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Novel *PTEN* mutations in patients with Cowden disease: absence of clear genotype–phenotype correlations

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Cowden disease (CD) is characterised by multiple hamartomas in a variety of tissues. The pathological hallmark is the presence of a number of trichilemmomas. Several neurological symptoms are also part of CD with megalencephaly and Lhermitte-Duclos disease (LDD) as the most important features. Early recognition of CD patients is important because of the increased risk of developing malignancies. Breast cancer is the most frequent malignancy, but also urogenital, digestive tract, and thyroid cancers are found with higher frequencies. CD was localised to chromosome 10q23 and the *PTEN* gene (also known as *MMAC1* or *TEP1*) was shown to be involved. Germline mutations were identified in both familial and sporadic CD patients. We identified eight *PTEN* mutations, of which seven were novel, in 13 CD patients. Combined with previous data we have identified 17 independent CD mutations. Gross DNA alterations in CD patients were not detected. Genotype–phenotype relations are discussed. The only correlation suggested to exist is that missense mutations are not detected in LDD patients. However, larger numbers are needed to confirm this. Association of *PTEN* mutations and the occurrence of malignant breast disease found in an earlier study cannot be confirmed. Clinical features of five CD patients without a *PTEN* mutation in the coding sequence do not differ from CD patients with a *PTEN* mutation. Furthermore, it is likely that we have identified the majority of CD patients in the Netherlands. From this we estimate that CD has a prevalence of about 1 in 250 000 in the Dutch population with a low mutation frequency.

Keywords: Cowden disease; multiple hamartoma syndrome; Lhermitte-Duclos disease; *PTEN/MMAC1*; tumour suppressor gene; chromosome 10

Introduction

Cowden disease (CD, MIM No. 158350) is a hereditary autosomal dominant cancer syndrome with a marked variable expression, between and even within families.[1,2] The main characteristic of this disorder is the formation of hamartomas in a variety of tissues. The majority of the patients (about 90%) develop characteristic skin and mucocutaneous lesions.[3–5] Multiple trichilemmomas, benign neoplasms of the hair follicle, are considered to be pathognomonic for this disease.[6] Other typical features include oral and facial papules, goitre and benign hamartomas of the thyroid and digestive tract.[7] A large number of CD patients (ie more than 80%) develops macrocephaly,[2] which is a true megalencephaly, but only a fraction of the patients develop Lhermitte-Duclos disease (LDD). This rare condition is believed to be a hamartomatous outgrowth of the cerebellum, characterised by hypertrophic ganglion cells. LDD was only recently identified as part of CD.[8,9] Megalencephaly and LDD are the most important neurological features of CD and the cause of symptoms like tremor, ataxia, and mental retardation.[8–10] In addition to these benign symptoms, CD patients have an increased risk of developing malignancies. They include follicular cell carcinoma of the thyroid, carcinomas of the breast and malignant cancers of the urogenital and digestive tract. The highest risk is of breast cancer in females, approximately 70%.[11]

We assigned the gene responsible for CD to chromosome 10q23 by an extensive linkage study in 12 families.[12] The gene encoding *PTEN* (alternatively named *MMAC1* or *TEP1*), a dual specificity phosphatase identified within the critical region,[13–15] was shown to be involved by the identification of germline mutations present in a number of CD patients.[16,17] Germline mutations were also identified in two other distinct but related syndromes, Bannayan-Riley-Ruvalcaba syndrome (BRR) and juvenile polyposis syndrome (JPS).[18–21] Symptoms found in BRR and CD are partly overlapping and comprise macrocephaly, intestinal hamartomatous polyps, lipomas and haemangiomas. The R233X mutation in the *PTEN* gene has been found in both a CD and a BRR patient.[19] This suggests that CD and BRR represent a variable spectrum of the same disorder. Alternatively, modifier genes might be responsible for the different phenotypes occurring in patients with an identical mutation. In JPS, characterised by hamartomatous polyps throughout the digestive tract and predisposition to digestive tract cancer, mutations have been identified which have so far not

been described for CD or BRR patients.[21] Genetic heterogeneity for JPS was suggested by a linkage study performed in eight informative JPS families. Linkage with the *PTEN* gene was excluded in these eight families and also mutation analysis of the coding region of the *PTEN* gene did not reveal any sequence alterations.[22] Recently it was shown that the *SMAD4* gene is the major gene involved in familial JPS.[23]

