

A Novel Loss-of-Function Mutation (N48K) in the PTEN Gene in a Spanish Patient with Cowden Disease

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Cowden disease, also known as multiple hamartoma syndrome, is a rare disease inherited in an autosomal dominant pattern, which confers a high risk of developing breast and thyroid carcinomas. Mutations in PTEN, a tumor suppressor gene located on chromosome 10q23, have been identified in patients with Cowden disease. In this work, the direct sequencing of all coding regions of the PTEN gene led us to the identification of N48K, a new germline PTEN missense mutation, in a patient suffering from Cowden disease. The genetic analysis of 200 chromosomes from healthy individuals revealed that the variant was not common in our population. Moreover, by functional analysis we found that the ability of PTEN N48K mutant protein to inhibit the activa-

tion of the proto-oncogene PKB/Akt was impaired, supporting the involvement of N48K mutation in Cowden disease. Loss of heterozygosity using three microsatellites (D10S215, D10S541, and D10S564) and the complete sequence analysis of PTEN exons in breast and endometrial tumor samples from the same patient were also carried out in an attempt to identify additional PTEN somatic mutations. The lack of loss of heterozygosity or additional mutations in tumor samples suggests that abnormalities of the regulatory regions of the PTEN gene or haplo-insufficiency might occur in tumors from Cowden disease patients. **Key words:** Cowden disease/functional analysis/genetic analysis/loss of heterozygosity/N48K/PTEN. *J Invest Dermatol* 121:1356–1359, 2003

Cowden disease (CD; MIM 158350), also known as multiple hamartoma syndrome, is a rare disease inherited in an autosomal dominant pattern. CD includes trichilemmomas (benign tumors of the hair follicle infundibulum), acral keratoses, papillomatous lesions, and mucosal lesions (Starink, 1984; Starink *et al*, 1986; Hanssen and Fryns, 1995; Longy and Lacombe, 1996). In addition, affected women have a high risk of developing fibrocystic disease and carcinomas of the breast; goiter, adenomas, follicular cell carcinomas of the thyroid gland, and polyps of the digestive tract are also found in affected individuals (Brownstein *et al*, 1979; Starink *et al*, 1986; Schrager *et al*, 1998). These manifestations can be associated with several neurologic disorders such as dysplastic gangliocytoma of the cerebellum (Lhermitte–Duclos disease), megalencephaly (Padberg *et al*, 1991), and other neurologic signs such as tremor, ataxia, epilepsy, and mental retardation (reviewed by Longy and Lacombe, 1996).

Germline mutations on the tumor suppressor gene PTEN (phosphatase and tensin homolog deleted on chromosome 10) (MIM #601728), also called MMAC1 (mutated in multiple advanced cancers) or TEP1 (tumor growth factor β regulated and epithelial cell enriched phosphatase), have been established as the

cause of CD. Moreover, somatic PTEN mutations have been found in a large number of sporadic tumors, predominantly in endometrial carcinomas and glioblastomas (reviewed in Bonneau and Longy, 2000).

PTEN protein is a phosphatase with enzymatic activity towards both protein substrates and the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (Myers *et al*, 1997; Maehama and Dixon, 1998; Tamura *et al*, 1998). PTEN regulates distinct signal transduction pathways including the PKB/Akt cell survival and the integrin-triggered signaling pathways (reviewed in Cantley and Neel, 1999; Maehama and Dixon, 1999; Tamura *et al*, 1999; Di Cristofano and Pandolfi, 2000). In tumors and cell lines, an inverse correlation exists between the presence of a functional lipid phosphatase PTEN enzyme and the activity of the PKB/Akt proto-oncogene (Haas-Kogan *et al*, 1998; Stambolic *et al*, 1998; Wu *et al*, 1998; Dahia *et al*, 1999; Ermoian *et al*, 2002). The PTEN protein structure consists of a 179-residue N-terminal domain (residues 7–185) and a 166-residue C-terminal domain (residues 186–351). The N-terminal domain is a phosphatase domain that is similar to that of protein tyrosine phosphatases but has an enlarged active site important for the accommodation of the phosphoinositide substrate. The C-terminal domain is a C2 domain that binds phospholipid membranes *in vitro* (Lee *et al*, 1999).

