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Germline Epigenetic Regulation of *KILLIN* in Cowden and Cowden-Like Syndromes

Kristi L. Bennett, Ph.D., **Jessica Mester, M.S., C.G.C.**, and **Charis Eng, M.D., Ph.D.**
Genomic Medicine Institute (K.L.B., J.M., C.E.), Lerner Research Institute, Taussig Cancer Institute (C.E.), and the Stanley Shalom Zielony Nursing Institute (C.E.), Cleveland Clinic, Cleveland, OH 44195; Department of Genetics and CASE Comprehensive Cancer Center, Case Western Reserve University School of Medicine, Cleveland, OH 44116 (C.E.)

Abstract

Context—Germline loss-of-function *PTEN* mutations cause 80% of Cowden syndrome (CS), an autosomal dominant disorder characterized by high risks of breast, thyroid, and other cancers. A large heterogenous group of CS-like (CSL) individuals, who have various combinations of CS features but who do not meet CS diagnostic criteria, have *PTEN* mutations <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 under-expression of *PTEN*, or of *KILLIN*, a novel tumor suppressor transcribed in the opposite direction, may account for the remainder of CS/CSL individuals. Screening for such promoter methylation, may improve the sensitivity of molecular diagnosis.

Objective—To determine whether germline methylation is found in CS/CSL individuals lacking germline *PTEN* mutations.

Design/Setting/Participants—Nucleic-acids from prospective nested series of 123 CS/CSL patients and 50 unaffected individuals without *PTEN* germline variants analyzed for germline methylation and expression of *PTEN* and *KILLIN* (Cleveland Clinic, 2008/8–2010/6). Prevalence of component cancers between groups was compared with Fisher's exact-test.

Main outcome measures—Frequency of germline methylation in *PTEN* mutation negative CS/CS-like individuals. Prevalence of component cancers in methylation-positive and *PTEN* mutation-positive patients.

Results—Of 123 CS/CS-like patients, 45 (37%, 95%CI 29–45%) showed hypermethylation upstream of *PTEN* but no transcriptional repression. The germline methylation was found to transcriptionally downregulate *KILLIN* 250-fold (95%CI 45–14286, P=0.007); and exclusively disrupted p53-activation of *KILLIN* by 30% (95%CI 7–45% (P=0.008). Demethylation treatment increased only *KILLIN* expression 4.88-fold (95%CI 1.4–18.1, P<0.05). Individuals with *KILLIN*-promoter methylation had 3-fold increased prevalence of breast cancer (35/42 vs 24/64,

Address reprint requests to: Dr. Eng at the Genomic Medicine Institute, Cleveland Clinic, 9500 Euclid Avenue, NE-50, Cleveland, OH 44195, or at engc@ccf.org.

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P<0.0001) and a >2-fold increase of kidney cancer (4/45 vs 6/155, P=0.004) over those with germline *PTEN* mutations.

Conclusions—Germline *KILLIN*- methylation is common among CS/CSL patients, and associated with increased risks of breast and renal cancer over *PTEN* mutation-positive patients. These observations need to be replicated.

Keywords

Cowden syndrome; *PTEN*; DNA methylation; p53; bidirectional promoter

INTRODUCTION

Germline mutations of *PTEN* (UCSCuc001kfb.2; RefSeqNM000314), encoding Phosphatase and TENsin homologue deleted on chromosome 10[1], cause 80% of autosomal-dominant Cowden syndrome (CS), and occurs 1:200,000 live-births. It is characterized by macrocephaly and typical mucocutaneous features (trichilemmomas, papillomatous papules), and hamartomas, with increased risk of various malignancies, ~10% lifetime risk for thyroid cancer and up to 50% lifetime risk for female breast cancer over the general population.[1–6,7,8] However, only 5% of this heterogeneous group referred to as CS-like (CSL) individuals, who have some features of CS but do not meet diagnostic criteria, have germline *PTEN* mutations. In the absence of germline *PTEN* mutations, approximately 10% CS/CSL individuals harbor germline *SDHB* (UCSCuc001bae.2; RefSeq NM003000) and *SDHD* (UCSCuc001pmz.2; RefSeqNM 003002) variants.[9] Overall, germline *PTEN* mutations/deletions and *SDHx* variants account for 82–85% of CS and 6–11% of individuals with CS-like phenotypic features.