The identification of *PTEN* as a tumour suppressor has created a great deal of interest in the field of cancer research. Studies have shown the involvement of *PTEN* in a large number of sporadic tumours. Mutations have been identified in glioblastomas, prostate cancer, melanomas, thyroid and endometrial tumours.[13,14,24–26] In some of these cancers, eg glioblastomas, melanomas and prostate cancer, *PTEN* mutations seem to be associated with tumour progression.[24,27,28] In endometrial carcinomas *PTEN* turned out to be the most frequently mutated gene so far.[26,29] Recent studies suggest that at least part of the function of *PTEN* is the regulation of cell migration and interactions with the extracellular matrix.[30]

Here we describe a detailed mutation analysis of the coding sequence of the *PTEN* gene in CD families and sporadic CD patients. We identified mutations in eight unrelated CD patients. One mutation was detected previously. Genotype–phenotype correlations are discussed for these eight mutations and nine mutations described previously.[17]

Materials and Methods

Patients

In total, 13 patients were included in the mutation analysis – seven families and six sporadic patients. Dutch CD patients, both familial and sporadic, were diagnosed mainly on the presence of the dermatological lesions.[5,6] Family anamnesis of the six sporadic patients was suspect in three cases. Parents of these patients were not examined for clinical signs. All patients fulfil the criteria of the international Cowden Consortium.[12] Phenotypes of the CD patients and CD families with a *PTEN* mutation can be found in Table 1. Of all patients combined, eight were diagnosed with LDD, four of which are familial. The five CD cases in which no *PTEN* mutation was detected were ascertained because of the presence of multiple features seen in CD. Family N1 has been used in the original linkage study and was previously described by Starink *et al*.[2] Trichilemmomas, macrocephaly, and goitre were present in the proband analysed in CD family N9. The proband's mother died of thyroid carcinoma. Also her brother has clinical symptoms of CD. Patient 5899 has an extensive clinical history, including oral papillomatosis, goitre, endometrium carcinoma, and breast cancer. Patient n354 was ascertained because of the presence of skin lesions, goitre,

Table 1 CD-mutations and their phenotype

Patient	Sex	Skin	Thyroid	Breast	I	UG	LDD	Head	Neurol. signs	Mutation	
N6(F)	3f				n.e.					I33del, exon 2	NT
n331	f									159insTTAC, exon 2	
N3(F)	2m/1f									IVS3+1G>C, intron 3	
N4(F)	3m/2f				n.e.					IVS4+1G>T, intron 4	
ddW	f						n.e.			IVS4+1G>T, intron 4	
dd14443	f									Q97X, exon 5	
n40	m				n.e.	n.e.				H123R, exon 5	NT
n130	f					n.e.				C124R, exon 5	NT
N2(F)	2m/7f									R130X, exon 5	
n264	f				n.e.	n.e.				R130X, exon 5	
n275	m					n.e.				E157X, exon 5	
n342	m									545insA, exon 6	
N8(F)	3f				n.e.					G165E, exon 6	NT
n269	m				n.e.	n.e.				783delGA, exon 7	
N5(F)	1m/6f									IVS7+1G>T, intron 7	
N7(F)	6m/3f									937delA, exon 8	
WvL	f									L320X, exon 8	

F: familial; I: Intestine; UG: urogenital; m: male; f: female; n.e.: not examined; white bar: normal; grey bar: benign lesions present or (skin) trichilemmomas and/or oral papilomatosis or (head) macrocephaly; black bar: malignancies; NT: non truncating.

and mamma-carcinoma. Family anamnesis of both patients was suspect. The fifth patient (n1420) has skin lesions although no trichilemmomas were detected on pathological examination. Furthermore, this patient has macrocephaly and is known to have epileptic seizures. Family anamnesis was negative.

Mutation Analysis
Sequence analysis was performed on PCR products, using intronic primers designed to amplify the nine different exons and the corresponding exon/intron boundaries of the *PTEN* gene.[13] Genomic DNA of patients was isolated using standard procedures. Products were gel purified using a PCR purification kit (Qiagen, Westburg, Reusden, The Netherlands).

Cycle sequencing was performed in the 9600 Thermal Cycler (ABI, Nieuwe Kerk ald Ysel, The Netherlands) with the dRhodamine dye terminator kit (ABI) for 28 cycles consisting of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. Sequencing products were precipitated with 70% ethanol, 0.5 mM MgCl$_2$ and resuspended in 2.5 μl loading buffer. The samples (2 μl) were run and analysed on an ABI 377 automated sequencer.