A recent review of germline mutations of human PTEN gene (Bonneau and Longy, 2000) described 82 different mutations and two gross deletions located along the whole gene, with the exception of exon 9. Interestingly, a great number of the mutations were found in exon 5 ($n = 35$, 31.5%), which encodes for the phosphatase core motif.

In this study, we have sequenced all the PTEN coding region from peripheral blood leukocytes of a woman diagnosed with

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Abbreviations: CD, Cowden disease; LOH, loss of heterozygosity; SSCP, single strand conformation polymorphism.

CD. The identification of a new missense mutation in exon 2 led us to perform a functional analysis of the PTEN mutated protein, as well as other genetic analysis on breast and endometrial tumors from the patient, to shed light on the pathogenicity of the mutation.

MATERIALS AND METHODS

All experiments and the informed patient consent protocols are correct from the ethical point of view and previously reviewed by the Ethical Committee of the Faculty of Medicine of the University of Santiago de Compostela. The work was conducted according to the Spanish law including adherence to the Helsinki Principles.

Phenotypic description We studied a 38-year-old woman with CD. The patient presents the clinical features of the disease, such as thyroid affection (multinodular goiter), skin lesions (trichilemmomas, oral lesions such as verrucous lesions of the palate, cobblestone-like papules of the gingiva, palmoplantar keratoses), invasive and intraductal breast carcinoma, gastrointestinal alterations (one adenomatous polyp of the colon), and also endometrial carcinoma and ovarian cyst. Other features are obesity, hirsutism, and fibromyalgia.

The patient's mother was diagnosed with a squamous cell carcinoma *in situ* (CIN III) of the uterine cervix at 70 y of age. The patient has no brothers or sisters, and in the mother's family there are patients with goiter and breast cancer (unfortunately, it was not possible to obtain samples from them), although the patient studied here has the stronger symptoms in the family.

DNA extraction DNA was extracted from 5 ml of peripheral blood using a standard proteinase K/phenol/chloroform procedure. The samples were incubated in 50 mM Tris-HCl, 150 mM NaCl, 100 mM Na₂ ethylenediamine tetraacetate, sodium dodecyl sulfate 1.25%, and 0.3 mg per ml proteinase K. DNA was precipitated with absolute ethanol after two extractions with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1).

Tumor tissues from breast and endometrial carcinomas of the patient were studied. The breast tumor was an invasive ductal carcinoma of grade I (Nottingham modification of the Bloom-Richardson system) with extensive intraductal carcinoma of cribriform type. Metastatic lymph nodes were not found. Tumor cells were positive for estrogen (clone GF11, dilution 1:10, Novocastra, Newcastle upon Tyne, UK) and progesterone receptors (clone 1⁴⁶, dilution 1:5, Novocastra). The endometrial tumor was a well-differentiated endometrioid carcinoma with squamous metaplasia (adenocarcinoma). DNA was obtained from paraffin-embedded tissue. Briefly, to dissolve the paraffin the tissue section was rapidly washed with xylene and centrifuged and the pellet was washed three times with ethanol at decreasing concentrations. After that it was then resuspended in 50 μ L of digestion buffer and incubated at 37°C overnight.

