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A Novel Phosphatase Gene on 10q23, MINPP, in Hereditary and Sporadic Breast Cancer

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PTEN is a tumor suppressor gene on 10q23 and encodes a dual specificity phosphatase. One of the major substrates for PTEN is phosphotidylinositol (3,4,5) triphosphate in the PI3 kinase pathway. When PTEN is dysfunctional or absent, P-Akt is high and hence, anti-apoptotic. PTEN is a major susceptibility gene for Cowden syndrome (CS), a hereditary disorder with a high risk of breast and thyroid cancer, and appears to be involved in a broad range of tumors. In addition, germline PTEN mutations have been found in a developmental disorder, Bannayan-Riley-Ruvalcaba syndrome (BRR) as well. This is an autosomal dominant disorder characterised by macrocephaly, lipomatosis, hemangiomatosis and speckled penis. Previously not thought to be associated with cancer risk, BRR families and cases with germline PTEN mutations have recently been shown to be at risk for cancers and especially breast tumors. Between 10-80% (mean 60%) of CS families and 60% of BRR individuals have germline PTEN mutations. Families that do not have germline PTEN mutations are not inconsistent with linkage to the 10q22-23 region. Thus, genes with related function to PTEN in the 10q21-2q5 region are good candidates genes for PTEN mutation negative CS, BRR and related sporadic tumors, eg, those of the breast and thyroid. MINPP1 lies no more than 1 Mb upstream of PTEN and encodes an inositol polyphosphate phosphatase. In Year 1 of this award, the PI has ascertained 14 unrelated CS probands, 22 BRR probands and 20 CS-like probands known not to harbor germline PTEN mutations. To date, they have not been found to carry germline MINPP1 mutations. We found 4 malignant and 3 benign thyroid tumors to harbor deletion or intragenic mutation of MINPP1. More interestingly, IVS3+34T>A was found in about 15% of FA cases and normal controls but not in patients with FTC. These results suggest a role for MINPP1 in the pathogenesis of at least a subset of malignant follicular thyroid tumors, and that MINPP1 might act as a low penetrance predisposition allele for FTC.
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Year 1 Annual Progress Report

Proposal Title: A novel phosphatase gene on 10q23, MINPP, in hereditary and sporadic breast cancer (DAMD17-00-1-0390)

PI: Charis Eng, MD, PhD

INTRODUCTION

PTEN is a tumor suppressor gene on 10q23 and encodes a dual specificity phosphatase. One of the major substrates for PTEN is phosphatidylinositol (3,4,5) triphosphate in the PI3 kinase pathway. Downstream of this pathway lies Akt/PKB, a known cell survival factor. When PTEN is functional and abundant, Akt is hypophosphorylated and hence, pro-apoptotic. Conversely, when PTEN is dysfunctional or absent, P-Akt is high and hence, anti-apoptotic. PTEN is a major susceptibility gene for Cowden syndrome (CS), a hereditary disorder with a high risk of breast and thyroid cancer, and appears to be involved in a broad range of tumors. In addition, germline PTEN mutations have been found in a developmental disorder, Bannayan-Riley-Ruvalcaba syndrome (BRR) as well. This is an autosomal dominant disorder characterised by macrocephaly, lipomatosis, hemangiomatosis and speckled penis. Previously not thought to be associated with cancer risk, BRR families and cases with germline PTEN mutations have recently been shown to be at risk for cancers and especially breast tumors. Between 10-80% (mean 60%) of CS families and 60% of BRR individuals have germline PTEN mutations. Families that do not have germline PTEN mutations are not inconsistent with linkage to the 10q22-23 region. While breast cancer is a major component of CS and 30-50% of sporadic tumors carry hemizygous deletion in the 10q22-23 region, no or rare sporadic breast carcinomas have somatic intragenic PTEN mutations. A gene encoding a novel inositol polyphosphate phosphatase, MINPP, with overlapping function with PTEN, has been mapped to 10q23. We hypothesise that MINPP will be the susceptibility gene for the remainder of CS and BRR families and might likely be the major tumor suppressor gene on 10q23 which plays a role in the pathogenesis of sporadic breast carcinomas. We hope to explore whether MINPP is another CS and BRR susceptibility gene by looking for germline mutations in cases without germline PTEN mutations. We will also perform mutation and fine structure deletion analysis of MINPP in sporadic breast carcinomas. And finally, to prove that MINPP is a tumor suppressor and to begin to explore its relationship with PTEN in breast carcinogenesis, we will perform stable transfection experiments into two breast cancer lines with known genomic PTEN status (one PTEN wildtype and one PTEN null) as well as known PTEN protein and P-Akt levels. We will especially determine if MINPP is growth suppressive like PTEN, and determine if growth suppression is mediated by G1 arrest and/or apoptosis. Towards these ends, our specific aims were:

1. To determine if germline mutations of MINPP cause PTEN mutation negative CS, BRR and CS-like families.
2. To determine if somatic MINPP mutations and deletions are associated with sporadic breast carcinomas.
3. To determine if MINPP affects Akt activity and causes G1 arrest and/or cell death in breast cancer cell lines.
BODY

Task 1: Mutation analysis of MINPP in germline PTEN mutation negative CS, BRR and CS-like Cases
Fourteen unrelated CS probands, 22 unrelated BRR probands and 20 unrelated CS-like probands known not to harbor germline PTEN mutations have thus far been ascertained. CS and BRR were diagnosed stringently by the criteria of the International Cowden Consortium (1) and as documented previously (2), respectively. The criteria for the diagnosis of a CS-like individual or family is as previously described (3). Preliminary mutation analysis of all exons, exon-intron junctions and flanking intronic sequences of MINPP1 have been performed on these subjects. Among a total of 56 subjects, no germline MINPP1 mutations were found (4) (Eng, unpublished). Please see appended reprint for further details.