A *PTEN* cDNA probe, encompassing the complete coding sequence and part of the 3' and 5' UTR, was used to screen for the presence of gross DNA alterations in patients without an identified mutation. DNA (7 μg) was digested with HinDIII and PstI, blotted to GenescreenPlus and hybridised with the *PTEN* cDNA probe. As a control probe PMP22 cDNA (17p11.2–p12) was used.

Control Analysis
Identified mutations were confirmed in independently amplified PCR products. Each mutation was checked for its presence in the normal population by allele-specific oligonucleotide hybridisation. The appropriate exon was amplified in 50 unrelated unaffected individuals using genomic DNA. Allele-specific oligonucleotides of 14–16 bp were synthesised containing either the wild type or the mutant sequence. PCR products were dot-blotted on to GenescreenPlus. The oligonucleotides were labelled radioactively and hybridised in $5 \times$ SSPE/0.3% SDS at 30°C overnight. Blots were washed in $5 \times$ SSPE/0.3% SDS at the proper temperature. Insertions and small deletions were tested as length polymorphisms by amplifying the exons in the presence of α-(^{32}P)dCTP and separating them on a 6.6% denaturing acrylamide gel.

Results

We have screened 13 patients diagnosed with CD for mutations in the coding region of the *PTEN* gene and eight mutations were identified (Table 2). All mutations were heterozygously present in genomic DNA. One mutation was detected previously. The patient group consists of six sporadic cases and seven familial cases for which the corresponding families were linked to 10q23. The mutations included two small deletions located in exon 2 and 8. Three splice site mutations were located in introns 3, 4, and 7. One missense mutation was found in exon 6. Two nonsense mutations were detected, one in exon 5 and the second in exon 8. All mutations identified in CD families were shown to cosegregate with the disorder. None of the detected mutations were present in 50 controls, excluding common polymorphisms, and all mutations were confirmed in independently amplified PCR fragments.

In three sporadic and two familial CD patients we did not identify a mutation. We were only able to analyse the *PTEN* gene in four of these patients for any gross alterations. Genomic DNA, digested with HinDIII and PstI, was hybridised with a *PTEN* cDNA probe. This probe contained the entire coding sequence

Table 2 Description of the identified CD mutations with their predicted effect

Patient	Mutation	Predicted effect
N6(F)	97-99delATT	I33del
N3(F)	IVS3+1G→C	splice-site mutation
ddW	IVS4+1G→T	splice-site mutation
dd14443	289C>T	Q97X
N8(F)	494G>A	G165E
N5(F)	IVS7+1G→T	splice-site mutation
N7(F)	937delA	frameshift, premature stop
WvL	959T>A	L320X

(F = family).

and a small portion of the 3' and 5' UTR. There were no indications of any gross alterations in the *PTEN* gene of these four patients.

Discussion

Cowden disease is an autosomal dominant cancer syndrome, caused by mutations in the recently cloned tumour suppressor gene *PTEN*, located at 10q23.1.[12,16,17] In this study we have performed an extensive mutation analysis in 13 unrelated patients, in which we detected eight mutations. Combined with our previous data[17] we have identified 17 mutations (Figure 1) in 22 patients. Two mutations were detected twice in our patient group. Mutations included missense (18%), nonsense (29%) and splice-site mutations (24%), small insertions (12%) and deletions (18%). The mutations were dispersed throughout the gene with a clustering in exon 5. The mutations and a summary of clinical data are presented in Table 1.

