A reinvestigation of somatic hypermethylation at the PTEN CpG island in cancer cell lines

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Abstract

Background: PTEN is an important tumour suppressor gene that is mutated in Cowden syndrome as well as various sporadic cancers. CpG island hypermethylation is another route to tumour suppressor gene inactivation, however, the literature regarding PTEN hypermethylation in cancer is controversial. Furthermore, investigation of the methylation status of the PTEN CpG island is challenging due to sequence homology with the PTEN pseudogene, PTENP1. PTEN shares a CpG island promoter with another gene known as KLLN. Here we present a thorough reinvestigation of the methylation status of the PTEN CpG island in DNA from colorectal, breast, ovarian, glioma, lung and haematological cancer cell lines.

Results: Using a range of bisulphite-based PCR assays we investigated 6 regions across the PTEN CpG island. We found that regions 1-4 were not methylated in cancer cell lines (0/36). By allelic bisulphite sequencing and pyrosequencing methylation was detected in regions 5 and 6 in colorectal, breast and haematological cancer cell lines. However, methylation detected in this region was associated with the PTENP1 promoter and not the PTEN CpG island.

Conclusions: We show that methylation of the PTEN CpG island is a rare event in cancer cell lines and that apparent methylation most likely originates from homologous regions of the PTENP1 pseudogene promoter. Future studies should utilize assays that reliably discriminate between PTEN and PTENP1 to avoid data misinterpretation.

Keywords: DNA methylation, Epigenetic, PTEN, KILLIN, PTENP1, Pseudogene, Cowden syndrome

Background

Phosphatase and tensin homologue (PTEN) is a tumour suppressor gene with dual protein and lipid phosphatase activity. The main mechanism by which PTEN functions as a tumour suppressor is by negatively regulating the PI3K-AKT-mTOR pathway [1,2]. Inactivating germline mutations of PTEN result in a group of rare syndromes collectively known as the PTEN hamartoma tumour syndromes (PHTS), which includes Cowden syndrome and Bannayan-Zonana syndrome [3]. Germline mutations of PTEN account for approximately 80% of Cowden syndrome cases [4]. This syndrome is characterised by a range of clinical features including an increased risk of breast, thyroid and endometrial cancers [3]. Mutation of the PTEN tumour suppressor gene also occurs in various sporadic cancers, including 38% of endometrial, 14% of prostate, 7% of colorectal and 5% of lung carcinomas [5].

Promoter CpG island hypermethylation, which can result in the transcriptional silencing of gene expression, is an alternative mechanism of gene inactivation. The importance of PTEN inactivation in PHTS and several types of sporadic cancers makes the gene an attractive candidate for epigenetic inactivation. The PTEN CpG island is shared with another gene, known as KLLN, which is transcribed from the negative DNA strand in the opposite direction. There are a number of methodological challenges associated with the detection of methylation at the PTEN CpG island and in determining the consequences of methylation on the expression of PTEN. These challenges arise due to the fact that the PTENP1 pseudogene (also known as PTEN2), shares 97.8% sequence identity with the PTEN mRNA sequence, and 91% identity with a 921 bp region of the PTEN CpG island. Consequently, without careful consideration of assay design there is a possibility of
amplification of PTENP1 rather than the PTEN gene. The PTENP1 pseudogene is a single exon gene located at 9p13.3 (genomic coordinates chr9:33,663,502-33,667,418, NCBI36/hg18, March 2006 freeze). The existence of the PTENP1 pseudogene has been known for some time [6,7] and is thought to have arisen due to retroposition to form an intronless copy of the PTEN gene. Though some earlier reports suggested PTENP1 is not transcribed [7], more recent data suggests that the PTENP1 mRNA is ubiquitously expressed in both normal and cancer specimens [8-11]. One study showed that PTENP1 mRNA levels may be as high as 70% of PTEN mRNA levels [8]. As a consequence of PTENP1 expression, accurate analysis of PTEN mRNA levels can be problematic and careful consideration of assay design is required. Most previous studies have chosen to analyse PTEN expression using immunohistochemistry [12,13], however, PTEN-specific RT-PCR assays that utilise sequence variations between the PTEN and PTENP1 genes have been described [14].