CS is a great clinical mimic and is difficult to recognize because every patient shows variable expression and penetrance. Importantly, many individuals in the general population share one or a few features of CS but may not have CS and may not even harbor alterations in any predisposition genes. Many such patients present to primary care and other specialty clinicians who are called upon to recognize such individuals because individuals with specific gene mutations have increased risks of different spectra of neoplasias. In the context of a difficult-to-recognize syndrome, identification of additional cancer predisposition genes would facilitate molecular diagnosis, genotype-specific predictive testing of family members who are as yet clinically unaffected, genetic counseling, and medical management. Relevant to primary care, once a mutation or alteration is found, primary care physicians must have a basic understanding of gene-specific cancer risks as they do play and will increase their role as the coordinator of gene-specific personalized management, surveillance and so on.

PTEN is a well-characterized tumor suppressor phosphatase involved in cellular regulation[10] via G1 cell cycle arrest and apoptosis.[11] Interestingly, a novel gene, *KILLIN* (UCSCuc009xti.2; RefSeqNM 001126049), also residing in the 10q23.31 chromosomal region is involved in cell cycle arrest and is regulated by p53 (UCSCuc002gig.1; RefSeqNM 000546), similar to *PTEN*.[12] *PTEN* and *KILLIN* share the same transcription start site but are transcribed in opposite directions. *KILLIN* has been shown to be necessary and sufficient for p53-induced apoptosis.[12] This high-affinity DNA-binding protein inhibits eukaryotic DNA synthesis *in vitro* and causes S phase arrest before apoptosis *in vivo*.[12] Because of similar function to *PTEN*, we investigated *KILLIN* as a predisposition gene in CS/CSL patients.

Epigenetic alterations play an important role in cancer progression through hypermethylation and silencing of tumor suppressor genes,[13,14] and somatic *PTEN* hypermethylation has been recognized as a means of *PTEN* downregulation in a subset of

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malignancies. We sought to address the hypothesis that germline methylation of the 10q23.31 bidirectional promoter CpG island (a region of at least 200bp with a GC content of ≥50% and an observed/expected CpG ratio of >60%) silences *PTEN*, *KILLIN*, or both. This, consequently, would account for patients with CS or CS-like features but without germline *PTEN* mutations/deletions.

METHODS

Patients

Between October 2005 and December 2009, 2000 CS or CS-like patients were prospectively enrolled mainly regionall and nationally by the Cleveland Clinic Genomic Medicine Institute in accordance with research protocol IRB8458-PTEN, 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 he/she meets the full CS diagnostic criteria established by the International Cowden Consortium (i.e. major criteria includes breast cancer, thyroid cancer, macrocephaly, endometrial carcinoma, Lhermitte-Duclos disease) according to version 2000 (Supplemental Table 1).[18] Patients meeting the relaxed criteria are referred to as individuals with CS-like phenotypes or CSL. Of the 2,000 prospectively enrolled subjects meeting the criteria for protocol 8458-PTEN, <400 lacked germline *PTEN* pathogenic mutations, large deletions, variants of unknown significance, and polymorphisms by sequencing analysis of all 9 exons and the promoter. 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 CS, 75 CSL, and 50 unaffected individuals (population controls resident in the region), for the purposes of this study. Sample sizes were selected to ensure power (P>0.9) to detect a 5% prevalence of the methylation, as well as to detect a three-fold difference between cases and controls. All specimens from study participants and controls were prepared and analyzed within the Genomic Medicine Institute. The majority of the subjects were isolated cases, with the exception of 3 individuals each of whom had at least one 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 study population included 48 CS patients, 75 CSL patients, and 50 unaffected individuals as controls. The Combined Bisulfite Restriction Analysis (COBRA)[19] and the bisulfite sequencing were performed as previously described.[20] The bisulfite PCR primer sequences are shown in Supplemental Table 2. In order to provide a comprehensive analysis of the methylation status across the CpG islands upstream of *PTEN*, we screened four different regions (+400bp to +700bp; −188bp to −477bp; −425bp to −640bp; −806bp to −1043bp, 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 (see http://www.lerner.ccf.org/gmi/gmb/methods.php) of the Cleveland Clinic Genomic Medicine Institute.

Promoter luciferase assay was performed in order to validate transcriptional repression by DNA promoter methylation and differential inhibition of p53 binding. Breast cancer cell line MDA-MB-453 (ATCC) was used for the luciferase assay. Lymphoblastoid cell lines and MDA-MB-453 were maintained in RPMI with 10% FBS and 2% antibiotics. The antibody used in the ChIP experiment (see below) was mouse monoclonal p53 (Santa Cruz; sc-126).

