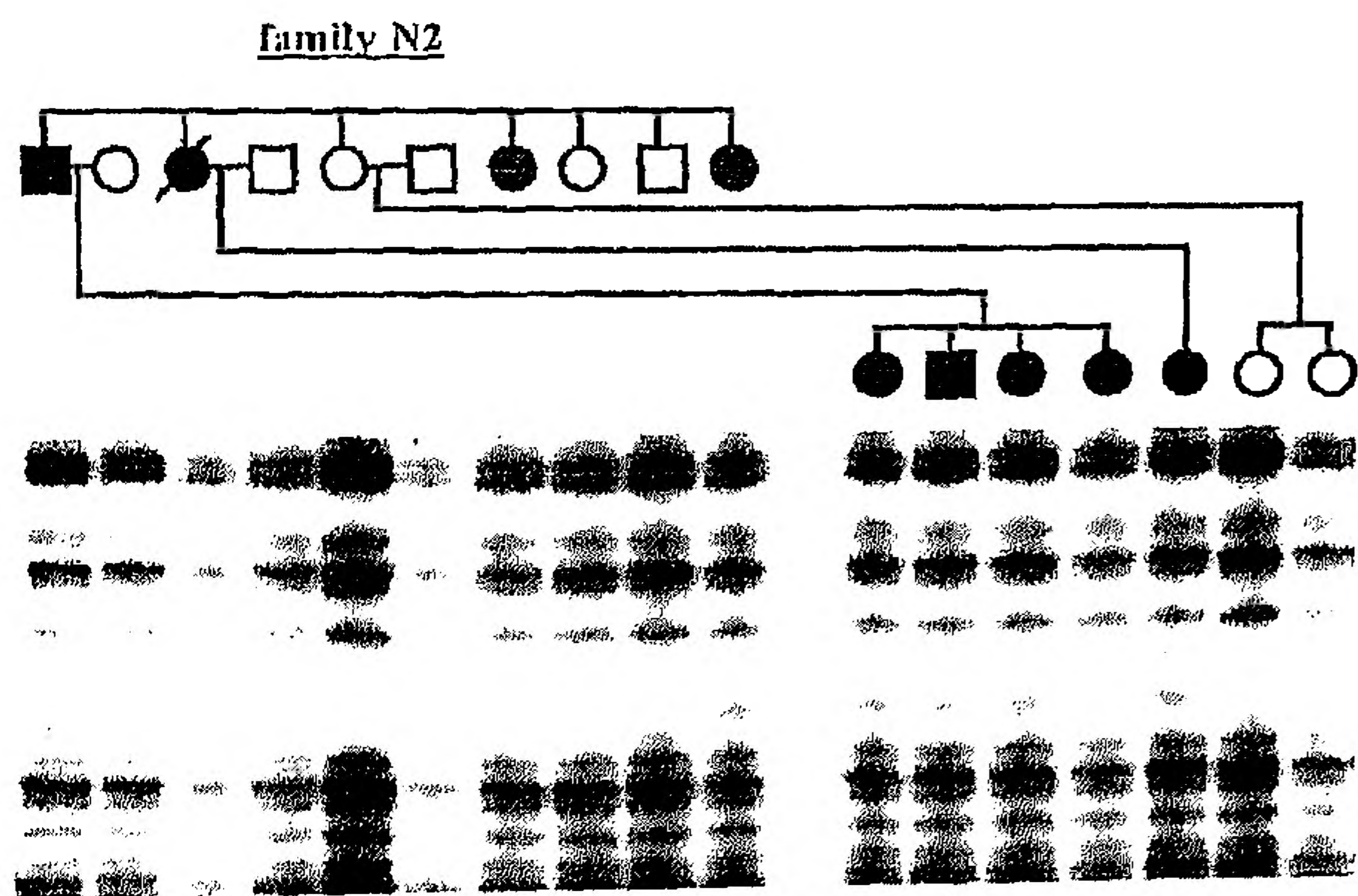


Figure 3. Analysis of the segregation of the Arg130Stop mutation in family N2. Sequencing of the A-lane was performed using the radioactively labeled reversed primer of exon 5.

and patient n269 with a frameshift mutation in exon 7. Patient D described by Liaw and co-workers (15) also has the Glu157Stop mutation and no thyroid symptoms too, suggesting that this is specific for this mutation. A larger number of patients is necessary to confirm a phenotype-genotype correlation for thyroid symptoms in CD.

Breast abnormalities are found only in females with the exception of one male patient. So far, all affected females have a mutation in the N-terminal half of the protein. Within this group of females, there are no indications for specific mutations leading to breast involvement. Also for intestinal polyps, urogenital involvement and neurological signs, there are no indications for phenotype-genotype correlations.