There have been occasional reports of PTEN promoter hypermethylation in cancer. Marsit et al. reported hypermethylation of a region of the PTEN CpG island that is not homologous to PTENP1 in 26% (39/151) of non-small cell lung cancers (NSCLC), however promoter methylation was not a strong predictor of reduced or absent PTEN protein expression. Interestingly, in the same study, PTENP1 promoter hypermethylation was found in 66% (112/169) of NSCLCs, but not in normal peripheral blood DNA [12]. Hypermethylation of two CpG sites within the PTEN CpG island was also reported in the peripheral blood of up to 62% of patients with metastatic melanoma [15], however, methylation and expression of the PTENP1 gene was not addressed in this study.

Recently, a study using constitutional DNA from 123 Cowden and Cowden-like syndrome patients without PTEN mutations reported hypermethylation of a specific region of the PTEN CpG island located within the 5′ UTR of the PTEN gene [16]. Interestingly, this study suggested that hypermethylation of this region correlated with downregulation of KLLN expression rather than PTEN expression. Inactivation of KLLN expression in patients with sporadic renal cell carcinomas by CpG island hypermethylation was later proposed in another study from the same laboratory [17]. Recent evidence suggests the KLLN gene is necessary for p53-induced apoptosis and may therefore possess tumour suppressor function [18].

Several studies have described how differences in the sequences of PTEN and PTENP1 can be misinterpreted as PTEN mutations [19]. Furthermore, one previous study demonstrated that methylation of the PTENP1 gene can also be misinterpreted as methylation of the PTEN CpG island [20]. In the current study we present a thorough reinvestigation of the PTEN CpG island to assess whether hypermethylation occurs in cancer and to specifically address some of the methodological challenges associated with the analysis of methylation at this CpG island.

**Results**

**Methylation analysis of the PTEN CpG island**

We designed bisulphite-based PCR assays to determine the methylation status of six regions (designated regions 1-6) within the PTEN CpG island. The regions examined for methylation, the position of these regions relative to the PTEN and KLLN genes, and the type of assay used are described in Figure 1 and Table 1. These regions encompassed the majority of the PTEN CpG island and included the KLLN and PTEN transcription start sites (TSS). Regions 1-4 are not homologous to the PTENP1 pseudogene whereas regions 5 and 6 show 97.6% and 95.8% homology with the 5′ region of the PTENP1 pseudogene. The methylation status of these regions was investigated using a range of methods, each with different sensitivities for methylation detection. These included combined bisulphite restriction analysis (COBRA), allelic bisulphite sequencing, bisulphite pyrosequencing and quantitative real-time methylation-specific PCR (qMSP). Using COBRA we found that the KLLN gene body (region 1), as well as the KLLN and PTEN TSS (region 3) were completely unmethylated in all 36 cancer cell lines analysed (Figure 2). We confirmed
these results using allelic bisulphite sequencing (data not shown). We then examined the regions of the PTEN CpG island most similar in sequence to the promoter region of the PTEN pseudogene (regions 5 and 6). Region 5 was examined using allelic bisulphite sequencing whereas region 6 was examined using a bisulphite pyrosequencing assay encompassing six CpG sites. Interestingly, using these assays we found methylation in five out of eight cancer cell lines (Figure 3B). These cell lines were RKO and SW48 (colorectal), Raji

| Region | Method | Position (bp) | Size (bp) | Forward primer (5'-3') | Reverse primer (5'-3') | Pyrosequencing primer (5'-3') |
|--------|--------|--------------|-----------|------------------------|------------------------|------------------------------|
| 1      | COBRA  | -966 to -733 | 233       | AGTTTGTTYGGY/GATTATTAT  | CCCAAAAACCTATCTAAATA   | AACT                          |
| 2      | Bisulphite pyrosequencing | -110 to +217 | 327       | TTATGGTTTGGTYGGGYGAGAGAGA | CTACAAAAACCAACAATAAC  |
|        |        |              |           |                         | AACTGAAAAAATTAACAAACAT | ACTCAA (nested)              |
| 3      | COBRA  | -80 to +234  | 314       | TTTAGTAGG/TCGTTGGTGTT GGAGAGGAGAGAAT | CAAAACCTCATCAAACTACA A  |
| 4      | qMSP   | +237 to +368 | 132       | AGTTGAGTCGTT/ GAGGCGA GTTCCG | CAAACCGACGACTccccGAAACG |
| 5      | Bisulphite sequencing | +554 to +836 | 283       | GTTGATTTGGGAGGGGGG G | CRCCRTTCAACACTCTAACTACTA |
| 6      | Bisulphite sequencing | +801 to +971 | 171       | GGGTTTT/TTTGGATGATGAAAA | CRCCRTTCAACACTCTACTACTAA | AAATGTTTGGGATTT |

