Germline Epigenetic Regulation of KILLIN in Cowden and Cowden-like Syndrome

Kristi L. Bennett, PhD
Jessica Mester, MS, CGC
Charis Eng, MD, PhD

Context Germline loss-of-function phosphatase and tensin homolog gene (PTEN) mutations cause 80% of Cowden syndrome, a rare autosomal-dominant disorder (1 in 200,000 live births), characterized by high risks of breast, thyroid, and other cancers. A large heterogeneous group of individuals with Cowden-like syndrome, who have various combinations of Cowden syndrome features but who do not meet Cowden syndrome diagnostic criteria, have PTEN mutations less than 10% of the time, making molecular diagnosis, prediction, genetic counseling, and risk management challenging. Other mechanisms of loss of function such as hypermethylation, which should result in underexpression of PTEN or of KILLIN, a novel tumor suppressor transcribed in the opposite direction, may account for the remainder of Cowden syndrome and Cowden-like syndrome.

Objective To determine whether germline methylation is found in Cowden syndrome or Cowden-like syndrome in individuals lacking germline PTEN mutations.

Design, Setting, and Participants Nucleic acids from prospective nested series of 123 patients with Cowden syndrome or Cowden-like syndrome and 50 unaffected individuals without PTEN variants were analyzed for germline methylation and expression of PTEN and KILLIN at the Cleveland Clinic, August 2008-June 2010. Prevalence of component cancers between groups was compared using the Fisher exact test.

Main Outcome Measures Frequency of germline methylation in PTEN mutation-negative Cowden syndrome and Cowden syndrome-like individuals. Prevalence of component cancers in methylation-positive and PTEN mutation-positive individuals.

Results Of 123 patients with Cowden syndrome or Cowden-like syndrome, 45 (37%; 95% confidence interval [CI], 29%-45%) showed hypermethylation upstream of PTEN but no transcriptional repression. The germline methylation was found to transcriptionally down-regulate KILLIN by 250-fold (95% CI, 45-14 286; \( P = .007 \)) and exclusively disrupted TP53 activation of KILLIN by 30% (95% CI, 7%-45%; \( P = .008 \)). Demethylation treatment increased only KILLIN expression 4.88-fold (95% CI, 1.4-18.1; \( P = .05 \)). Individuals with KILLIN-promoter methylation had a 3-fold increased prevalence of breast cancer (35/42 vs 24/64; \( P < .0001 \)) and a greater than 2-fold increase of kidney cancer (4/45 vs 6/155; \( P = .004 \)) over individuals with germline PTEN mutations.

Conclusions Germline KILLIN methylation is common among patients with Cowden syndrome or Cowden-like syndrome and is associated with increased risks of breast and renal cancer over PTEN mutation-positive individuals. These observations need to be replicated.
Because of similar function to PTEN, we investigated KILLIN as a predisposition gene in patients with Cowden syndrome or Cowden-like syndrome.

Epigenetic alterations play an important role in cancer progression through hypermethylation and silencing of tumor suppressor genes, and somatic PTEN hypermethylation has been recognized as a means of PTEN down-regulation in a subset of malignancies. We sought to address the hypothesis that germline methylation of the 10q23.31 bidirectional promoter CpG island (a region of at least 200 base pairs [bp] with a GC content of ≥50% and an observed and expected CpG ratio of >60%) silences PTEN, KILLIN, or both. This, consequently, would account for patients with Cowden syndrome or Cowden-like syndrome features but without germline PTEN mutations or deletions.

**METHODS**

**Patients**

Between October 2005 and December 2009, 2000 patients with Cowden syndrome or Cowden-like syndrome were prospectively enrolled mainly regionally and also nationally by the Cleveland Clinic Genomic Medicine Institute in accordance with research protocol (IRB8458-PTEN) and approved by the respective institutional review boards for human subjects protection. All research participants provided written informed consent. To be enrolled in the IRB8458-PTEN, individuals are eligible if they meet the full Cowden syndrome or Cowden-like syndrome diagnostic criteria.