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The *in vitro* methylated constructs used for the luciferase assay were generated by first digesting 90μg of the original *PTEN* and *KILLIN* promoter constructs (containing 1 to 1344bp upstream of the *PTEN* translational start site cloned in either direction) with BglII (NEB) and BbvCI (NEB). The linearized, digested inserts and vectors were gel extracted. The insert DNAs, which contain the sequence that is methylated *in vivo* in CS/CSL patients, were then methylated with CpG SssI methylase (NEB) for 4 hours. Following *in vitro* methylation, the insert was re-ligated with its corresponding vector using a 3:1 insert to vector ratio with 2μg total DNA. For comparison, the unmethylated counterpart was digested and re-ligated in parallel.

Chromatin Immunoprecipitation Analysis (ChIP)

ChIP analysis was performed in order to validate that p53 binding is differentially affected by DNA methylation and performed as previously described,[19] according to the Upstate Cell Signaling Solutions protocol. ChIP analysis utilized 2 controls and 4 patients (patients 21, 31, 32, and 40 from eTable 1) that were selected based on methylation status, representation of CS and CSL, and similar levels of *KILLIN* mRNA downreglation. Sequences of the primers used for the quantitative ChIP PCRs can be found in Supplemental Table 2.

Luciferase Assays

Luciferase assays were performed as previously described using MDA-MB453 cells.[21]

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The quantitative RT-PCRs were performed as previously described.[19] The study population included 4 controls and 8 patients (patients 21, 31, 32, 40, 366, 397, 446, and 1350 from eTable 1) that were selected based on confirmed methylation status by bisulfite sequencing analysis and representation of CS and CSL condition.

Demethylation and Histone Deacetylation Inhibition Treatment

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

Statistical Analysis

The statistical significance of the results from qRT-PCR and luciferase assays was calculated by unpaired Student's t test, with $P<0.05$ being considered statistically significant, using the 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's 2-tailed exact test with $P<0.05$ considered to be significant.

RESULTS

Germline Methylation in *PTEN* Mutation Negative Cowden Syndrome (CS) and Cowden Syndrome-like (CSL) Patients

We analyzed germline genomic DNA from CS/CSL patients and from population controls for methylation upstream of *PTEN* using COBRA. Differential germline methylation was

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detected between 188 and 477bp upstream of the translation start site for *PTEN* (Figure 1A). All normal controls showed no methylation (Figure 1B). Among the 123 CS/CSL patient samples analyzed, 45 (37%) were hypermethylated compared to all 50 controls (Figure 1B). Twenty of the 48 (42%) classic CS patients without germline *PTEN* mutations showed germline hypermethylation. Of the 75 *PTEN* mutation negative CSL patients, 25 (33%) were found to have germline hypermethylation. Bisulfite sequencing analysis confirmed these differences in a set of CS and CSL patients (Figure 1C).

We then investigated whether methylation segregates with disease in family members of a proband with germline methylation. Of the 45 participants with methylation, only one proband (#616, eTable 1) had >1 affected family member and >1 unaffected family member who agreed to enroll in our study. We found germline methylation in 4/6 of the family members, and three of these four had documented CS/CSL features (with one unknown phenotype). The two remaining unaffected family members did not have germline methylation.

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 2, top panel). The *PTEN* 5'UTR and coding region analyzed for methylation overlaps with the putative promoter for *KILLIN*, a newly characterized tumor suppressor gene (Figure 1A).[12] 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 under-expression of *KILLIN* was observed compared to the controls (Figure 2, bottom panel; ** P=0.007).

If, in fact, germline methylation downregulates *KILLIN* expression, then demethylation should restore *KILLIN* expression. DNA methylation and histone deacetylation of the promoter often work together to achieve gene silencing,[22] and histone acetylation has previously been shown to be transcriptionally relevant in the vicinity of the *PTEN-KILLIN* bidirectional promoter.[23] 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-aza-2'-deoxycytidine and/or the histone deacetylase inhibitor Trichostatin A (TSA). Demethylation and/or inhibition of histone deacetylation led to a significant decrease in *PTEN* expression for 7 of the 8 (88%) patient cell lines (Figure 3, top panel). In contrast to *PTEN*, *KILLIN* expression was restored in 88% (7 of the 8) analyzed patient cell lines following exposure to 5-aza-2'-deoxycytidine and/or TSA (Figure 3, bottom panel).

Germline Methylation Affects p53 Binding to the *KILLIN* Promoter

Because the methylation of the shared bidirectional promoter had a differential impact on transcription for these two genes, we sought to mechanistically explain what might account for the differential epigenetic control. Both genes are transcriptionally regulated by p53, and there appears to be two distinct p53-binding sites - one for *KILLIN* and the other for *PTEN*. The p53-binding site for transcriptional activation of *PTEN* lies outside of our germline methylated region (Figure 4A),[27] whereas, the putative p53 binding site for *KILLIN* lies within the methylated region identified in this study. Therefore, if we are correct that the methylation downregulates only *KILLIN* expression, then the methylation should

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exclusively inhibit p53 binding and activation for *KILLIN* alone, without affecting *PTEN* transcription (Figure 4A).