Mutation screening Each of the nine exons of the PTEN gene was amplified under standard PCR conditions using 100 ng of genomic DNA obtained from a blood sample or 2 μ L of DNA solution obtained from breast and from endometrial tumor extractions. Amplifications were made in 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M of each dNTP, 10 pmol of each primer, and 1.25 units Taq polymerase (Invitrogen Life Technologies, Carlsbad, CA), in a final volume of 25 μ L. Reactions were performed in a PerkinElmer Cetus 2400 thermocycler, consisting of an initial denaturation step at 95°C for 5 min followed by 35 cycles of 30 s at 94°C, 45 s at the appropriate annealing temperature (50°C for exons 2, 3, 4, 5, 6; 60°C for exons 1, 7, 8, 9), and 1 min at 72°C. The PCR product was purified using ExoSapI (Amersham Pharmacia Biotech, Uppsala, Sweden) previous to the sequence reaction. Sequence reactions were carried out using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). The sequencing reaction was 30 cycles at 95°C for 20 s, 55°C for 20 s, and 60°C for 1 min. Sequences were run in a Megabac capillary sequencer (Amersham Pharmacia Biotech) using forward and reverse primers. (Primer sequences are available upon request.) Electrophoresis conditions were as follows: sample injection voltage 2 s; sample injection time 40 s; run voltage 7 kV; run time 100 min.

Analysis of control individuals 200 chromosomes from healthy individuals of our population were genotyped by single strand conformation polymorphism (SSCP) analysis at the variant N48K. Exon 2 PCR products were diluted 1:10 in loading buffer (deionized formamide and blue dextran), heated at 95°C for 5 min, and then cooled on ice.

SSCP analysis was performed in a semiautomatic electrophoretic system (PhastSystem, Amersham Pharmacia Biotech) using PhastGel (*T* = 20% polyacrylamide gels) and Native buffer strips. One microliter of the denatured product was applied to the cathodic end. The running conditions during the separation step were 400 V, 5.0 mA, 1.0 W at 15°C for a total of 600 V h. A prerun with 400 V, 5.0 mA, 1.0 W at 15°C for a total of 20 V h was added. After electrophoresis PhastGels were silver stained.

Comparative sequence analysis between species and related phosphatases The PTEN phosphatase domain, containing the Asn⁴⁸ residue, was compared by amino acid sequence alignment between *Homo sapiens*, *Xenopus laevis*, *Rattus norvegicus*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Dictyostelium discoideum*, and *Arabidopsis thaliana* (Genbank accession numbers NM0000314, AF144732, AF017185, AF144232, AF126286, AF483827, and AJ490172, respectively). A comparison of human PTEN with TPTE (AF00718) and TPIP (NM130785), two human related phosphatases, was also carried out.

Loss of heterozygosity (LOH) analysis For LOH analysis three dinucleotide microsatellite markers (D10S215, D10S541, and D10S564) and the exon 2 of the PTEN gene that contained the mutation N48K were considered. Genomic DNA corresponding to DNA extracted from endometrial and breast tumor, as well as the blood sample of the patient, was subjected to PCR amplification with the previously described conditions and using an annealing temperature of 55°C for all three microsatellites. For microsatellite LOH analysis PCR products were detected in an automated fluorescent system: 5 μ L of loading buffer (5 mg per ml dextran blue/formamide) was added to 1 μ L of each PCR product and combined with internal lane standards. The samples were heat denatured at 94°C for 4 min before being loaded onto a gel (ReproGel High Resolution, Amersham Pharmacia Biotech) with a final concentration of 8% (wt/vol) acrylamide/bisacrylamide monomers and 1 \times TBE (Tris-Borate-EDTA). Electrophoresis was carried out at 1500 V, 30 W, and 60 mA for 2 h on the Automatic Laser Fluorescent DNA sequencer (Amersham Pharmacia Biotech).

LOH at exon 2 was studied by sequence comparison of tumor (breast and endometrial) with the blood sample from the patient.

Cell culture, transfections, and plasmids The U87MG cell line was grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. Cells were transfected with the indicated plasmids using FuGENE 6 (Roche Diagnostics, Basel, Switzerland) following the recommendations of the manufacturer. The pRK5 PTEN wild-type, pSG5 HA-PKB, and pEF-BOS-Ras V12 have been described previously (Jimenez *et al*, 1998; Torres and Pulido, 2001). The pRK5 PTEN N48K and pRK5 PTEN G129E mutations were obtained by PCR oligonucleotide site-directed mutagenesis, and the mutations were confirmed by DNA sequencing.

