

Activation of a Cryptic Splice Site of *PTEN* and Loss of Heterozygosity in Benign Skin Lesions in Cowden Disease

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Cowden disease is an autosomal dominant syndrome characterized by facial trichilemmomas, acral keratoses, papillomatous papules, mucosal lesions, and an increased risk for breast and nonmedullary thyroid cancer. Here, we describe a novel *PTEN* splicing site mutation in a family with classical Cowden disease and we studied benign skin lesions typical for Cowden disease for loss of heterozygosity. We found a *PTEN* IVS2 + 1G > A 5'-splicing acceptor mutation resulting in activation of a cryptic splice site. Activation of this cryptic splice site is predicted to result in a frameshift with a premature stop codon,

thus disrupting the phosphatase core motif of *PTEN*. Loss of heterozygosity analysis of two trichilemmomas, one fibroma, and three acanthomas of the index patient demonstrated loss of heterozygosity at the *PTEN* locus in four of these lesions. In conclusion, our data demonstrate that a *PTEN* splicing site mutation causes activation of a cryptic splice site, which results in aberrant transcripts. **Key words:** genes, suppressor, tumor/germline mutation/hamartoma syndrome, multiple/keratosis/loss of heterozygosity/RNA splicing. *J Invest Dermatol* 117:1650–1653, 2001

Cowden disease (CD) is an autosomal dominant familial cancer syndrome characterized by the development of hamartomas in multiple organs, including skin, gastrointestinal tract, central nervous system, breast, and thyroid. Diagnostic criteria have been developed for the clinical identification of CD: facial trichilemmomas, acral keratoses, papillomatous papules, and mucosal lesions are pathognomonic criteria for CD. Breast cancer, nonmedullary thyroid cancer, macrocephaly, and dysplastic gangliocytoma of the cerebellum (Lhermitte–Duclos disease) are considered as major criteria, whereas multinodular goiter, thyroid adenoma, mental retardation, gastrointestinal hamartomatous polyposis, fibrocystic breast disease, lipomas, fibromas, and genitourinary tumors or malformation are considered as minor criteria (Nelen *et al*, 1996; Eng, 2000).

A gene responsible for CD was localized to chromosome 10q22–23 (Nelen *et al*, 1999b) and germline mutations in this gene, named *PTEN* (Li *et al*, 1997), *MMAC1* (Steck *et al*, 1997), or *TEP1* (Li and Sun, 1997), were identified in patients with CD (Liaw *et al*, 1997). *PTEN* encodes a ubiquitously expressed dual-specificity phosphatase and is mutated in a variety of sporadic malignancies (Li *et al*, 1997; Steck *et al*, 1997). *PTEN* is important for the induction of cell cycle arrest, apoptosis, but also affects cell adhesion, migration, and differentiation, and thus is considered as a tumor suppressor (Di Cristofano and Pandolfi, 2000).

In this report, we describe a novel *PTEN* 5'-splicing acceptor site mutation in a family with classical CD. We show that this mutation resulted in activation of cryptic splicing at the 3' site of exon 2. These aberrant *PTEN* transcripts are predicted to be less abundant than normally spliced *PTEN* transcripts. Furthermore, loss of heterozygosity (LOH) at the *PTEN* gene locus in benign skin lesions typical for CD was demonstrated.

MATERIALS AND METHODS

Case report A 73-y-old female patient (II.1) with a history of multinodular goiter and ductal carcinoma *in situ* of the breast was referred to our department for endoscopy because of familial intestinal polyposis. Moderate macrocephaly, facial papules, small polypoid mucosal lesions of the mouth, nasal papillomatous papules, and acral keratoses were noted. Three further members of the patient's family were also affected by mucocutaneous lesions and/or gastrointestinal polyposis (Fig 1a). Endoscopy revealed multiple hyperplastic antral, duodenal, and colorectal polyps with a maximum size of 5–10 mm. Because the diagnosis of Cowden disease was suspected, mucocutaneous lesions were biopsied and histologically classified as facial trichilemmomas, fibromas, and mucocutaneous acanthomas. After genetic counseling and written informed consent genetic testing for a *PTEN* germline mutation was initiated.

DNA extraction and mutational analysis of *PTEN* Genomic DNA was extracted from the index patient's peripheral blood mononuclear cells using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) amplification of the coding region of *PTEN* was carried out using published primer sets (Steck *et al*, 1997; Marsh *et al*, 1998). Different primers were designed for exon 1 (forward, 5'-CAGCTACCGCCAAGTCCAGAGCC-3'), exon 3 (forward, 5'-TTTTTGTTAATGGTGGCTTTTTG-3'), exon 6 (reverse, 5'-GATATGGTTAAGAAAAGTGTCC-3'), exon 8 (reverse, 5'-CAAGTACCAACCCCAAAAATG-3'), and exon 9 (forward, 5'-GATGATCATATTTGTGGGTTTC-3'; reverse, 5'-TCATGGTTGTTTATCCCTCTTG-3'). PCR products were directly sequenced

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Abbreviations: CD, Cowden disease; LOH, loss of heterozygosity.