We are continuing to accrue germline PTEN mutation negative, CS, BRR and CS-like probands for MINPP analysis. In addition, we are also examining PTEN mutation negative, MINPP1 mutation negative probands for mutations in genes in the 10q22-24 interval.

Task 2: Mutation and deletion analysis of MINPP in sporadic primary human breast carcinomas
To further understand the role of MINPP in sporadic counterparts of CS component cancers, we are accruing two series of sporadic tumors, primary adenocarcinomas of the breast and primary follicular thyroid neoplasias. Currently, we have accrued 50 breast cancers and have examined the first 10 for somatic MINPP mutations. To date, no obvious pathogenic mutations have been found but N=10 is a small subset of the entire series. Accrual of breast tumors and MINPP mutation analysis continues.

We then turned to examining the sporadic counterpart of common CS component neoplasias, namely, sporadic follicular thyroid adenomas (FA) and follicular thyroid carcinomas (FTC). We analyzed DNA from tumor and corresponding normal tissue from 23 patients with FA and 15 patients with FTC for LOH and mutations at the MINPP1 locus. LOH was identified in 4 malignant and 3 benign tumors. One of these FTC’s with LOH was found to harbor a somatic c.122C>T or S41L mutation. We also found two germline sequence variants, c.809A>G (Q270R) and IVS3+34T>A. The c.809A>G variant was only found in one patient with FA but not in patients with FTC or normal controls. More interestingly, IVS3+34T>A was found in about 15% of FA cases and normal controls but not in patients with FTC. These results suggest a role for MINPP1 in the pathogenesis of at least a subset of malignant follicular thyroid tumors, and that MINPP1 might act as a low penetrance predisposition allele for FTC. See appended reprint for details.
Task 3: Functional studies of MINPP in PTEN+/+ and PTEN null breast cancer cell lines
MINPP1 cDNA constructs are being made in pCR2.1 and in the mammalian expression system pUHD10-3 which contains a tetracycline-suppressible (Tet-off) promoter, as previously described for PTEN expression constructs (5).

KEY RESEARCH ACCOMPLISHMENTS

• Germline MINPP1 mutations likely do not account for a large proportion of germline PTEN mutation negative CS, BRR and CS-like probands, although accrual is not sufficient to draw any conclusions at this time.

• Somatic MINPP1 alterations play some role in the genesis of sporadic follicular thyroid neoplasias.

• Germline MINPP1 variation may be considered low penetrance alleles for predisposition to FTC.

REPORTABLE OUTCOMES


CONCLUSIONS
The first year of work exploring MINPP as an alternative phosphatase in playing a role in the etiology and pathogenesis of CS and sporadic tumors remain inconclusive for reasons of sample size. Our work to date has shown that germline mutations in MINPP1 do not account for the majority of germline PTEN mutation negative CS, BRR and CS-like probands. However, sample size for each category is too small to draw any conclusions at present whether germline MINPP1 mutations play any role in these syndromes. As originally proposed, accrual of further PTEN mutation negative CS, BRR and CS-like individuals continues. Similarly, the somatic analysis of MINPP1 in sporadic breast carcinomas is still in its infancy with a sample size tested to date of 10. Further samples are being accrued for MINPP1 analysis. We have shown that somatic MINPP1 alterations does play a role in the pathogenesis of sporadic FA and FTC. Further, we have some evidence that MINPP1 might serve as a common low penetrance gene for susceptibility to isolated FTC.
REFERENCES
APPENDIX

Reprints of Articles:


NIH-Style Modular Biosketch
of LDLR as FH causing, as they appear to have a modest effect on LDL receptor function.

Karen Heath is a PhD student sponsored by the John Pinto Foundation and financial support is from British Heart Foundation grants (RG95007 and RG93008).

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11 Nicolaides NC, Stoeckell CJ. A simple, efficient method for the separate isolation of RNA and DNA from the same cells. Biotechniques 1990;8:154-6.

Absence of germine mutations in MINPP1, a phosphatase encoding gene centromeric of PTEN, in patients with Cowden and Bannayan-Riley-Ruvalcaba syndrome without germine PTEN mutations