Six regions of the PTEN CpG island were investigated for methylation in this study using various methods. Listed are the regions assayed, the method used for the detection of DNA methylation, the position of the region analysed relative to the transcription start site of the PTEN gene (NM_000314) located at Chr10:89613175 (NCBI36/hg18, March 2006 freeze), the size of each amplicon, and primer sequences (including pyrosequencing primer sequences)

Figure 2 Methylation analysis of the PTEN CpG island using COBRA. Two regions of the PTEN CpG island were investigated for methylation using COBRA and were designated regions 1 and 3 as indicated in Figure 1. A, Region 1 was assayed for methylation using the restriction endonuclease AatII. Identical results were also obtained using the restriction endonuclease ClaI. B, Region 3 was assayed for methylation using the restriction endonuclease TaqI. Identical results were also obtained using the restriction endonuclease MluI. No methylation was found in any sample tested. For each region we used M.SssI in vitro methylated normal blood DNA as a positive (+ve) control. -, undigested PCR product; +, digested PCR product.

Figure 3 Bisulphite pyrosequencing analysis of the PTEN CpG island. Shown are representative pyrograms of region 2 (panel A) and region 6 (panel B) of the PTEN CpG island from the colorectal cancer cell line RKO. A, Region 2 was completely unmethylated in all samples tested. B, Region 6 showed a mean of 12% methylation across the 6 CpG sites tested in the colorectal cancer cell line RKO. Indicated by the black arrow is the presence of an A nucleotide variant specific to the PTENP1 pseudogene suggesting non-specific amplification of PTENP1. Blue boxes indicate the percent methylation at each CpG site. Grey shading indicates the CpG sites tested. Yellow shading indicates bisulphite conversion control dispensations, which indicated complete conversion in all samples tested.
and U937 (haematological) and MDA-MB-231 (breast). However, given the similarity of these regions to the promoter region of the PTENP1 pseudogene we next determined whether the methylation observed was attributable to non-specific amplification of the homologous PTENP1 region. Indeed, closer inspection of the pyrograms revealed the presence of an adenine nucleotide peak at the exact position of a thymine to adenine transition specific to the PTENP1 pseudogene (shown by the black arrow in Figure 3B). The presence of this adenine peak suggested that PTENP1 alleles were also amplified using these assays. To confirm whether this was the case, and to determine whether PTENP1 alleles could account for the observed methylation, we performed allelic bisulphite sequencing at both regions. We utilised several single nucleotide sequence differences between PTEN and PTENP1 within these regions to discriminate PTEN alleles from PTENP1 alleles. We found that non-specific amplification of the PTENP1 pseudogene accounted for up to 44% and 22% of alleles amplified using the assays at regions 5 and 6 respectively. Furthermore, methylation was specifically associated with PTENP1 alleles whereas all PTEN alleles were unmethylated (Figure 4).

Discussion
In this study, we present a thorough reinvestigation of the methylation status of the PTEN CpG island in an extensive cohort of cancer cell lines. Using a range of methods including COBRA, bisulphite pyrosequencing, allelic bisulphite sequencing and qMSP we show that hypermethylation of this CpG island in cancer cell lines is rare. These techniques allow the detection of methylation within individual alleles, as well as the quantitative measurement of methylation at individual CpG dinucleotides with extremely high sensitivity. Despite this meticulous analysis, all cell lines analysed were unmethylated. Our assays spanned the majority of the PTEN CpG island including the PTEN and KLLN TSS, the PTEN and KLLN gene bodies, as well as regions previously reported as hypermethylated [16,17]. We utilised allelic bisulphite sequencing and sequence variations between the PTEN and PTENP1 gene to unequivocally demonstrate that apparent methylation of the PTEN CpG island is actually attributable to the non-specific amplification of the highly homologous PTENP1 gene.

The challenges posed by sequence homology with pseudogenes are by no means particular to the PTEN gene. Another example is the DNA mismatch repair gene postmeiotic segregation increased 2 (PMS2) gene, which shares at least 95.2% sequence identity with six other genes (PMS2CL, PMS2L2, PMS2P4, PMS2P5, PMS2P1, PMS2P11) as well as a further 10 sites, all on chromosome 7. Importantly, the PMS2 CpG island shares over 87.5% sequence identity encompassing at least 849 bp of the 5’ regions of the PMS2L5, PMS2P4, PMS2L2 and PMS2P1 genes. This makes the methylation analysis of the PMS2 CpG island particularly challenging.