In total, we identified in seven of the nine familial CD cases a *PTEN* mutation. Family N1, in which we did not find a mutation, has been used in the original linkage study and the data are compatible with linkage to 10q23.[12] However, since the size of this family is small the data are not statistically significant. Clinical symptoms include skin lesions, macrocephaly, mamacarcinoma and goitre. Recently, three papers described the presence of a processed pseudogene or homologue *PTH2*,[31–33] located on chromosome 9. To exclude involvement of *PTH2* in this family we tested markers, including D9S165, D9S1878 and D9S15. These markers flank the interval containing this gene. Haplotype analysis indicated that the *PTH2* locus is not linked to CD in this family (data not shown). So far, only the coding region of the *PTEN* gene is analysed for mutations and gross alterations are excluded. Mutations in the 5' regulatory regions, other regions that influence proper *PTEN* functioning or intronic mutations that create alternative splice sites might be present in this family. The second family (N9) without a detected mutation in *PTEN* does show the typical skin lesions, confirmed by pathological examination. Also, goitre and macrocephaly are present. The proband's mother died of a metastasised thyroid carcinoma. We performed mutation analysis in the proband and are now collecting blood samples of other family members to either prove or exclude involvement of the *PTEN* gene.
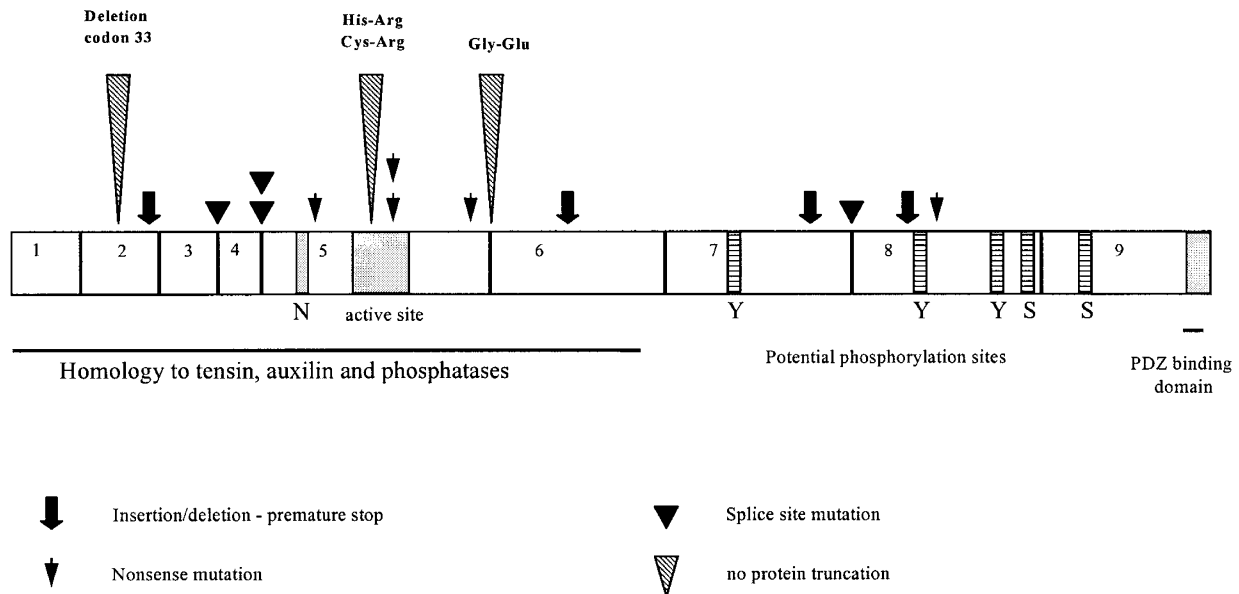
Figure 1 *Shown are the locations of the alterations detected in the* PTEN *gene in relation to the functional domains of the protein. Corresponding phenotypes are depicted in Table 1. The N-terminal part of* PTEN *is homologous to other phosphatases and also to tensin and auxilin. The C-terminal part of* PTEN *shares no homology with other proteins but contains three putative tyrosine (Y) and two putative serine phosphorylation (S) sites and a PDZ binding domain at the 3' end of the protein*

In 10 of the 13 sporadic patients a mutation in the *PTEN* gene was detected. The three patients in which no *PTEN* mutation was detected had typical features of CD. These cases might have mutations in the same, not yet analysed, regions of the gene as mentioned above. Such mutations are not detectable by the methods used in this study. Another explanation for not finding mutations in both these sporadic and familial patients is genetic heterogeneity, suggested by the existence of CD families not linked to 10q23.1.[34] We also tested patients, offered to us for DNA diagnosis, with features commonly seen in CD but without the pathognomonic skin lesions. So far, we have not detected a mutation or a gross DNA alteration in any of these patients. The absence of mutations in this set of patients might underline the importance of skin lesions in CD diagnosis.