Editor—Germine mutations in the dual specificity phosphatase gene PTEN (also known as MMAC1 or TEP1) have been associated with susceptibility to two related hamartomatous disorders, Cowden syndrome (CS, MIM 158350) and Bannayan-Riley-Ruvalcaba syndrome (BRR, MIM 133480). It has recently been established that PTEN functions as a 3-phosphatase towards phospholipid substrates in the phosphatidylinositol 3-kinase (PI-3 kinase) pathway. Lack of PTEN results in the accumulation of phosphatidylinositol-3,4,5-P3, which is required for activation of protein kinase B (PKB/Akt), a downstream target of PI-3-kinase and a known cell survival factor. While up to 8% of CS and approximately 60% of BRR cases have detectable PTEN germine mutations, no mutations in the coding region or exon-intron boundaries of PTEN have been found in the remaining affected subjects. Informative PTEN mutation negative families have been shown to be linked to the 10q23 region, where PTEN lies. Although recently there has been a report of two CS families in which linkage to 10q23 has been excluded. This has raised the possibility that either a regulator of the PTEN gene not included in previous studies, such as the promoter region, or another, closely located gene might be responsible for the CS and BRR cases in which no PTEN mutation has been found. The first alternative is unlikely to represent the majority of such cases, as no evidence of PTEN transcriptional silencing has been detected in the tissue of affected CS subjects in which no PTEN mutation was identified (Dahia and Eng, unpublished observations). Transcription levels of PTEN were found to be similar in affected and unaffected tissues of at least three unrelated CS patients and were equivalent to those of normal subjects. This suggests that methylation of the promoter or mutation within the promoter affecting transcription of PTEN does not occur in at least a subset of these PTEN mutation negative CS and BRR cases. To investigate the possibility that a closely mapped gene was the target of such mutations, we examined the coding region of a recently identified gene mapping to 10q23, next to D10S579, a marker estimated to lie no more than 1 Mb centromeric of PTEN. The multiple inositol polyphosphate phosphatase, known as MINPP1 or MIPP, has been cloned and shown to encode a conserved domain common to histidine phosphatases. MINPP1 codes for an approximately 52 kDa enzyme with the ability to remove the 3-phosphate from inositol phosphate substrates, such as Ins (1,3,4,5)P4, as well as other inositol moieties. It has been shown that human MINPP1 has a wide tissue distribution pattern and its subcellular localisation appears to be targeted to the endoplasmic reticulum (ER). While little is known about the human MINPP1 function, its most well studied homologue, chick HIPER1, has a more restricted tissue distribution and appears to be critical to regulate the transition
of growth plate chondrocytes from proliferation to hypertrophy. It is presumed that human MINNP1 plays a role in differentiation and apoptosis, although details on the pathways involved in such signalling are as yet unknown. Thus, owing to its chromosomal location and to the fact that, like PTEN, it encodes a phosphatase with activity towards lipid substrates, we sought to investigate whether mutations in MINNP1 would account for cases of CS and BRR without detectable PTEN mutations.

We obtained DNA from 36 subjects who met stringent criteria for the diagnosis of CS (n=14) and BRR (n=22) and in whom no PTEN mutation had been detected. In at least one of the families, linkage data were compatible with linkage of the CS phenotype with the 10q23 region. The rest of the cases were isolated or belonged to small families where linkage analysis was impossible. Informed consent was obtained from all subjects enrolled in this study, according to institutional Human Subjects Protection Committee protocols. All samples were screened for mutations in the coding region of MINNP1 and most intron-exon boundaries of the gene by PCR based (primer sequences and PCR conditions in table 1) direct sequence analysis, as previously described. No MINNP1 mutations were found in germline DNA from any of the subjects examined in the present study. In particular, no mutations were found at the highly conserved histidine phosphatase motif, RHGxxRxP, which defines members of the histidine acid phosphatase family. In addition, a second highly conserved site in this group of phosphatases comprising a histidine residue located at position 370 was found to be intact in all samples examined. This represents a proton donor site at the carboxy-terminal region of the protein which appears to be critical for full catalytic activity of this group of enzymes. We identified five variants from the reference MINNP1 sequence from the database in all samples, as well as in three normal controls (GenBank accession number AF046914). All of these sequence variants were identical to the reference MINNP2 sequence (GenBank accession number AF084943). A sixth variant, c.444A→G, was noted in all our sequences which is in agreement with the MINNP1 reference sequence, but at odds with that of MINNP2. It is likely, therefore, that these variations might represent errors in sequence entry on the database, rather than being associated with any particular phenotype, as they were identical in all samples, including the normal controls.

While described as independent hamartoma syndromes with shared clinical features until recently, it has been generally accepted that only CS bears a higher susceptibility to malignancies. A broad analysis of genotype-phenotype data in the largest series of both CS and BRR recently undertaken in our laboratory has suggested that they might in fact represent distinct spectra of the same primary disorder. These findings have clear implications for the follow up of affected subjects, in which systematic cancer surveillance is now recommended for both disorders, and not only for patients with CS.

In several human malignancies, such as breast, prostate, and thyroid cancer with loss of heterozygosity of 10q and in which no PTEN mutations have been found, it has been suggested that a region proximal to PTEN might be the main target in the tumorigenesis pathway. In several hamartoma syndromes, these studies have been replicated and extended.

This work was partially supported by the US Army Breast Cancer Research Program (to CE) and P30CA16058 from the National Cancer Institute. Bethesda, MD (Ohio State University Comprehensive Cancer Center). PLMID is a Susan G Komen Breast Cancer Research Foundation Postdoctoral Research Fellow (to CE) and O2 Fellow of the DFG.

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Mosaicism in Alport syndrome and genetic counselling

Editor—Alport syndrome is characterised by a progressive glomerulonephritis with typical ultrastructural changes in the glomerular basement membrane. The most frequent, semidominant, X linked type is the result of a variety of mutations (either point mutations or intragenic deletions) of the COL4A5 gene encoding the α5 chain of type IV collagen.

During SSCP scanning of the COL4A5 gene, a shift in a segment including exon 44 and flanking intronic sequences was found in a 19 year old proband showing typical ultrastructural changes of the glomerular basement membrane (III.3 in fig 1). Sequence analysis showed a G→C transition in the 5’ splice site of intron 44 (position 4271+1). The mutation introduced an A+1 restriction site which divided a 66 bp fragment into two fragments of 39 + 27 bp. All 18 family members were tested using this restriction assay and the mutation was found in the proband’s affected brother, his cousin, his mother, and two maternal aunts. Surprisingly, the proband’s grandmother was a normal homozygote. The proband’s grandfather was dead, but true paternity of all daughters could be (indirectly) ascertained by polymorphic markers.