Analysis of PKB activation Transfected U87MG cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 1 mM phenylmethylsulfonyl fluoride; 1 mg per ml aprotinin; 2 mM Na₃VO₄; 20 mM Na₄P₂O₇). The activation of HA-PKB was assessed by immunoblot using the antiphospho-active-PKB/Akt (Ser⁴⁷³) antibody (Cell Signalling Technologies Beverly, MA), following immunoprecipitation of HA-PKB from the lysates with the anti-HA monoclonal antibody 12CA5. The anti-PKB/Akt and anti-PTEN antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA) and Cascade Bioscience, (Winchester, MA) respectively. Immunoblot and stripping of the membranes were performed as described previously (Torres and Pulido, 2001).

RESULTS

Mutation analysis We sequenced all coding regions of the PTEN gene from a blood sample as well as from breast and endometrial tumor samples of a patient with CD. A heterozygous variant in exon 2 of the gene, which was confirmed in a second independent PCR and sequence reaction, was found at the germline (**Fig 1**) and was also present in both tumor samples. The variant was a single nucleotide substitution at nucleotide 144, and originates a change at codon 48 of the PTEN protein. The amino acid asparagine (AAC) at codon 48, which is polar and uncharged, turns to lysine (AAA), which is a positively charged amino acid, originating the missense mutation N48K. No other change was found at tumor level.

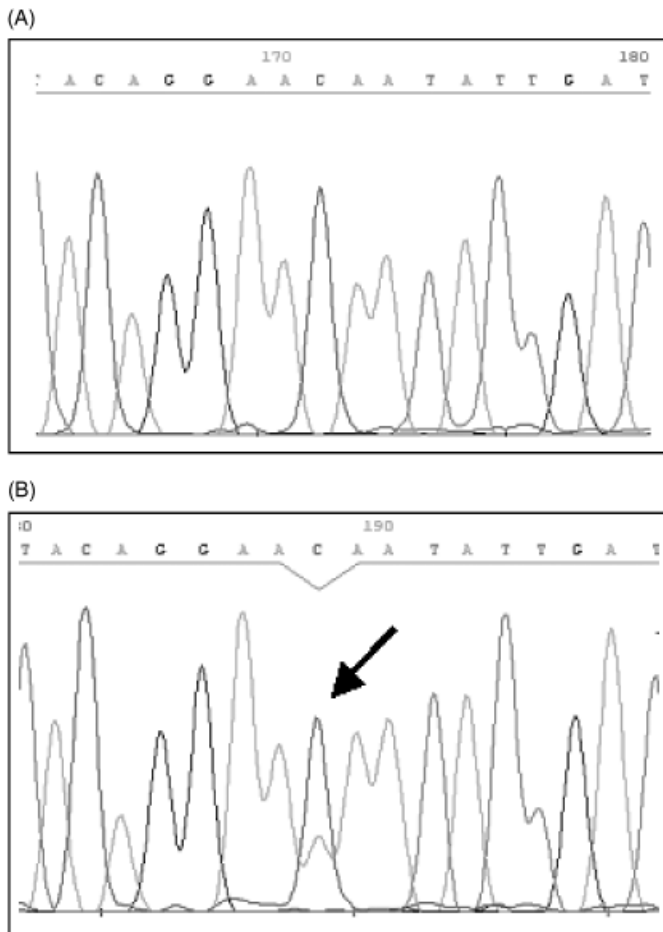
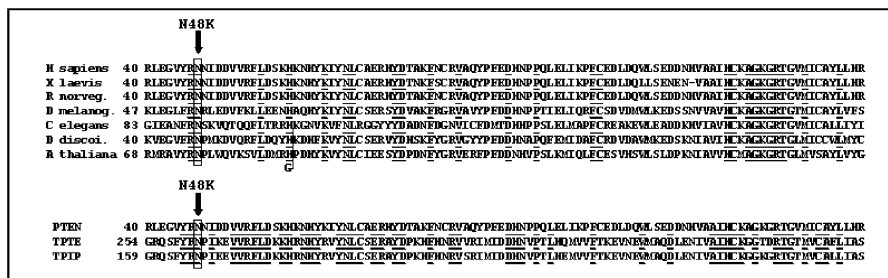