One powerful way to interrogate this is by ChIP analysis: if there is no methylation "blocking" the relevant p53-binding sites, then ChIP should reveal the p53-associated regions of DNA by "pulling down" the sites bound by p53 protein via the use of a p53 antibody. Accordingly, we utilized 4 lines from patients (patients #21, 31, 32, and 40, eTable 1) who exhibited germline methylation of the *KILLIN* promoter and found that in 3, p53 bound more strongly to its *PTEN* binding site and relatively poorly to its *KILLIN* binding site, which was blocked by methylation (Figure 4B). As controls, ChIP analysis revealed no difference of p53 binding to both the *PTEN* and *KILLIN* p53-binding sites in the unmethylated control cell lines tested (Figure 4B).

To further address whether the differential p53 binding of these two 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 p53 in these cells, we observed a significant increase in *PTEN* promoter activity, without significant differences in the level of activation between the unmethylated and methylated *PTEN* constructs (Figure 4C). Although both the unmethylated and methylated *KILLIN* constructs also provided an increase in transcriptional activation with p53 overexpression, the methylated *KILLIN* construct showed significantly less transcriptional activation by p53 compared to the unmethylated *KILLIN* construct or to the *PTEN* constructs (Figure 4C; P=0.008).

Prevalent Cancers in Pathogenic *PTEN* Mutation Positive versus *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 to 24 of 64 women (from the same IRB8458-PTEN series) with germline *PTEN* pathogenic mutations (P<0.0001). Renal cell carcinoma was over-represented in the methylation positive subjects over *PTEN* mutation positive individuals (4/45 vs 6/155, P=0.004). However, no differences in prevalent thyroid cancers or endometrial cancers were found between the two groups (P=0.2 and 0.4, respectively). Among the 12 epithelial thyroid carcinomas in *KILLIN* methylation positive CS/CSL individuals, 7 are classic papillary thyroid carcinomas (cPTC), compared to the 5 cPTC to 10 FTC/FvPTC ratio seen in *PTEN* mutation positive individuals.

COMMENT