In this family the mutation is associated with juvenile Alport syndrome in males, suggesting that the splicing defect results in a low level or absence of the protein, in agreement with our previous findings on genotype-phenotype correlations. Interestingly, we noted considerable clinical variability among heterozygous females (n=4), ranging from ESRD at 27 years to absence of microscopic haematuria at 37 years.

Our data strongly suggest mosaicism in the germ cells of either grandparent. Mosaicism in germ cells may be the result of either a mutation in a germ cell that thereafter undergoes mitotic divisions (giving rise to mosaicicism confined to germ cells), or an early postzygotic mutation before separation of the somatic/germ cells (giving rise to mosaicism in both the tissues and germine). In the latter case, the phenotype may or may not be expressed in the mosaic subjects, depending on the proportion of mutant cells in the relevant tissues. In order to verify mosaicism in somatic tissues of the living grandmother (I.1 in fig 1), we used Amplification Refractory Mutation System (ARMS-PCR), a tool able to detect known mutations even when present in a low fraction of template molecules. The primer sense for exon 44 was used in combination with the following specific antisense primers: normal (5'-GGTATAACTCTTCGAGAATAAGCTTAC-3') and mutant (5'-GGTATAACTCTTCGAGAATAAGCTTG-3'). We performed ARMS-PCR on DNA extracted from grandmaternal peripheral blood using progressively lower stringency by lowering the temperature or increasing the PCR cycle number or both, with the aim of reaching a condition where even the very few mutated molecules present in the blood sample would be amplified. This condition was never reached, as the grandmother’s DNA always gave the same results as normal homzygous female controls (data not shown).

On analysis of Xq22 DNA polymorphisms, the three carrier females in the second generation were homozygous for one of the maternal haplotypes, which therefore must have been present in the dead grandfather as well, while the single non-carrier female and the unaffected male carried the other maternal haplotype. These data might suggest that the mutation was present in the grandmaternal gonads on
Somatic Mutation and Germline Variants of MINPP1, a Phosphatase Gene Located in Proximity to PTEN on 10q23.3, in Follicular Thyroid Carcinomas*

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ABSTRACT
Various genes have been identified to play a role in the pathogenesis of follicular thyroid tumors. Cowden syndrome is the only known familial syndrome with an increased risk of both follicular thyroid adenoma (PTA) and carcinoma (PTC). Germline mutations in the tumor suppressor gene PTEN, which encodes a dual-specificity phosphatase, have been found in up to 80% of patients with Cowden syndrome suggesting a role of PTEN in the pathogenesis of follicular thyroid tumors. Although somatic intragenic mutations in PTEN, which maps to 10q23.3, are rarely found in follicular tumors, loss of heterozygosity (LOH) of markers within 10q22-24 occurs in about 25%. Recently, another phosphatase gene, MINPP1, has been localized to 10q23.3. MINPP1 has the ability to remove 3-phosphate from inositol phosphate substrates, a function that overlaps with that of PTEN. Overexpression of MINPP1 might act as a low penetrance predisposition allele for PTC in patients with follicular thyroid tumors, we considered it to be an excellent candidate gene that could contribute to the pathogenesis of follicular thyroid tumors. We analyzed DNA from tumor and corresponding normal tissue from 23 patients with PTA and 15 patients with PTC for LOH and mutations at the MINPP1 locus. LOH was identified in four malignant and three benign tumors. One of these PTCs with LOH was found to harbor a somatic c.122C > T or S41L mutation. We also found two germline sequence variants, c.809A > G (Q270R) and IVS3 + 34T > A. The c.809A > G variant was found in only one patient with PTA but not in patients with PTC or normal controls. More interestingly, IVS3 + 34T > A was found in about 15% of PTA cases and normal controls but not in patients with PTC. These results suggest a role for MINPP1 in the pathogenesis of at least a subset of malignant follicular thyroid tumors, and MINPP1 might act as a low penetrance predisposition allele for PTC. (J Clin Endocrinol Metab 86: 1801-1805, 2001)

FOLLICULAR THYROID TUMORS are a common finding in iodine-deficient areas. By far, the most common tumors are benign follicular thyroid adenomas; only a minority of the tumors are carcinomas. Until today, it is unknown whether an adenoma-carcinoma sequence exists. Data supporting both theories exist (1-3).

The only known familial syndrome with an increased risk of both benign and malignant follicular thyroid tumors is Cowden syndrome (4). Germline mutations of PTEN, encoding a dual-specificity phosphatase, are found in up to 80% of patients with Cowden syndrome (5, 6), 60% of patients with Bannayan-Riley-Ruvalcaba syndrome (7), and an unknown proportion of patients with a Proteus-like syndrome (8). Although somatic intragenic PTEN mutations are found in only a minority of sporadic follicular thyroid carcinomas (9, 10), loss of heterozygosity (LOH) of markers within 10q23, especially including marker D10S579, has been found in up to 25% of either benign or malignant follicular tumors (9-11). In another study, fine structure deletion analysis of 10q22-24 demonstrated regions of loss that suggest that follicular adenomas and carcinomas develop along distinct parallel neoplastic pathways (11).

A new gene, MINPP1 (multiple inositol polyphosphate phosphatase), has recently been localized to 10q23.3 in close proximity to marker D10S579 (12). MINPP1, also known as MIPP, has been shown to encode a conserved domain common to histidine phosphatases (12, 13). This 52-kDa enzyme has the ability to remove 3-phosphate from inositol phosphate substrates, such as Ins(1,3,4,5)P_4, a function that overlaps that of PTEN even though the sequence similarity of PTEN and MINPP1 is only about 16%. MINPP1 is the only
enzyme known to hydrolyze the abundant metabolites inositol pentakisphosphate and inositol hexakisphosphate. Little is known about human MINPP1. It has been shown, however, to be expressed in a wide variety of tissues, including the human thyroid (Gimm, O., and C. Eng, unpublished data). Because of MINPP1's overlapping function with PTEN and its physical location within a region of LOH for thyroid tumors, it is an excellent candidate gene that could contribute to thyroid tumorigenesis.