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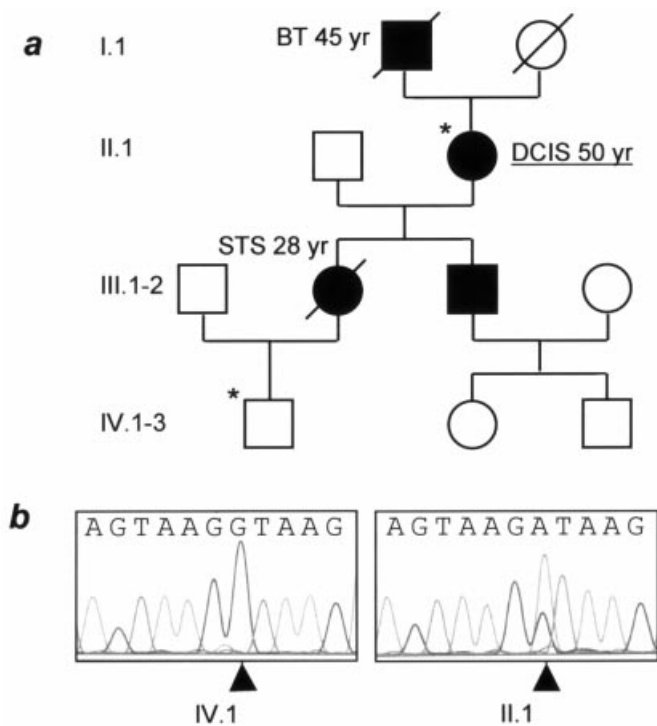


Figure 1. Mutation analysis of *PTEN*. (a) Pedigree of a family with classical Cowden disease. Phenotypically affected family members are denoted by filled symbols. Numbers indicate the age of diagnosis with a malignant tumor. Underlined, index patient; BT, brain tumor; DCIS, ductal carcinoma *in situ* of the breast; STS, soft-tissue sarcoma; *, blood available for *PTEN* mutation analysis. (b) A heterozygous *PTEN* germline mutation of the 5'-splice acceptor site of exon 2 (IVS2 + 1G > A) was identified in the index patient (II.1), whereas a phenotypically unaffected grandson (IV.1) carried only the wildtype sequence (arrowheads).

(BigDye Ready Reaction Terminators; Applied Biosystems, Weiterstadt, Germany).

Reverse transcription PCR (RT-PCR) and subcloning of *PTEN* transcripts Total RNA was extracted from peripheral blood mononuclear cells using TriStar reagent according to the manufacturer's protocol (Sigma, Deisenhofen, Germany). Because of the presence of a highly conserved *PTEN* pseudogene localized to chromosome 9p21 (Dahia *et al*, 1998; Whang *et al*, 1998), *PTEN* cDNA specific primers (forward, 5'-TTTCCATCCTGCAGAAGAAGC-3'; reverse, 5'-TAAA-TATGCACATATCATTAC-3') spanning exons 1–5 were designed. The pseudogene sequence showed a 4 bp deletion 65 bp upstream of the translation start of *PTEN* (GenBank accession: AF343912), which was used for the design of the forward primer. The reverse primer anneals to a region in exon 5 that differs between *PTEN* mRNA and *PTEN* pseudogene in three base pairs. PCR was carried out for 10 min at 95°C; followed by 45 cycles of 30 s at 95°C, 90 s at 55°C, and 2 min at 72°C; and 10 min at 72°C. RT-PCR products were cloned into pCRTM2.1-TOPOTM (Invitrogen, Leek, The Netherlands), amplified, and directly sequenced.

LOH analysis of *PTEN* in benign skin lesions The *PTEN* gene locus at chromosome 10q22–23 was studied for LOH by PCR amplification of the microsatellite markers D10S215, D10S249, and D10S541 using published primer sequences (Gyapay *et al*, 1994). DNA of paraffin-embedded tissues was extracted after microdissection from two trichilemmomas, one fibroma, and three acanthomas. PCR analysis was performed as previously described (Trojan *et al*, 2000). To confirm LOH at the *PTEN* locus in mucocutaneous lesions heterozygosity at *PTEN* exon 2 was analyzed by PCR amplification and direct sequencing.