**Figure 1. Schematic of the Genomic Structure of the KILLIN and PTEN Genes on Chromosome 10**

The region analyzed for DNA methylation is indicated, with the numbers showing the location of the bisulfite polymerase chain reaction product with respect to the translation start site (mRNA [messenger RNA]) of the PTEN gene. As depicted, the KILLIN promoter overlaps with the 5′UTR (untranslated region) and coding region of PTEN, bp indicates base pair.
syndrome diagnostic criteria established by the International Cowden Consortium (ie, major criteria include breast cancer, thyroid cancer, macrocephaly, endometrial carcinoma, Lhermitte-Duclos disease) according to version 2000 (eTable 1, available at http://www.jama.com).15 Patients meeting the relaxed criteria are referred to as individuals with Cowden-like syndrome phenotypes (or CSL).

Of the 2000 prospectively enrolled participants meeting the criteria for protocol IRB8458-PTEN, fewer than 400 lacked germline PTEN pathogenic mutations, large deletions, variants of unknown significance, and polymorphisms by sequencing analysis of all 9 exons and the promotor. Of these 400, we selected a nested series of the most recent 123 participants who also were found not to have SDHB/D variation, regardless of family history status, comprising 48 with Cowden syndrome, 75 with Cowden-like syndrome, and 50 unaffected individuals (population controls resident in the region), for the purposes of this study. Sample sizes were selected to ensure power (P > .90) to detect a 5% prevalence of the methylation, as well as to detect a 3-fold difference between case and control participants.

All specimens from study and control participants were prepared and analyzed within the Genomic Medicine Institute. The majority of the participants were isolated cases, with the exception of 3 individuals, each of whom had at least 1 family member who also agreed to be part of our study. All analyses were performed from August 2008 through June 2010.

Analysis of Germline Hypermethylation

The combined bisulfite restriction analysis (COBRA)16 and the bisulfite sequencing were performed as previously described.17 The bisulfite polymerase chain reaction (PCR) primer sequences are shown in eTable 2. To provide a comprehensive analysis of the methylation status across the CpG islands upstream of PTEN, we screened 4 different regions (+400 bp to +700 bp; −188 bp to −477 bp; −425 bp to −640 bp; −806 bp to −1043 bp, all with respect to the PTEN translation start site).

Cell Lines, Antibodies, and Plasmids

The patient and control lymphoblastoid cell lines used in this study were generated from peripheral blood samples by the Genomic Medicine Biorepository (http://www.lerner.ccf}

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**Figure 2.** Germline DNA Methylation of PTEN and KILLIN in Cowden Syndrome and Cowden-like Syndrome

A Example of COBRA of PCR product (−188 to 477 bp from PTEN translation start site)

B Bisulfite sequencing analysis of selected patients
Promoter luciferase assay was performed in order to validate transcriptional repression by DNA promoter methylation and differential inhibition of TP53 binding. Breast cancer cell line MDA-MB-453 (American Type Culture Collection) was used for the luciferase assay. Lymphoblastoid cell lines and MDA-MB-453 (M.D. Anderson metastatic breast cancer) were maintained in RPMI (Rosewell Park Memorial Institute) medium with 10% fetal bovine serum and 2% antibiotics. The antibody used in the chromatin immunoprecipitation analysis (ChIP) experiment was mouse monoclonal TP53 (Santa Cruz; sc-126). The in vitro methylated constructs used for the luciferase assay were generated by first digesting 90 µg of the original PTEN and KILLIN promoter constructs (containing 1 to 1344 bp upstream of the PTEN translational start site cloned in either direction) with BgIII (New England Biolabs), Ipswich, Massachusetts) and BbvCI (New England Biolabs). The linearized, digested inserts and vectors were gel extracted. The insert DNA, which contain the sequence that is methylated in vivo in patients with Cowden syndrome or Cowden-like syndrome, was then methylated with CpG 5x5I methylase (New England Biolabs) for 4 hours. Following in vitro methylation, the insert was religated with its corresponding vector using a 3:1 insert to vector ratio with 2 µg total DNA. For comparison, the unmethylated counterpart was digested and religated in parallel.

Chromatin Immunoprecipitation Analysis
ChIP analysis was performed in order to validate that TP53 binding is differentially affected by DNA methylation and performed as previously described, according to the Upstate Cell Signaling Solutions protocol. ChIP analysis utilized 2 controls and 4 patients (eTable 1, patients 21, 31, 32, and 40) that were selected based on methylation status, representation of Cowden syndrome and Cowden-like syndrome, and similar levels of KILLIN mRNA down-regulation. Sequences of the primers used for the quantitative ChIP PCRs can be found in eTable 2.