Here, we report the results of mutation analysis of MINPP1 in benign and malignant follicular thyroid tumors from an iodine-deficient area. Our data might tentatively suggest a role of MINPP1 in the tumorigenesis of at least a subset of malignant follicular thyroid tumors.

Materials and Methods

Patients and specimens

Paraffin blocks from 38 selected benign (n = 23) and malignant (n = 15) follicular thyroid tumors were ascertained from Germany and Switzerland. Three malignant tumors were classified as Hürthle cell carcinoma. All samples were obtained with informed consent. In all 38 samples, tumor tissue and corresponding normal tissue (either normal thyroid tissue from a different block or from an area not in proximity to the tumor, or adjacent muscle tissue distant to the tumor site) were available for extraction of paired somatic and "germline" genomic DNA. DNA extraction following microdissection was performed using standard protocols (14).

Mutation analysis

PCR amplification using genomic DNA as template was carried out in 1X PCR buffer (Perkin-Elmer Corp., Norwalk, CT) containing 200 μM dNTP (Life Technologies, Inc., Gaithersburg, MD), 1 μM of each primer (see Table 1), 2.5U Tag polymerase (QIAGEN, Valencia, CA), 0.9 mM MgCl₂, 1X Q-buffer (QIAGEN), and 50–100 ng of tumor DNA template in a 50 μl volume. PCR conditions were 35 cycles of 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C followed by 10 min at 72°C. All exons were at least divided into two (a and b) because of their large sizes. Exon 1 had to be divided into three fragments (a–c).

Mutation analysis for exon 2 (fragments 2a and 2b), exon 3 (fragments 3a and 3b), and exon 6 (fragments 6a and 6b) was performed with DGGE. Exon 1 and exon 4 of MINPP1 are very GC rich and therefore less suitable for DGGE. Hence, mutation analysis for these two exons was performed using SSCP (fragments 1b, 1c, 4a, and 4b). No optimal SSCP condition could be found for the 5' part of exon 1 (fragment 1a) and hence was subjected to direct semi-automated sequence analysis as previously described (5, 15, 16).

Before DGGE, 10 μl of the resulting PCR product were added to 1 μl of Ficoll-based loading buffer. This mixture was loaded onto 10% polyacrylamide gels carrying a 15–65% urea-formamide gradient and a 2–5% glycerol gradient in 0.3 X TAE. The amplicons were electrophoresed at 60°C and 105 V for 16 h. The fragments were visualized with ultraviolet transillumination after staining with ethidium bromide solution (15 μl in 500 ml dH₂O) for 30 min.

Before SSCP, 2 μl of the resulting PCR product were added to 3 μl of formamide buffer and then heated to 95°C for 10 min and subsequently cooled on dry ice. Immediately before SSCP, the samples were quickly thawed and then run through a 10% polyacrylamide/1X TBE gel. Gels were run either at 100 V for 14 h at room temperature (fragments 1b, 4a, 4b) or at 150 V for 16 h at 4°C (fragment 1c). Subsequent silver staining was performed as previously described (17).

Although DGGE is 100% sensitive and specific in this and other laboratory's hands (18–20), SSCP is acknowledged not to have the same high sensitivity and specificity (21). Routine quality control for both SSCP and DGGE in our laboratory takes the form of subjecting a known positive and known negative to electrophoresis along with the test samples. Further, three random SSCP negative samples are subjected to direct sequence analysis.

If variant DGGE/SSCP banding patterns were observed, the remaining PCR aliquot was subjected to purification and semi-automated sequencing using the above primers and dye terminator technology (see above). If sequencing revealed a variant, the corresponding germline DNA was examined in the same manner to determine whether the sequence variant is somatic or germline.

The frequencies of these sequence variants in patients with follicular thyroid tumors and in a race-matched control group were determined