There are a number of methodological strategies that can be employed to prevent the misinterpretation of data when investigating the methylation status of a gene with a pseudogene. When designing assays for a given gene, the existence of a pseudogene or other regions of high sequence homology should first be determined using in silico sequence analysis of the mRNA and CpG island sequences. Following sequence comparisons assays should utilise critical sequence variations that allow the specific amplification of the gene of interest, or regions of high homology can be avoided completely. Newly designed assays must always be validated using methods that can discriminate between individual alleles. This is most easily achieved using allelic bisulphite sequencing. It is also crucial to be aware of amplicon sizes given that multiple amplicons or unexpected amplicon sizes can indicate non-specific amplification of related sequences such as pseudogenes. Finally, we recommend that the methylation status of regions with particularly high homology to pseudogenes can only be determined using allelic bisulphite sequencing to allow the identification of pseudogene-specific alleles.

Conclusions
We show that somatic hypermethylation of the PTEN CpG island is rare in cancer cell lines. Our study demonstrates the technical challenges associated with analysing the methylation status of the PTEN CpG island and suggests methodological strategies for studying the methylation status of genes with pseudogenes, such as PTEN. It is imperative that the methodological challenges outlined in this study are taken into account when interpreting the data from previous and future studies of the PTEN CpG island.

Methods
Sample details and DNA extraction
DNA from 36 cancer cell lines was extracted using the standard phenol-chloroform method. These included 20 colorectal (RKO, SW48, HT-29, Lim1215, SW480, NUCC2B, Lim2412, SW620, Lim2405, DLD1, HCT116, HCT-15, LoVo, LS147T, Colo 320 DM, LS411, Caco-2, SW948, HCT8, COLO 205), 3 breast (MCF-7, SK-BR-3, MDA-MB-231), 6 haematological (Raji, U937, K562, KG1, MF-1, Kasumi-i), 4 ovarian (OVCAR-3, TOV-112D, TOV-21 G, EF027) one glioma (U251), one embryonal carcinoma (NCCIT) and one lung (A549) cancer cell line
DNA methylation is specifically associated with PTENP1 and not the PTEN CpG island. 

A. Representative sequencing data of region 6 from the haematological and colorectal cancer cell lines Raji and RKO. Only PTENP1 derived alleles showed DNA methylation. Each line represents a single allele. Circles indicate the positions of CpG dinucleotides; black circles indicate methylated CpG dinucleotides; white circles indicate unmethylated CpG dinucleotides; yellow diamonds indicate the positions of nucleotide variations within PTENP1 alleles used to discriminate between PTEN and PTENP1 alleles. 

B. Representative electropherograms from region 6 showing an unmethylated PTEN allele and a methylated PTENP1 allele. 

C. Allelic bisulphite sequencing of region 5 in the cancer cell lines Raji and RKO. Again, only PTENP1 derived alleles showed methylation. Sequence variations used to discriminate between PTEN and PTENP1 alleles are indicated by yellow diamonds. Black diamonds indicate the positions of additional CpG dinucleotides specific to PTENP1 alleles that were also methylated. 

D. Representative electropherograms from region 5 showing an unmethylated PTEN allele and a methylated PTENP1 allele from the cell line Raji. Indicated by the black arrow is the position of a nucleotide variation used to discriminate the PTEN and PTENP1 alleles.
Sodium bisulphite modification
Genomic DNA was treated with sodium bisulphite using the EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA, USA), according to the manufacturers’ instructions. A measure of 1-1.5 μg DNA was incubated for 10 min at 98°C, followed by 18 hr at 53°C. Bisulphite converted DNA was eluted in 20-25 μL M-elution buffer and stored at -20°C until required.

Primer sequences
All primer sequences used throughout this study are listed in Table 1.