Figure 1. Nucleotide sequence of a fragment of exon 2 of the PTEN gene containing the variant (arrow). (A) Wild-type sequence. (B) Heterozygous mutation N48K.

Analysis of control individuals To discard the variant N48K as a common polymorphism, 200 chromosomes from healthy individuals of our population were genotyped by SSCP analysis. The absence of N48K in the control population suggests that the variant is not a common polymorphism and points to a disease-associated mutation.

Comparative sequence analysis As the mutation N48K is located at the N-terminal catalytic domain of the PTEN protein, in the vicinity of the enzyme active center, we carried out a comparative amino acid sequence analysis between different species to know the conservation grade of the Asn⁴⁸ residue. As shown in **Fig 2(A)**, Asn⁴⁸ is present in all of the species, suggesting an important role for this residue in PTEN function. In addition, Asn⁴⁸ is also conserved in the two PTEN-related phosphatases, TPTE and TPIP (**Fig 2B**).



LOH Biopsy specimens from an invasive ductal carcinoma of grade I and well-differentiated endometrioid carcinoma with squamous metaplasia (adenoacanthoma) were evaluated for LOH at microsatellite markers D10S215, D10S541, and D10S564, and at the PTEN nucleotide 144. All the three microsatellites were heterozygous in the blood sample of the patient as well as in both tumor samples (breast and endometrial), showing no LOH. None of the tumor types was hemizygous for the mutation at nucleotide 144.

Analysis of PKB/Akt activation To test the implications of the N48K mutation on PTEN function, the capacity of a PTEN N48K mutant protein to inhibit the activation of the proto-oncogene PKB/Akt in the U87MG PTEN-null cell line was tested (**Fig 3**). As shown, inhibition of PKB/Akt activation by the PTEN N48K mutation was impaired (**Fig 3, lanes 3, 7**), as observed for the lipid phosphatase inactive G129E mutation (**Fig 3, lanes 4, 8**) (Myers *et al*, 1998). The impairment of PTEN function in the mutant proteins was observed both in normal cell growth conditions (**Fig 3, lanes 1–4**) and on stimulation of PKB/Akt by expression of the constitutively active form of Ras, RasV12 (**Fig 3, lanes 5–8**).

DISCUSSION

In this work the study of a female diagnosed with CD led us to the identification of a new missense mutation (N48K) in exon 2 of the PTEN gene. An extensive bibliographic and database search for PTEN mutations showed that N48K is a variant not previously reported.

As the implication of each missense mutation in the development of a disease has to be confirmed, additional analyses were performed to establish the pathogenicity of N48K in CD. First, a genotype analysis of 200 chromosomes from healthy individuals was performed by SSCP analysis of exon 2; it was found that the variant was not common in our population: none of the chromosomes analyzed presented the change N48K. Second, the capacity of a PTEN N48K mutant protein to inhibit the activation of the proto-oncogene PKB/Akt was tested. The ability of the PTEN N48K mutation to inhibit PKB/Akt activation was impaired, as shown for the lipid phosphatase inactive G129E mutation (**Fig 3**), which has been associated with CD (Liaw *et al*, 1997; Myers *et al*, 1998). Thus, the N48K mutation renders a PTEN enzyme inactive, probably by altering the conformation of the nearby catalytic site and/or by making the accessibility to the phosphatidylinositol-3,4,5-triphosphate substrate difficult.