Luciferase Assays
Luciferase assays were performed as previously described using MDA-MB-453 cells.

Reverse Transcription Polymerase Chain Reaction
The quantitative reverse transcription PCRs were performed as previously described. The study population included 4 controls and 8 patients (eTable 1, patients 21, 31, 32, 40, 366, 397, 446, and 1350) that were selected based on confirmed methylation status by bisulfite sequencing analysis and representation of the Cowden syndrome and Cowden-like syndrome condition.

Demethylation and Histone Deacetylation Inhibition Treatment
The study population included 8 patients (eTable 1, patients 21, 31, 32, 40, 366, 397, 446, and 1350) that were selected based on confirmed methylation status by bisulfite sequencing analysis and representation of Cowden syndrome and Cowden-like syndrome condition. Demethylation treatment was performed with a cytosine analog, 5-aza-2’-deoxycytidine (decitabine; Sigma), for 96 hours at 0.5 µM concentration with approximately 40% confluent suspension lymphoblastoid cells. Inhibition of histone deacetylation was performed with 200 nM concentration of Trichostatin A (TSA; Sigma) with approximately 40% confluent suspension lymphoblastoid cells.
for 48 hours, with or without 0.5 μM decitabine. The drug was changed daily, and the cells were collected for RNA isolation.

Statistical Analysis
The statistical significance of the results from reverse transcription PCR and luciferase assays was calculated by unpaired t test, with \( P < .05 \) being considered statistically significant, using Microsoft Excel version 12.2.5. The prevalence of component malignancies between KILLIN promoter methylation–positive patients and germline pathogenic PTEN mutation–positive patients was compared using the Fisher 2-tailed exact test, with \( P < .05 \) considered to be significant.

RESULTS
Germline Methylation in PTEN Mutation–Negative Cowden Syndrome and Cowden-like Syndrome
We analyzed germline genomic DNA from patients with Cowden syndrome or Cowden-like syndrome and from population controls for methylation upstream of PTEN using COBRA. Differential germline methylation was detected between 188 and 477 bp upstream of the translation start site for PTEN (Figure 1). All controls showed no methylation (Figure 2A). Among the 123 Cowden syndrome/Cowden-like syndrome samples analyzed, 45 (37%) were hypermethylated compared with all 50 controls (Figure 2A). Twenty of the 48 (42%) classic Cowden syndrome patients without germline PTEN mutations showed germline hypermethylation. Of the 75 PTEN mutation–negative Cowden-like syndrome patients, 25 (33%) were found to have germline hypermethylation. Bisulfite sequencing analysis confirmed these differences in a set of Cowden syndrome and Cowden-like syndrome samples (Figure 2B).

We then investigated whether methylation segregates with disease in family members of a proband with germline methylation. Of the 45 participants with methylation, only 1 proband (eTable 1, patient 616) had more than 1 affected family member and more than 1 unaffected family member who agreed to enroll in this study. We found germline methylation in 4 of 6 of the family members, and 3 of these 4 had documented Cowden syndrome/Cowden-like syndrome features (with 1 unknown phenotype). The 2 remaining unaffected family members did not have germline methylation (30%; 95% confidence interval, 7%-45%; \( P = .008 \)).

Germline Methylation and Effect on PTEN and KILLIN Expression
Promoter methylation should result in decreased expression of the relevant gene. In order to validate the pathogenic relevance of this methylation, the expression of PTEN was analyzed in 4 control and 8 patient cell lines as proof of principle. PTEN expression in the methylated patient samples was surprisingly not decreased, and instead, increased PTEN expression was noted (Figure 3). The PTEN 5′UTR and coding region analyzed for methylation overlaps with the putative promoter for KILLIN, a newly characterized tumor suppressor gene (Figure 1). Therefore, in order to address our hypothesis that germline methylation upstream of PTEN may, instead, be silencing KILLIN, we then analyzed KILLIN expression in the patient samples that showed germline methylation. In the methylated patient samples tested, significant underexpression of KILLIN was observed.