All but four mutations detected so far result in a premature truncation of the protein. This large proportion of protein truncating mutations is in agreement with the mutations described in the literature.[20,35,36] Of the four mutations that do not result in a protein truncation, three are missense mutations and one is an in-frame 3 bp deletion. Two missense mutations result in a substitution of two invariant amino acids in the active site, His123 and Cys124, by an arginine. A dramatic decrease of phosphatase activity was shown for a His123Tyr substitution in the active site[37] and one

might expect this to be also true for these two missense mutations. The third missense mutation, Gly165Glu, involves the first amino acid residue of exon 6. This mutation is localised in a structural element, an α-helix, conserved between *PTEN* and tensin. Therefore, this mutation is predicted to disrupt the PTEN activity.[38] The mutation is located at the second nucleotide in exon 6 at the splice acceptor site. However, it is unlikely that this mutation has a dramatic effect on RNA splicing since the second nucleotide of an exon is not part of the consensus sequence of the 3' splice site.[39] The 3 bp in-frame deletion (Ile33) does not result in protein truncation. Codon 33, however, is conserved in both tensin and auxilin. This mutation might affect interactions with other important molecules or the mutation might alter the stability of the RNA as is suggested by Lynch *et al.*[20]

The families and sporadic patients were considered for possible genotype–phenotype correlations. CD patients show involvement of multiple organ sites and all sites are involved in mutations throughout the protein. No specific mutation or part of the protein seems to be connected with a particular phenotype. We cannot exclude that there is a genotype–phenotype correlation for specific mutations. However, because only very few mutations are detected in more than one case a statistical analysis was not performed. Also, from family data we know that there is a marked variation in

expression and age of occurrence of the various symptoms. This is in agreement with *PTEN* functioning as a tumour suppressor gene, as a second hit randomly occurs in different tissues. LDD, however, is not seen in patients with a missense mutation (eight cases). This is also true of the three LDD cases described in the literature.[35] It is also notable that so far patients with mutations in exon 1, 8 and 9 have not been diagnosed with LDD.[20,35] Furthermore, the two mutations found in exon 2 do not result in the typical papillary skin lesions. Only one of the three patients in family N6 has oral papillomatosis. A larger number of patients is needed to confirm any of these associations. We cannot confirm the observed association between the presence of a *PTEN* mutation and malignant breast disease.[35] Four of the five female CD patients, of which two are mother and daughter, without a detected *PTEN* mutation do show malignancies of the breast. Furthermore, a brother of one of these female CD patients developed bilateral breast cancer. This is in contrast to the data reported in the literature, in which only one out of seven patients with no detected *PTEN* mutation had malignant breast disease.[35] This indicates that larger numbers are needed to address these clinically relevant observations.

We have used the PALGA database, the Dutch network and database for pathology, to screen for any unknown CD patients. This network contains information of more than 4.5 million individuals. In total, we have identified 45 CD patients in the age group of 25 years and older in the Dutch population, including patients not participating in this study. It is likely that we have identified the majority of patients in the Netherlands in this way. However, we cannot exclude possible misdiagnosis of CD patients. Although CD can be expressed with great variation, even within families, mutation analysis of both breast cancer patients and families indicates that the CD symptoms are highly expressed and well recognised by clinicians. Rarely is a *PTEN* mutation identified in such breast cancer cases.[40,41] Furthermore, CD can be attributed to a *PTEN* mutation in the majority of CD patients. Therefore, we estimate that the prevalence of CD is between 1 in 200 000 and 1 in 250 000 in the Dutch population. In four patients it is likely that CD is caused by a new mutation. Three of the four cases are proven 'de novo' mutations. In two patients (*n*269, *n*40) the expected mutation was not present in the parents and in family N3 the mutation could not be found in a healthy sister carrying the affected haplotype. This indicates a low mutation frequency, also shown by the rare finding of silent mutations in the coding sequence of *PTEN* in the normal population.

The identification of *in vivo PTEN* substrates might reveal the cellular signalling pathways that are normally regulated by *PTEN*. This strategy might also identify protein kinases that counteract the effects of *PTEN* and are thus potentially oncogenic. A good candidate for such a kinase is the recently identified *LKB1* gene, a serine threonine kinase, which is involved in Peutz-Jeghers syndrome.[42] Patients are predisposed to the formation of intestinal hamartomatous polyps, mucocutaneous pigmentation affecting lips, buccal mucosa and digits and to many different types of cancer. It remains to be seen if both these tumour susceptibility genes function in the same pathways.

Acknowledgements

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