**TABLE 1. MINPP1 primer sequences and PCR-product sizes**

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Primer name</th>
<th>Primer sequence (5' to 3')</th>
<th>PCR-product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-a</td>
<td>MINPP1-10F&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CGGCCTGCAGCTGTATAC</td>
<td>241</td>
</tr>
<tr>
<td></td>
<td>MINPP1-260R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CGCTGCCGAGTGCCTA</td>
<td></td>
</tr>
<tr>
<td>1-b</td>
<td>MINPP1-189F&lt;sup&gt;a&lt;/sup&gt;</td>
<td>AACCTGCTGCATGTGTCG</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>MINPP1-488R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CTCGCATATCTGTCGCG</td>
<td></td>
</tr>
<tr>
<td>1-c</td>
<td>MINPP1-444F</td>
<td>AGTGGAGCTCGGAGCGTC</td>
<td>251</td>
</tr>
<tr>
<td></td>
<td>MINPP1-1.1R</td>
<td>AGAGCCGCGACGACGCA</td>
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</tr>
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<td>2-a</td>
<td>MINPP1-1.2F</td>
<td>CGCTGCTGCGGATGATGGAG</td>
<td>292</td>
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<td></td>
<td>MINPP1-GC-2a&lt;sup&gt;b&lt;/sup&gt;</td>
<td>TCTGCTTCAGAAGTGGCG</td>
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<td>2-b</td>
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<td>TGAGCTTCAAGCACTTAAGTA</td>
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<td>3-a</td>
<td>MINPP1-I.2R</td>
<td>TACCTATGTGTTCGATTCC</td>
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<td>MINPP1-I-3F</td>
<td>TCCCAAGTAAAGTATTGCC</td>
<td>244</td>
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<td>3-b</td>
<td>MINPP1-GC-3a&lt;sup&gt;b&lt;/sup&gt;</td>
<td>TCAAACCATCAGAAGAGG</td>
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<td>MINPP1-GC-3b&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CTGCTGAGTGCCGCAA</td>
<td>185</td>
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<tr>
<td>4-a</td>
<td>MINPP1-1.3R</td>
<td>AAGCTGAGTGCCGAAGGAG</td>
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<td>MINPP1-1.4F</td>
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<td>177</td>
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<td>4-b</td>
<td>MINPP1-1071R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CTGCTTATGCGCGGAGG</td>
<td></td>
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<tr>
<td>6-a&lt;sup&gt;c&lt;/sup&gt;</td>
<td>MINPP1-1027F&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GCCTGCGCTGCGGAGGAT</td>
<td>251</td>
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<tr>
<td>6-b&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>TCCTGCGCTGCGGAGG</td>
<td></td>
</tr>
<tr>
<td>6-b&lt;sup&gt;c&lt;/sup&gt;</td>
<td>MINPP1-1538R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GCAGTCTAATGCTGACTC</td>
<td>298</td>
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</table>

<sup>a</sup> The number refers to the number of the position of the 5' end of the primer in the sequence available under accession number AF084943; it is not equal to the nucleotide number within the translated coding region.

<sup>b</sup> These primers have in addition a GC-rich clamp (5'-CGCGCGGCGGCGGCGGCGGCGGCGTCCGCGCGGCGGCCGCGGCGGCGGCCGCG-3') on their 5' end in order to perform DGGE mutation analysis.

<sup>c</sup> In the chick MINPP1 homolog, HiPER1, an extra exon, dubbed exon 5, and not seen in humans, precedes the final exon, named therefore exon 6.
using peripheral blood leukocyte DNA. This race-matched control
group consisted of patients who were admitted to the Department of
General Surgery, Halle, Germany, for nonthyroid-related diseases. In-
formed consent was given in all cases.

**LOH analysis**

For every germline-tumor pair, PCR reactions were carried out using
0.6 μM each of forward and reverse primer in 1x PCR buffer (QIAGEN),
4.5 mM MgCl₂ (QIAGEN), 1x Q-buffer (QIAGEN), 2.5 U HotStarTag
polymerase (QIAGEN), and 200 μM dNTP (Life Technologies, Inc.) in a
final volume of 50 μL. Reactions were subjected to 35 cycles of 94°C
for 1 min, 55–60°C for 1 min and 72°C for 1 min followed by 10 min at 72
°C. LOH analysis for each germline-tumor pair was performed as pre-
aviously described using markers flanking MINPP1, D10S541 (telomeric),
D10S2491 (telomeric) (5, 22), and D10S1686 (centromeric) as well as the
marker D10S579 that lies in close proximity to MINPP1 (12). All forward
primers were 5’-labeled with either HEX or 6-FAM fluorescent dye
(RESEARCH Genetics, Inc., Huntsville, AL).

**Statistical analysis**

Differences in allele frequencies were calculated using the standard
Chi-square test. A P value less than 0.05 was considered significant.

**Results**

Mutation analysis of all 5 exons of MINPP1 from 38 follic-
ular thyroid tumors revealed variants in 3 exons (frag-
ments 1a, 2b, and 3b). Sequencing revealed one sequence
variant each (Table 2). Corresponding germline DNA was
examined for the presence of each of these variants.

We detected a sequence variant in one carcinoma,
c.122C > T (S41L), in the 5’ end of exon 1 (fragment 1a) (Fig.
1A). This variant was absent in the corresponding germline
(DNA from muscle) (Fig. 1B) and most likely represents a
somatic missense mutation. Repeat PCR and sequencing
confirmed the variant and excluded PCR errors. Thus, somatic
S41L was found in 1 out of 15 carcinomas (7%).

The variants in exon 2 (fragment 2b), c.809A > G (Q270R)
(Fig. 1C and D) and fragment 3b, IVS3 + 34T > A (Fig. 1, F and G)
were also present in the germline (data not shown).
The heterozygous c.809A > G variant was seen in a patient
with follicular thyroid adenoma and was never seen in pa-
patients with follicular thyroid carcinoma or in a race-matched
control group (Fig. 1E and Table 2a).

The germline IVS3 + 34T > A variant was underrepre-
resented in cases with follicular thyroid carcinoma (0%), com-
pared with those with adenoma (15%; P < 0.03, Table 2b) or
normal controls (14%; P < 0.04, Table 2b).

LOH analysis within 10q22–24 was performed for all
tumor-germline pairs. We found LOH in seven follicular
tumors, four carcinomas (27%), and three adenomas (13%).
None of the seven follicular adenomas with IVS3 + 34T > A
had LOH. Interestingly, the one carcinoma harboring the
somatic mutation S41L showed LOH at D10S579, and the
flanking markers D10S2491 and D10S1686 were not
informative.

**Discussion**

In the present study, we detected a somatic S41L mutation in
MINPP1 together with loss of the corresponding wild-type allele
in one follicular thyroid carcinoma. We also found two
previously unreported germline sequence variants in
MINPP1; one, an intronic variant, is underrepresented in
cases with follicular thyroid carcinomas, compared with
those with follicular thyroid adenomas or normal controls.