Figure 4. Quantitative mRNA Analysis of PTEN and KILLIN Expression With Germline Methylation, With and Without Demethylation and Histone Deacetylase Inhibition Treatment

Quantitative reverse transcription polymerase chain reaction analysis was performed on the complementary DNA from cells with (+) and without (−) drug exposure (see “Methods” section) to detect changes in expression from the demethylation and histone deacetylase inhibition treatment. All values were first normalized to their internal control (GAPDH). The fold increase or decrease in expression in the drug-treated samples is derived by normalizing to its untreated counterpart, which was set as 1. PTEN expression is shown on the top panel and reveals a significant decrease in PTEN expression following demethylation in all but 1 cell line. Patient 397 that showed an increase in PTEN expression following demethylation treatment alone was not significant (\( P = .42 \)). The bottom panel shows KILLIN expression following demethylation and/or inhibition of histone deacetylation, which shows a significant increase in expression in 7 of 8 cell lines (patient 446 was the exception). mRNA indicates messenger RNA. Error bars indicate 95% confidence intervals.
compared with the control samples (250-fold; 95% confidence interval, 45-14,286; P = .007) (Figure 3).

If, in fact, germline methylation down-regulates KILLIN expression, then demethylation should restore KILLIN expression. DNA methylation and histone deacetylation of the promoter often work together to achieve gene silencing,19 and histone acetylation has previously been shown to be transcriptionally relevant in the vicinity of the PTEN-KILLIN bidirectional promoter.20 Therefore, we investigated whether reversal of these epigenetic modifications, via demethylation and/or inhibition of histone deacetylation, would restore only KILLIN expression. KILLIN-methylated patient lymphoblastoid cell lines were treated with the demethylating drug 5-azacytidine and/or TSA. Demethylation of the shared methylated region identified in this study (250-fold; 95% confidence interval, 45–1,141) (Figure 3).

Germline Methylation Affects TP53 Binding to the KILLIN Promoter

Because the methylation of the shared bidirectional promoter had a differential impact on transcription for these 2 genes, we sought to mechanistically explain what might account for the differential epigenetic control. Both genes are transcriptionally regulated by TP53, and there appears to be 2 distinct TP53 binding sites—one for KILLIN and the other for PTEN. The TP53 binding site for transcriptional activation of PTEN lies outside of our germline methylated region,21 whereas the putative TP53 binding site for KILLIN lies within the methylated region identified in this study (Figure 5). Therefore, if we are correct that the methylation down-regulates only KILLIN expression, then the methylation should exclusively inhibit TP53 binding and activation for KILLIN alone, without affecting PTEN transcription (Figure 5).

One powerful way to interrogate this is by ChIP analysis: if there is no methylation “blocking” the relevant TP53 binding sites, then ChIP should reveal the TP53-associated regions of DNA by “pulling down” the sites bound by TP53 protein via the use of a TP53 antibody. Accordingly, we utilized 4 lines from patients (eTable 1, patients 21, 31, 32, and 40) who exhibited germline methylation of the KILLIN promoter and found that in 3 (patients 21, 31, and 40), TP53 bound more strongly to its PTEN binding site and relatively poorly to its KILLIN binding site, which was blocked by methylation (Figure 6). As controls, ChIP analysis revealed no difference of TP53 binding to both the PTEN and KILLIN TP53 binding sites in the unmethylated control cell lines tested (Figure 6).

To further address whether the differential TP53 binding of these 2 regions is due to methylation seen in the patient samples, we artificially and purposefully methylated the same CpG region in a PTEN or KILLIN promoter construct. By overexpressing TP53 in these cells, we observed a significant increase in PTEN promoter activity, without significant differences in the level

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of activation between the unmethylated and methylated PTEN constructs (Figure 7). Although both the unmethylated and methylated KILLIN constructs also provided an increase in transcriptional activation with TP53 overexpression, the methylated KILLIN construct showed significantly less transcriptional activation (30%; 95% confidence interval, 7%-45%; P = .008) by TP53 compared with the unmethylated KILLIN construct or the PTEN constructs (Figure 7).