Individuals with heritable syndromes, such as hereditary nonpolyposis colorectal cancer, that 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.[29, 30] This guided our initial hypothesis that a subset of CS/CSL patients without *PTEN* mutations would possibly have *PTEN* promoter hypermethylation. Instead, our alternative hypothesis was proven correct, resulting in our uncovering a novel CS/CSL predisposition gene, *KILLIN*, and a new mechanism of epimutation that contributes to the pathogenesis of CS/CSL in those without germline *PTEN* mutations. The bidirectional promoter is affected by the distinct mechanism of exclusive disruption of p53 binding and activation of *KILLIN*, while p53 regulation of *PTEN* (latter is outside of the methylated region) remains unaffected.

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The germline *KILLIN* promoter epigenetic modification mechanism described here accounts for 1/3 of germline *PTEN* mutation negative CS and of those whose phenotypic features resemble CS, prominently those with breast and thyroid disease. In our current series, >40% of *PTEN* mutation negative classic CS and 33% of mutation negative CSL 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. Individuals with classic CS should be offered *PTEN* testing first; those found not to have germline *PTEN* mutations should then be offered *KILLIN* epigenetic analysis, in the setting of genetic counseling. Finally, classic CS individuals 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 CS. Patients with CS-like features, especially where breast cancer and/or renal carcinomas are present in the individual and/or family, should be offered *KILLIN* methylation analysis first because it accounts for 30% of such patients compared to *PTEN* mutations which only accounts for 5–10% 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 those 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 two- to three-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 CS/CSL patients, 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 type II error. Second, this study is preliminary in nature and the assumption that *KILLIN* surveillance will improve CS/CSL diagnosis may be overly optimistic until further validation in a larger patient set can be performed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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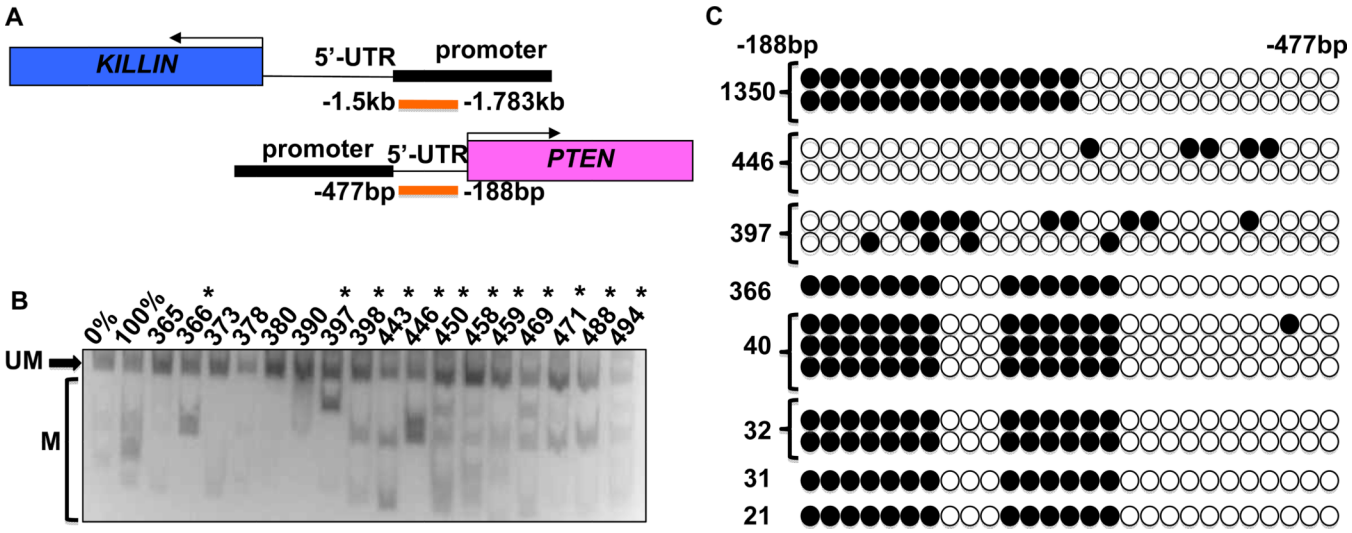