The somatic mutation c.122C > T in tumor DNA from one
patient with follicular thyroid carcinoma changes serine, a
neutral and polar amino acid, at position 41, to leucine, which
is also neutral but hydrophobic. This region is highly con-
served among several species (human, rat, mouse) (12, 13).
Hence, one can speculate that this polar for hydrophobic
amino acid substitution changes the structure of MINPP1.
Postulating that MINPP1 might act as a tumor suppressor, its
functional activity might subsequently be lost or at least
decreased. However, functional analysis would be necessary
to confirm this premature hypothesis. Nonetheless, loss of the
respective wild-type allele in this sample lends credence
that the somatic S41L mutation is pathogenic and both
MINPP1 alleles inactivated.

The finding of a rare germline sequence variant in one
patient with follicular thyroid adenoma is intriguing. This
variant was neither observed in 78 control alleles nor found
in 36 patients with Cowden syndrome or Bannayan-Riley-
Ruvalca's syndrome (23). One may speculate that this vari-
ant, which leads to substitution of a neutral and polar amino
acid for a basic amino acid, affects the function of MINPP1.
Whether this hypothetical change in MINPP1 function plays
a role in the pathogenesis of follicular thyroid adenomas
must remain unresolved at this point. Histological appear-
ance did not show any unusual features. Also, there was no
family history of follicular thyroid adenomas, but no germ-
line DNA was available from any relative.

The absence of the relatively frequent intronic polymorph-
omic sequence variant IVS3 + 34T > A in follicular thyroid
carcinoma patients is intriguing. Even though our numbers
are small, at least one or two follicular thyroid carcinomas
harboring this sequence variant should have been detected:
power calculations reveal that if only 10% of 30 alleles have
this variant, our power to detect this in at least one case
would exceed 0.92. We also screened 30 patients with breast
cancer for variation in MINPP1 and found about the same
frequency (12%) of this polymorphism as found in patients
with FA (15%) and controls (14%) (Gimmel, O., and C. Eng, un-
published data). Of note, this intronic polymorphism lies
on the border of a poly-T/poly-A/poly-T tract. There is some

**TABLE 2. Allele frequency of MINPP1 polymorphic sequence
variants in patients with follicular thyroid adenoma and follicular
thyroid carcinoma and race-matched controls**

<table>
<thead>
<tr>
<th>Codon</th>
<th>Nucleotide (amino acid)</th>
<th>(a) Exon 2</th>
<th>FA</th>
<th>FTC</th>
<th>Controls</th>
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<tbody>
<tr>
<td>270</td>
<td>c.809G (Arg)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>270</td>
<td>c.809A (Gln)</td>
<td>45</td>
<td>30</td>
<td>78</td>
<td></td>
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</table>

FA vs. FTC, P = n.s.; FA vs. controls, P = n.s.

<table>
<thead>
<tr>
<th>Codon</th>
<th>Nucleotide (amino acid)</th>
<th>(b) Exon 3</th>
<th>FA</th>
<th>FTC</th>
<th>Controls</th>
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</thead>
<tbody>
<tr>
<td>N/A</td>
<td>IVS3 + 34A (N/A)</td>
<td>7</td>
<td>0</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>N/A</td>
<td>IVS3 + 34T (N/A)</td>
<td>39</td>
<td>30</td>
<td>57</td>
<td></td>
</tr>
</tbody>
</table>

FTC vs. FA, P < 0.03; FTC vs. controls, P < 0.04. n.s., not signif-
ificant; N/A, not applicable; FA, follicular thyroid adenoma; FTC, fol-
licular thyroid carcinoma.
evidence that poly-N tracts may play important roles in RNA splicing and processing (24, 25). Recently, it has become more evident that development of a cancer can result from an interplay of either a few "high penetrance" mutations in key genes or from several, or many, sequence variants presently of unknown significance. For example, overrepresentation of a rare sequence variant of RET has been observed in patients with sporadic medullary thyroid carcinoma (26). Similar observations have been made for polymorphic sequence variants of RET in patients with HSCR (27, 28), Cul2 in sporadic pheochromocytomas (29), and PPARgamma in isolated gliblastoma multiforme cases (30). However, the precise mechanism to explain how the intronic sequence variant could "protect" or at least lower the chance of developing follicular thyroid carcinoma is unknown and open to speculation. Possibly, this intronic change affects a splice-donor or splice-acceptor site or enhances a cryptic splice site, which would subsequently lead to a different protein. Unfortunately, complementary DNA was not available to test this hypothesis. The absence of this polymorphism in patients with follicular thyroid carcinoma may also support the hypothesis that follicular thyroid adenomas and carcinomas do not adhere to an adenoma-carcinoma sequence. Fine-structure deletion mapping of 10q22–24 also suggests that sporadic follicular thyroid adenomas and follicular thyroid carcinomas develop along distinct neoplastic pathways (11).

In conclusion, our observations suggest a role for MINPP1 in the tumorigenesis of malignant follicular thyroid carcinoma. Although it may infrequently contribute to follicular carcinogenesis via the traditional pathway of somatic high penetrance, two-hit (31) mutations, this gene seems to harbor a variant that could act as a common low penetrance susceptibility allele for follicular thyroid carcinoma. Further, the DGGE and SSCP conditions reported here together with the knowledge of the frequency of various sequence variants may help in future mutation analyses of DNA from other cancers with LOH in the 10q23 region in which PTEN does not seem to play a major role, such as head and neck carcinomas, lung cancer, and melanomas (32).