The recent observation of germline mutations of the PTEN gene in patients with CD provides strong evidence that PTEN functions as a tumor suppressor gene in this disease and related inherited disorders (Nelen *et al*, 1996; Liaw *et al*, 1997; Steck *et al*, 1997). It is well known that inactivation of a tumor suppressor gene generally involves independent inactivation of both alleles, usually by a point mutation of one allele and deletion of the other wild-type allele (Knudson *et al*, 1975). Nevertheless, in our analysis, and using markers D10S215, D10S541, and D10S564, we detected the presence of both PTEN alleles, as well as the variant N48K in heterozygous status, in breast and endometrial tumors

Figure 2. PTEN protein inter and intra specific homologies. (A) PTEN phosphatase domain amino acid sequence homology across species. Sequence homologies are underlined. Asparagine at codon 48 is indicated with a vertical rectangle. In *C. elegans* an insertion is indicated with a line and the amino acid letter. (B) Phosphatase domain amino acid sequence homology between human PTEN and human phosphatases TPTE and TPIP. Codon 48 is indicated with an arrow.

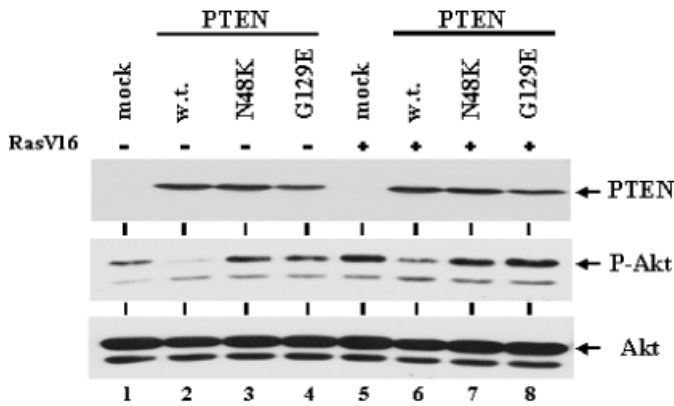


Figure 3. Phospho-active PKB/Akt in the presence of PTEN wild-type or mutations. U87MG cells were transfected with pSG5 HA-PKB/Akt plus pRK5 vector alone (mock) or pRK5 PTEN wild-type (w.t.) or mutations, as indicated. In lanes 5–8, pEF-BOS-Ras V12 was cotransfected. After 48 h, cells were lysed and HA-PKB/Akt was immunoprecipitated with the anti-HA 12CA5 monoclonal antibody, followed by immunoblotting with the antiphospho-PKB (Ser⁴⁷³) antibody (middle panel). After stripping of the membrane, immunoblotting with anti-PKB/Akt was performed (lower panel). In the upper panel, 50 μ g of total cell lysate were loaded and subjected to immunoblotting with anti-PTEN antibody.

from the patient under study. Moreover, the complete sequence analysis of PTEN exons in breast and endometrial tumor samples revealed the absence of a second point mutation. This finding is similar to that reported by Cairns *et al* (1998) who in a recent study of PTEN mutations and deletions in primary bladder cancers demonstrated a low frequency of biallelic inactivation, suggesting other mechanisms of PTEN inactivation such as hypermethylation or even the involvement of another gene. Other recent works also support the implication of abnormal methylation in the PTEN promoter region in the tumorigenesis process (Chi *et al*, 1998; Whang *et al*, 1998; Salvesen *et al*, 2001).

In summary, we report here the identification of N48K, a new germline PTEN missense mutation, in a patient suffering from CD. By both genetic and functional analysis we confirm the pathogenicity of the mutation in CD. As we did not find a second mutation at tumor level, we speculate that abnormalities of the regulatory regions of the PTEN gene not targeted by our study, such as the promoter region, transcriptional silencing (e.g., via methylation), or even haplo-insufficiency might occur in cases where a coding mutation of this gene is seen in the absence of LOH at the wild-type allele.

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