**Prevalent Cancers in Pathogenic PTEN Mutation Positive vs KILLIN Methylation-Positive Patients**

We then turned our attention to the prevalence of component malignancies in those with germline KILLIN promoter methylation and those with proven pathogenic germline PTEN mutations. We found a significant association between the KILLIN methylation status and prevalence of female breast cancer. In our 42 women with methylation, 35 had invasive breast cancers compared with 24 of 64 women (from the same IRB8458-PTEN series) with germline PTEN pathogenic mutations (P < .0001). Renal cell carcinoma was overrepresented in the methylation-positive participants over PTEN mutation–positive individuals (4/45 vs 6/155; P = .004). However, no differences in prevalent thyroid cancers or endometrial cancers were found between the 2 groups (P = .2 and .4, respectively). Among the 12 epithelial thyroid carcinomas in individuals with KILLIN methylation–positive Cowden syndrome/Cowden-like syndrome, 7 were classic papillary thyroid carcinomas compared with the 5 classic papillary thyroid carcinomas to 10 follicular thyroid carcinoma/follicular variant of papillary thyroid carcinoma ratio seen in PTEN mutation–positive individuals.

**COMMENT**

Individuals with heritable syndromes, such as hereditary nonpolyposis colorectal cancer, who are negative for mutations in the known predisposition genes have rarely been shown to have heritable hypermethylation (also known as epimutation) of the respective promoters of these genes. This guided our initial hypothesis that a subset of patients with Cowden syndrome/Cowden-like syndrome without PTEN mutations would possibly have PTEN promoter hypermethylation. Instead, our alternative hypothesis was proven correct, resulting in our uncovering a novel Cowden syndrome/Cowden-like syndrome predisposition gene, KILLIN, and a new mechanism of epimutation that contributes to the pathogenesis of Cowden syndrome/Cowden-like syndrome features in individuals without germline PTEN mutations. The bidirectional promoter is affected by the distinct mechanism of exclusive disruption of TP53 binding and activation of KILLIN, while TP53 regulation of PTEN (the latter is outside of the methylated region) remains unaffected.

The germline KILLIN promoter epigenetic modification mechanism described here accounts for one-third of germline PTEN mutation–negative Cowden syndrome and of those whose phenotypic features resemble Cowden syndrome, prominently those with breast and thyroid disease. In our current series, more than 40% of PTEN mutation–negative classic Cowden syndrome and 33% of mutation-negative Cowden-like syndrome patients have germline epigenetic inactivation of the KILLIN promoter. If these data can be and must be replicated independently, then a hypothetical schema for prioritizing gene testing could be as follows: (1) individuals with classic Cowden syndrome should be offered PTEN testing first; (2) those found not to have germline PTEN mutations should then be offered KILLIN epigenetic analysis, in the setting of genetic counseling; and (3) individuals with classic Cowden syndrome without germline PTEN mutation (80% are mutation–positive) and without KILLIN epigenetic inactivation (half of the 20% should have KILLIN epigenetic inactivation) should then be offered SDHB/D testing (10% of the 20% should have SDHB/D mutation). Altogether, therefore, PTEN, KILLIN, and SDHB/D should then account for 92% of all classic Cowden syndrome. Patients with Cowden-like syndrome features, especially where breast cancer and/or renal carcinomas are present in the individual or family (or both) should be
offered KILLIN methylation analysis first because it accounts for 30% of such patients compared with PTEN mutations, which only account for 5% to 10% of such individuals.

By discovering another cancer predisposition gene, we have added to the sensitivity of molecular diagnosis and predictive testing becomes possible. Importantly, genetic counseling and gene-informed risk assessment and management become evidence based. In contrast to germline PTEN mutations, germline methylation of the KILLIN promoter confers a significantly higher prevalence of female invasive breast cancer and renal cell carcinomas. The current national practice guidelines for individuals with PTEN germline mutations includes heightened surveillance of the female breasts and thyroid, but do not have awareness of renal cancer risk. If our observations of 2- to 3-fold increased risks of renal and/or breast cancer with KILLIN germline methylation over those of PTEN mutation holds, then extra vigilance for the organs at risk, breast, and kidneys, is warranted. The KILLIN-associated breast cancer risks would parallel those conferred by germline BRCA1/2 mutations.

CONCLUSIONS

Among patients with Cowden syndrome or Cowden-like syndrome, presence of germline KILLIN gene promoter hypermethylation was common and was associated with increased risk of breast and renal cancer compared with PTEN mutation-positive patients.

Two limitations of this study must be considered. First, the relatively small sample size may result in a type II error. Second, this study is preliminary in nature and the assumption that KILLIN surveillance will improve the diagnosis of Cowden syndrome and Cowden-like syndrome may be overly optimistic until further validation in a larger patient set can be performed.

REFERENCES