Figure 1. Germline DNA methylation of *PTEN* and *KILLIN* in Cowden and Cowden-like syndrome patients

a) A schematic of the genomic structure of the *PTEN* and *KILLIN* genes in relationship to one another at 10q23. The region analyzed for DNA methylation is depicted by the orange bars, with the numbers showing the location of the bisulfite PCR product with respect to the translation start site of each gene. As depicted, the *KILLIN* promoter overlaps with the 5'UTR and coding region of *PTEN*. b) Example of Combined Bisulfite Restriction Analysis (COBRA) of DNA from a subset of controls and CS/CSL patients (numbers refer to patient IDs in eTable 1). The 0% and 100% are from peripheral blood DNA, the 100% having been *in vitro* methylated with SssI methylase. These serve as negative and positive controls for methylation pattern analysis of the patients. An increase in the intensity of smaller, digested bands compared to the adjacent normal indicate increased methylation in the tumor DNA. * = methylation in that patient sample. c) Results from bisulfite sequencing analysis of 8 of the patient samples. Each circle represents a CpG dinucleotide (black = methylated, white = unmethylated), with the percentage filled reflecting the percentage of methylation seen at that CpG. "M"=digested bands representing methylated DNA, "UM"=undigested PCR product representing unmethylated DNA.

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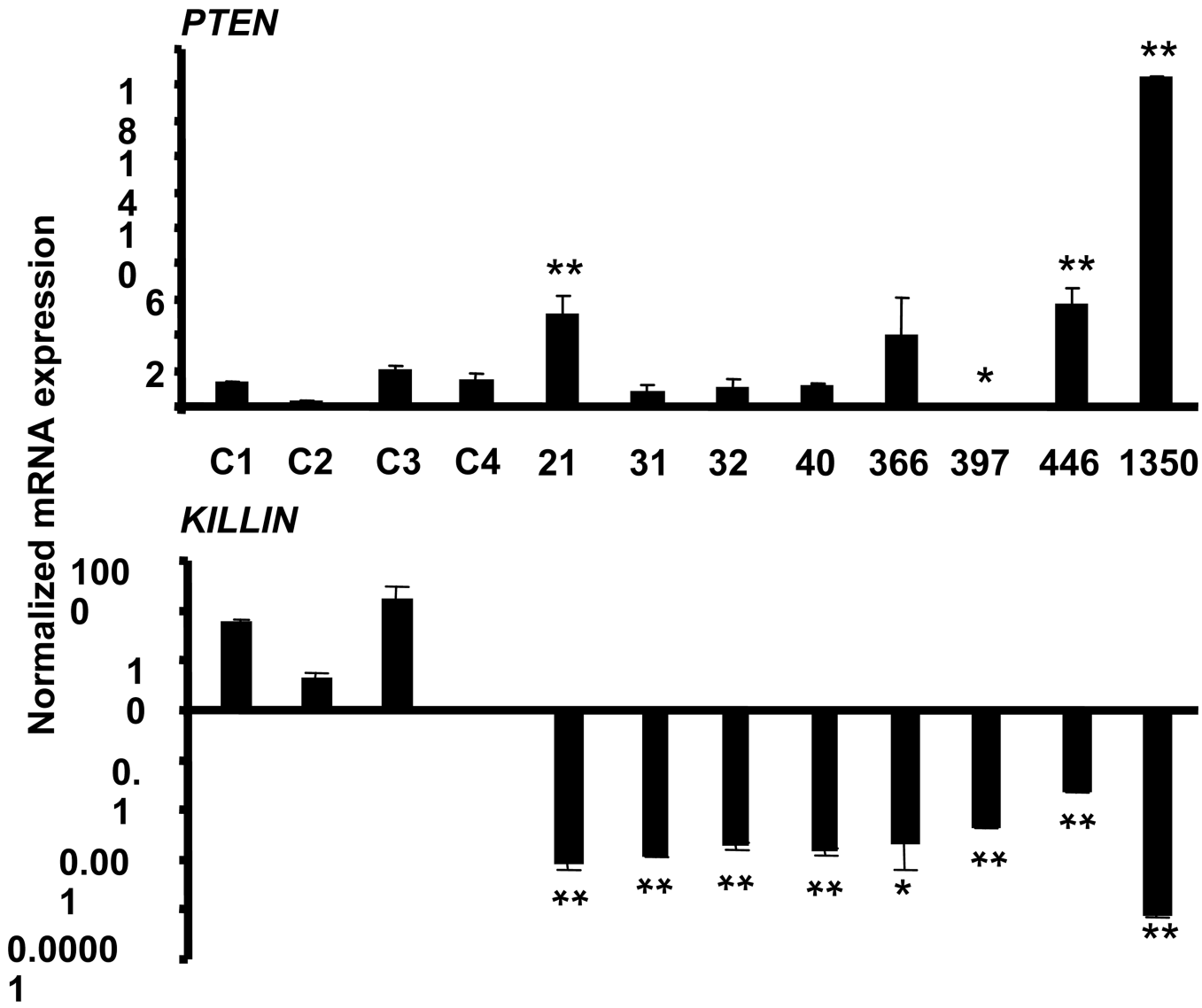

Figure 2. Quantitative mRNA analysis of *PTEN* and *KILLIN* expression in 8 CS/CSL patients with germline methylation

qRT-PCR analysis of 4 controls and 8 patient samples. All samples were first normalized to their own internal control (*GAPDH*). The average of the controls, set to 1, was used for normalization for all samples. The top panel displays the expression for *PTEN*, which reveals significantly increased expression in three patient samples compared to the normals, while only one sample showed significantly decreased expression. P=0.013 for samples 21 and 397, P<0.0001 for samples 446 and 1350. The bottom panel reveals significantly decreased *KILLIN* expression in all patient samples analyzed. P=0.007 for sample 366, P<0.0001 for samples 21, 31, 32, 40, 397, 446, and 1350.