References


SOMATIC MUTATION AND GERMLINE VARIANTS OF MINPP1


20. Dahia PLM, Aguilar RCJ, Albert J, et al. 1999 PTEN is inversely correlated with the cell survival factor PKB/Akt and is inactivated by diverse mechanisms in haematologic malignancies. Hum Mol Genet. 8:185–193.
BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2. Photocopy this page or follow this format for each person.

<table>
<thead>
<tr>
<th>NAME</th>
<th>POSITION TITLE</th>
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<tbody>
<tr>
<td>ENG, Charis, MD, PhD</td>
<td>Associate Professor of Medicine &amp; Human Genetics</td>
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EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

<table>
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<th>INSTITUTION AND LOCATION</th>
<th>DEGREE (if applicable)</th>
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<tr>
<td>University of Chicago, Chicago, IL</td>
<td>B.A.</td>
<td>1978-82</td>
<td>Biological Sci</td>
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<tr>
<td>University of Chicago, Chicago, IL</td>
<td>Ph.D.</td>
<td>1982-86</td>
<td>Development. Bio</td>
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<tr>
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<td>M.D.</td>
<td>1982-88</td>
<td>Medicine</td>
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<tr>
<td>University of Cambridge, Cambridge, UK</td>
<td>(Post-Doc)</td>
<td>1992-95</td>
<td>Cancer Genetics</td>
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RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds three pages, select the most pertinent publications. DO NOT EXCEED THREE PAGES.

**Professional Positions**

1988-91 Residency in Internal Medicine, Beth Israel Hospital, Boston, MA
1991-94 Clinical Fellowship, Medical Oncology, Dana-Farber Cancer Institute, Boston, MA
1992-95 CRC Dana-Farber Fellowship in Human Cancer Genetics, University of Cambridge, UK
1992-95 Senior Registrar in Clinical Cancer Genetics, University of Cambridge Addenbrooke’s Hospital, Cambridge, UK and Royal Marsden Hospital, London, UK
1994-95 Instructor in Medicine, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA
1995-98 Assistant Professor of Medicine, Dana-Farber Cancer Institute and Harvard Medical School, Boston
1995-98 Active Staff Physician, Adult Oncology, Dana-Farber Cancer Institute, Boston
1995-98 Associate Physician, Brigham and Women’s Hospital, Boston
1998- North American Editor and Cancer Genetics Editor, Journal of Medical Genetics
1999- Associate Professor (with tenure) of Medicine, The Ohio State University, Columbus, OH
1999- Director, Clinical Cancer Genetics Program, James Cancer Hospital and Solove Research Institute, Comprehensive Cancer Center, Ohio State University, Columbus, OH

**Awards and Other Professional Activities**

1982 Phi Beta Kappa
1982 Sigma Xi Associate Membership and Sigma Xi Prize
1987 Sigma Xi Promotion to Full Membership
1988 Alpha Omega Alpha
1995 First Lawrence and Susan Marx Investigator in Human Cancer Genetics, Dana-Farber Cancer Institute
1997-99 National Cancer Institute of Canada Panel J Member
1997-99 MBY3 Study Section, Department of Defence Breast Cancer Research Program Grants
1998 NCCN Genetics/High Risk Screening Panel
1999 American College of Physicians, Promotion to Fellowship
2001 American Society for Clinical Investigation, Elected Membership

**Selected Research Projects On-Going or Completed In Last 3 Years**

*“Genetics of PTEN in Cowden syndrome and sporadic breast cancer”*

**PI:** Charis Eng, MD, PhD

**Agency:** American Cancer Society

**Research Project Grant (RPG-98-211-01-CCE) 7/1/98-6/30/01**

The goal of this project is to determine genotype-phenotype associations in Cowden syndrome and to determine if low penetrance mutations in PTEN predispose to apparently isolated breast cancer.
"Genetics of PTEN in Cowden-like syndromes"
PI: Charis Eng, MD, PhD
Agency: Department of Defence
Idea Award (DAMD17-98-1-8058) 10/1/98-9/30/01
The goal of this project is to genetically delineate the function of PTEN in non-Cowden syndrome hereditary breast cancer and multiple primaries. The focus will be on breast and thyroid as well as breast and uterine cancer cases and families that do not meet the Consortium diagnostic criteria for Cowden syndrome.

"Dissecting out the bifurcation of lipid and protein phosphatase activities in PTEN-mediated growth arrest in a breast cancer model"
PI: Charis Eng, MD, PhD
Agency: Susan G. Komen Breast Cancer Research Foundation
Grant 10/1/00-9/30/02
The goal of this project is to determine the pathways downstream of PTEN's lipid and protein phosphatase activities as they related to breast carcinogenesis

"Genetic analysis of the role of the microenvironment in epithelial tumor progression"
PI's: Charis Eng, MD, PhD, Gustavo Leone, PhD and Michael C. Ostrowski, PhD
Agency: V Foundation Jimmy V Golf Classic Research Award 3/1/01-2/28/04
The goal of this award is to provide seed moneys to gather preliminary data and make reagents related to tumor-microenvironmental interactions so that a group grant, e.g. PPG, may result from such work, as well as novel targets for therapy and prevention

"RET complex polymorphisms in Hirschsprung disease"
PI: Charis Eng, MD, PhD
Agency: National Institutes of Health R01 Research Project Grant 7/1/01-6/30/05
The goal of this project is to identify and characterise common low penetrance alleles within RET and the genes which encode its ligands and co-ligands in "sporadic" medullary thyroid carcinoma as well as sporadic Hirschsprung disease

Selected Publications (selected from a total of 167 published and in press peer reviewed original articles)