RESULTS

Complete sequence analysis of *PTEN* of the index patient (II.1) demonstrated a heterozygous G to A transversion at the 5'-splice

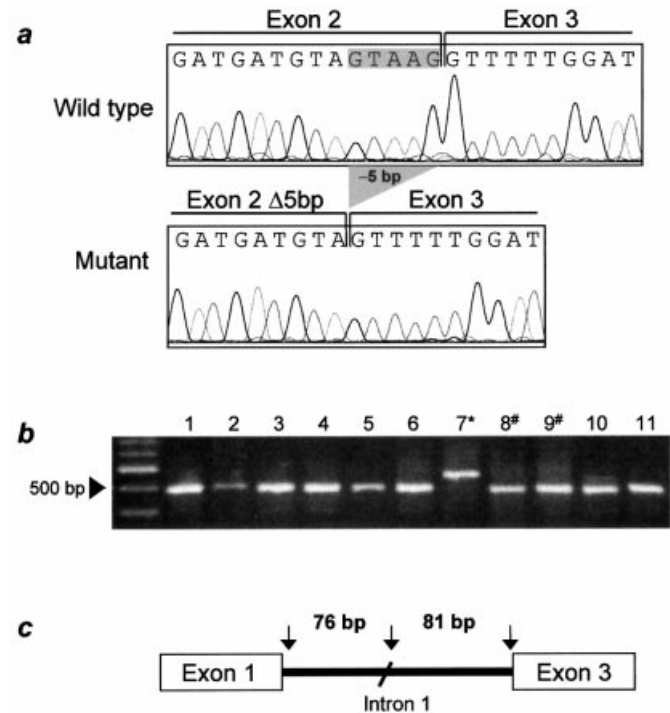


Figure 2. RT-PCR amplification and subcloning of *PTEN* transcripts from a patient with Cowden disease. (a) *PTEN* wildtype transcript sequence was found in eight of 10 clones (upper panel), but two clones carried a 5 bp deletion at the 3' site of *PTEN* exon 2 (lower panel), which is predicted to result in a truncated protein. (b) Size differences between wildtype (lanes 1–6, 10, and 11) and cryptic spliced *PTEN* transcripts (lanes 8[#] and 9[#]) were not detected. One (lane 7^{*}) out of 60 clones carried an insert of larger size. (c) Sequence analysis of this clone showed a complex rearrangement with partial retention of *PTEN* intron 1 and skipping of exon 2. The retained intronic sequence contained a 76 bp sequence of the 5' site of intron 1 followed by 81 bp of a further downstream "exon-like" sequence of intron 1.

acceptor site of exon 2 (IVS2 + 1G > A) (Fig 1b) with no further mutation found in the entire coding sequence. This mutation was absent in DNA from 20 unrelated healthy individuals. To test for possible consequences of this splicing site mutation on *PTEN* pre-mRNA splicing, RT-PCR was performed and *PTEN* transcripts were sequenced. Sequencing of the *PTEN* pseudogene was performed to design specific primers for *PTEN* transcripts. Direct sequence analysis detected only *PTEN* wildtype sequence, suggesting absence of aberrant transcripts. Because less abundant aberrantly spliced *PTEN* transcripts could not constantly be detected by dye-terminator sequencing techniques (Raizis *et al*, 2000), PCR products were cloned and sequenced. Two out of 10 clones carried a 5 bp deletion at the 3' site of *PTEN* exon 2 (Fig 2a), which is predicted to lead to a premature translational stop at codon 60, whereas none of the 20 clones from healthy probands carried this transcript. This internal deletion is anticipated to result from the described 5'-splice acceptor site mutation, which activates a cryptic splice site resembling the 5'-splicing site consensus sequence (Maquat, 1996). An additional 50 clones were PCR amplified and analyzed for further aberrant transcripts carrying larger deletions or insertions. Only one clone carried an insert of larger size (Fig 2b). Sequence analysis revealed a complex rearrangement with partial retention of *PTEN* intron 1 and skipping of exon 2 (Fig 2c).

DNA extracted from microdissected tissue sections histologically characterized as perifollicular fibroma and acanthomas, respectively, demonstrated LOH at the microsatellite marker D10S541, which is located distal to the *PTEN* locus. Two other acanthomas demonstrated LOH at the intragenic *PTEN* microsatellite marker