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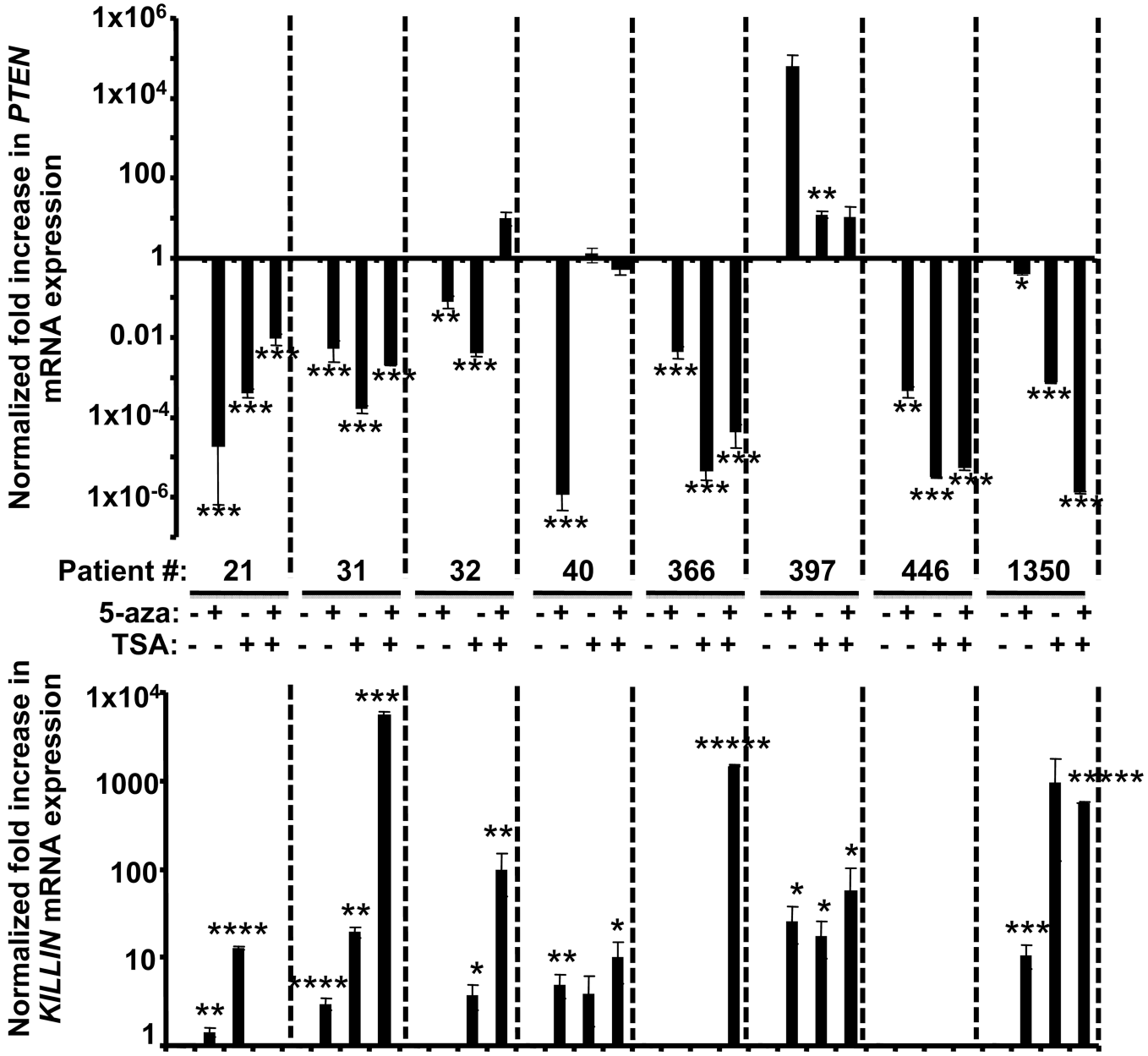

Figure 3. Quantitative mRNA analysis of *PTEN* and *KILLIN* expression in 8 CS/CSL patients with germline methylation, with and without demethylation and histone deacetlyase inhibition treatment

Eight patient cell lines with germline methylation in the region analyzed were subjected to 5-aza-2'-deoxycytidine for 96 hours at 0.5mM concentration and/or 200nM Trichostatin A. Quantitative RT-PCR analysis was performed on the cDNA from cells with and without drug treatment 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 one cell line. * P=0.018, ** P=0.001, *** P<0.0001. Patient #397 that showed an increase in *PTEN* expression following demethylation treatment alone was

not significant (P=0.42). The bottom panel shows *KILLIN* expression following demethylation and/or inhibition of histone deacetylation, which shows a significant increase in expression in 7/8 of the cell lines. *P=0.050, ** P=0.029, *** P=0.018, ****P=0.006, *****P<6.84×10^{-6}.