LDD is associated with mutations leading to a premature termination of the protein and a splice site mutation but not with the detected missense mutations. This is also true for the patients described by Liaw *et al.* (15). We cannot confirm their suggestion that LDD is associated with the more N-terminal truncations since patient n269 has a frameshift mutation in codon 262. However, one could argue that the long stretch of 35 changed amino acids between the frameshift and the termination might have a more deteriorating effect on the protein in comparison to the described Arg233 nonsense mutation which is not associated with LDD (15).

The identification of the gene involved in CD makes early, presymptomatic diagnosis of the disorder possible not only in the familial cases but also in the large fraction of sporadic cases in whom diagnosis based on linkage analysis cannot be performed. This is of great clinical importance since accurate surveillance for the occurrence of neoplasms associated with CD can be offered now to the carriers. Our study underlines difficulties in the diagnosis of CD based on clinical symptoms since we had two sibs from CD families suspected for the disorder because of macrocephaly: N4-II-10, who has been described in the results section, and a 5 year old girl (N5-IV-1, Fig. 1) with macrocephaly and a haemangioma on her ankle occurring regularly in CD. Both individuals did not have the affected haplotype in the interval of the *PTEN/MMAC1* gene (Fig. 1) and for N4-II-10 we could show the absence of the mutation cosegregating with the disorder in the family. The mutation in family N5 has not yet been detected. These results indicate that in families, macrocephaly as an early

indication for CD has to be treated with caution. In the Dutch families N1–N5, macrocephaly is present in 24 of 25 patients but also in five of 12 unaffecteds (E.A.J. Peeters, in preparation).

Mutations in the *PTEN/MMAC1* gene causing CD can give some clues as to the function of the gene. The presence of megalencephaly, and the occasional hypertrophy of breasts and increased size of hands and feet in patients with CD (18,19) suggest that haploinsufficiency of the gene leads to disturbance of growth restriction in development. Furthermore, as already suggested by Liaw *et al.* (15), inactivation of the second allele causes disorganization and proliferation resulting in hamartomas, the hallmark of CD. Somatic mutations in other tumour suppressor genes or oncogenes could then cause malignant transformation. The rare occurrence of glioblastoma multiforme in CD patients supports the notion that the appearance of this tumour is the result of a cascade of events in which a mutation in the *PTEN/MMAC1* gene probably is the last step. Furthermore, the putative nature of the gene i.e. a protein tyrosine or dual specificity phosphatase is in agreement with the observed features of CD: these phosphatases are involved in cell proliferation and cell differentiation (17). The occurrence of acquired mutations in the *PTEN/MMAC1* gene in a number of tumour types (13,14) and the germline mutations causing the rare CD makes this gene comparable to p53 showing mutations in many tumour types and in the rare Li–Fraumeni syndrome, promising interesting future results on the *PTEN/MMAC1* gene.

MATERIALS AND METHODS

Patients

Diagnoses of patients were based on dermatological criteria with the exception of patient n331 (see Table 1). In this patient diagnosis was based on the oncological features and LDD. Fine-mapping was performed in the families N1–N5. The families N1 and N2 have been described by Starink *et al.* (4) and the families N4 and N5 by Padberg *et al.* (8). Patient n275 is described by Lindboe *et al.* (20). Clinical features of patients in which a mutation was detected are summarized in Table 1.

Typing of CA repeat markers

Genomic DNA used for the typing of the DNA polymorphisms was isolated as reported before (21). Amplification and separation of the polymorphic CA-repeat fragments were performed as described (22). The markers and their order were as given by Genethon (23) with the exception of *D10S1761* and *D10S532* which are ordered according to recombinations in our families.

Mutation analysis

For mutation analysis, DNA fragments containing the exons were amplified using the inner primers described in (14). PCR products were purified from the reaction mixtures using QIAquick spin columns (Qiagen) according to the gel extraction protocol. The fragments were sequenced using the ds cycle-sequencing system (Gibco-BRL) following the manufacturer's protocol.

Analysis of control individuals

The presence of the detected mutations in 50 unrelated and unaffected individuals was tested in lymphocyte DNA isolated as

described (21). For the mutations in the exons 4, 5 and 6, oligonucleotides of 14–16 bp were developed with approximately in the middle either the wildtype or mutated nucleotide. Exons were amplified as described and blotted onto geneScreen Plus. Ten nanograms of the oligonucleotides were radioactively labeled and used for hybridization in 5× SSPE (0.15 M NaCl, 10 mM NaH₂PO₄, 10 mM EDTA, pH 7.4), 0.3% SDS at 37°C for at least 6 h. After hybridization blots were washed in 5× SSPE, 0.3% SDS for 15 min at room temperature and 2–5 min at 37°C. The insertions in exons 2 and 7 were tested as length polymorphisms by amplifying the exons as described and separating them as described for the CA repeat markers (22).

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