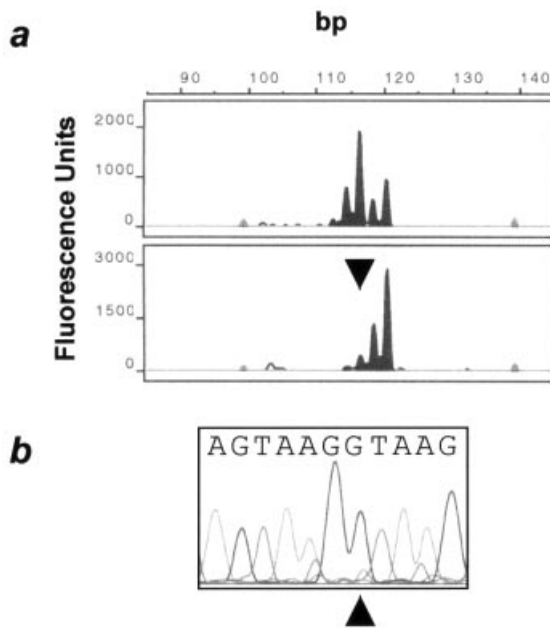


Figure 3. LOH analysis in benign mucocutaneous lesions of a patient with CD. (a) Fluorescent microsatellite analysis of the intragenic *PTEN* microsatellite marker D10S249. Compared with PCR products generated from peripheral blood mononuclear cells (upper panel) DNA extracted from a paraffin-embedded acanthoma demonstrated LOH (lower panel, arrowhead). (b) Sequencing analysis showed hemizygosity for the wildtype allele at *PTEN* IVS2 + 1, indicating loss of the mutant allele.

D10S249 (Fig 3a). Sequence analysis demonstrated hemizygosity for the wildtype allele at *PTEN* IVS2 + 1 in the two acanthomas with LOH at D10S249, indicating complete loss of the mutant allele (Fig 3b). The remaining five mucocutaneous lesions were heterozygous at *PTEN* IVS2 + 1 (data not shown).

DISCUSSION

In this study, we describe a novel *PTEN* splicing site mutation in a family with classical CD. Ten different *PTEN* splicing site mutations in patients with CD have been reported (Bonneau and Longy, 2000; Celebi *et al*, 2000). The described *PTEN* IVS2 + 1G > A splicing acceptor site mutation resulted in activation of a cryptic splice site and a complex rearrangement of *PTEN* with a partial retention of *PTEN* intron 1 and skipping of exon 2. This finding, which has not yet been described in patients with CD, is in accordance with current knowledge of splicing site defects of other genes, causing either exon skipping, activation of a cryptic splice site, intron retention as part of the mRNA, or a combination of these events (Maquat, 1996). Although splicing variants of *PTEN* have been described in healthy subjects (Sharrard and Maitland, 2000), the reported aberrantly spliced transcripts were exclusively detected in the patient with CD.

Our initial strategy to directly detect mutant *PTEN* transcripts failed, indicating that the mutated *PTEN* mRNA is not stable or cannot be detected using dye-based sequencing techniques in the presence of wildtype transcripts (Raizis *et al*, 2000). After subcloning of the RT-PCR products aberrant *PTEN* transcripts resulting from activation of a cryptic splice site were detected. The aberrantly spliced transcripts are predicted to result in a frameshift with a premature stop codon, which will probably lead to nonsense-mediated decay of the mRNA (Maquat, 1996). Because the phosphatase core motif encoded by exon 5 is disrupted by the resulting premature termination codon, we speculate that the *PTEN* phosphatase activity is abolished.

It has been established that *PTEN* acts as a tumor suppressor in different malignant tumors (Di Cristofano and Pandolfi, 2000).

Nevertheless, the role of *PTEN* in the development of benign, CD-associated epidermal lesions without potential to progress toward a malignant tumor was not previously investigated. In this study, we detected LOH at the *PTEN* locus in some acanthomas and in a perifollicular fibroma. This finding is in accordance with other studies, reporting LOH in fibroadenomas of the breast, hamartomas of the lung and stomach, juvenile colonic polyps, and colonic adenomas of patients with CD (Lynch *et al*, 1997; Chi *et al*, 1998; Marsh *et al*, 1998a). We observed that LOH at the *PTEN* locus was also associated with loss of the mutant allele in two hyperplastic skin lesions, however, which seems to be in contradiction with current ideas on tumor development in cases of *PTEN* haplo-insufficiency. Although it cannot be excluded that this finding is coincidental or artificial because of the tissue fixation process, others have also reported loss of the mutant *PTEN* allele from apparently normal tissue adjacent to a ductal carcinoma *in situ* of the breast and a thyroid adenoma in patients with CD harboring a *PTEN* germline mutation (Lynch *et al*, 1997). Studies with *Pten*^{+/-} mice suggest that in *Pten* haplo-insufficiency Fas-dependent apoptosis is impaired, which increases survival and proliferation of certain cell types leading to a hyperplastic-dysplastic phenotype (Di Cristofano *et al*, 1999). In the case of accumulation of DNA damage, including LOH at the *Pten* locus, neoplastic transformation in mice might ultimately arise from total *PTEN* loss (Di Cristofano and Pandolfi, 2000). Our finding that hyperplastic epidermal lesions in a patient with CD are associated with LOH at the *PTEN* locus, partially involving the mutant allele, further supports the role of *PTEN* insufficiency for increased proliferation of epidermal cells. It remains to be established why *PTEN* loss in these epidermal lesions is not associated with neoplastic transformation, however.

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