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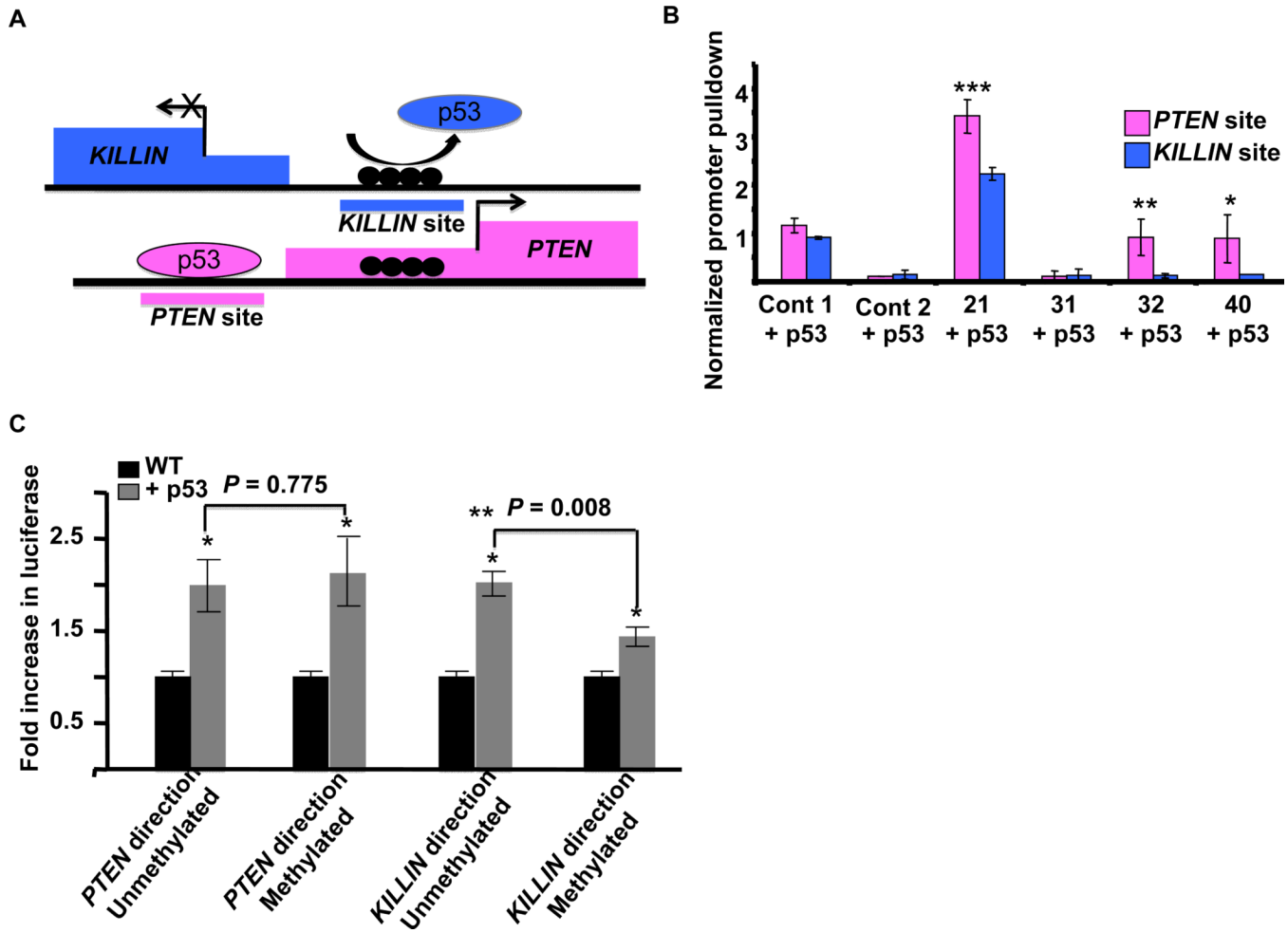

Figure 4. Methylation selectively inhibits p53 binding and transcriptional activation of *KILLIN* while not affecting p53 binding for *PTEN*

a) Model depicts where the observed methylation (closed, small circles) resides with respect to both *PTEN* and *KILLIN* and shows the two regions where p53 binds for *PTEN* and is blocked from binding (swooped arrow) for *KILLIN* transcriptional activation. "*PTEN* site" and "*KILLIN* site" represent the PCR products amplified from the eluate to analyze for p53 binding following ChIP analysis. The thinner pink and blue boxes indicate the transcribed region that is not translated, whereas the wider pink and blue boxes indicate the translation start site for the given gene. b) Chromatin immunoprecipitation analysis of p53 pulldown of either *KILLIN*'s or *PTEN*'s p53 binding element in controls and Cowden syndrome patients. All samples are normalized to their negative control, IgG. Varied enrichment of the *KILLIN* and *PTEN* p53 binding sites was observed in the control samples, whereas a significantly greater amount of Region 1 (*PTEN*'s p53 binding site) was pulled down in 75% of the patient cell lines. * P=0.032, ** P=0.012, *** P=0.002. c) *In vitro* methylation with SssI methylase was performed for both the *PTEN* and *KILLIN* luciferase promoter constructs. The constructs contained the same promoter sequence (either in orientation for *KILLIN* or in the opposite orientation for *PTEN*), which includes -1 to -1344 of sequence upstream of the translation start site of *PTEN* (-745 to +600 in respect to the *KILLIN* translation start site). Luciferase promoter analysis of *PTEN* and *KILLIN* promoter activity was done using the MDA-MB-453 breast cancer cells in the absence ("WT") or presence ("+ p53") of p53 transfection. All values were first normalized to their internal control, Renilla luciferase. The

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fold increase in the samples with p53 overexpression was attained by normalization to those without p53 transfection, which was set as 1. The *PTEN* constructs showed significant activation by p53 regardless of methylation status (* P=0.012). However, the *KILLIN* methylated construct showed significantly less activation by p53 compared to the unmethylated *KILLIN* luciferase construct (** P=0.008).