Combined bisulphite restriction analysis
The methylation of two regions of the PTEN CpG island (regions 1 and 3, Table 1 and Figure 1) were analysed by COBRA, consisting of sodium bisulphite modification followed by PCR and restriction digestion [21]. For each assay, the PCR mixture contained 1x PCR buffer, 0.25 mM each dNTP, 1.5 mM MgCl₂, 0.4 μM forward and reverse primers (Geneworks), and 1.25 U Platinum Taq polymerase (Invitrogen), and 80-225 ng bisulphite modified DNA. The PCR mixture for region 1 was subject to thermocycling at 95°C for 5 min, 40 cycles of 95°C for 30 sec/51°C for 30 sec/72°C for 30 sec, followed by a final extension of 72°C for 10 min. The PCR mixture for region 3 was subjected to thermocycling at 95°C for 5 min, 10 cycles of touchdown at 95°C for 30 sec/65°C for 30 sec, 30 cycles at 95°C for 30 sec/55°C for 30 sec/72°C for 30 sec, followed by a final extension of 72°C for 10 min. The PCR mixture for region 3 was subjected to thermocycling at 95°C for 5 min, 45 cycles of 95°C for 30 sec/62°C for 30 sec/72°C for 30 sec, followed by a final extension of 72°C for 10 min. Prior to restriction digestion 3 μL of each amplicon was resolved on a 1.5% agarose gel for quality control. Restriction digest reactions were carried out in a total volume of 15 μL containing the appropriate 1x restriction enzyme buffer, 1x BSA if required, 10 U restriction enzyme (New England Biolabs) and 9.5 μL amplicon. To assay for methylation the restriction enzymes AatII and Clal (region 1) or TaqI and MluI (region 3) were used, each in separate reactions. For each restriction digest, an equal amount of amplicon was subject to the same conditions without the addition of restriction enzyme. Digest reactions with and without restriction enzyme were resolved in adjacent lanes on a 2% high resolution agarose gel to prevent background bands from the PCR reaction from being misinterpreted as digestion, and therefore methylation. Each assay included a fully methylated positive control (genomic DNA from a normal individual methylated with CpG M.Sssl methylase), as well as normal blood DNA.

Quantitative real-time methylation specific PCR
Quantitative real-time methylation-specific PCR was used to assay for methylation at region 4 (Table 1 and Figure 1). This assay consists of sodium bisulphite modification followed by PCR with primers specific to methylated template. The PCR mixture contained 1x iQ™ SYBR Green® Supermix buffer (Bio-Rad), 0.3 μM forward and reverse primers (Geneworks) and 80-120 ng bisulphite modified DNA. This PCR mixture was subjected to thermocycling at 95°C for 2 min, 42 cycles at 95°C for 30 sec/70°C for sec/72°C for 30 sec, followed by melt curve analysis from 70°C to 94°C. In addition, a bisulphite specific control reaction using MyoD measured the loading of bisulphite converted DNA. The MyoD reaction is not methylation specific but is specific for bisulphite converted DNA. The MyoD specific primers used were 5’- CCAACTCCAATCCCTCTCCTC- TAT-3’ and 5’-TGATTAAATTAGATTGGGTATTTAGAAGGA-3’. MyoD MSP was subject to thermocycling at 95°C for 2 min, 42 cycles at 95°C for 30 sec/58.5°C for 30 sec/72°C for 30 sec/77.5°C for 30 sec, followed by melt curve analysis from 70°C to 88.5°C. Absolute values for experimental samples were calculated from the PCR cycle number at which the fluorescence crossed the threshold with the use of the standard curve. Values were normalised against MyoD and the percentage of methylated alleles was calculated with reference to 100% in vitro methylated human DNA (Zymo Research, Orange, CA, USA).

Pyrosequencing
We used pyrosequencing to assay for methylation at two regions of the PTEN CpG island (regions 2 and 5, Table 1 and Figure 1). Pyrosequencing consisted of sodium bisulphite modification followed by PCR and pyrosequencing with an additional internal primer. In all cases the reverse primer was labelled with biotin. For region 2 we used semi-nested PCR. Both rounds of PCR contained 1x PCR buffer, 0.25 mM each dNTP, 1.5 mM MgCl₂, 0.4 μM forward and reverse primers and 1.25 U Platinum Taq polymerase, and 80-120 ng bisulphite modified DNA. This PCR mixture was subjected to thermocycling at 95°C for 5 min, 30 cycles at 95°C for 30 sec/61°C for 30 sec/72°C for 30 sec, followed by a final extension of 72°C for 10 min. For the second round, 1 μL of the first round PCR was added to a second PCR reaction containing the same ingredients but with a nested reverse primer and subjected to the same thermocycle as above. For region 5, the PCR mixture contained 1x PCR buffer, 0.25 mM each dNTP, 1.5 mM MgCl₂, 0.4 μM forward and reverse primers and 1.25 U Platinum Taq polymerase, and 80-120 ng bisulphite modified DNA. This PCR mixture was subjected to
thermocycling at 95°C for 5 min, 45 cycles of 95°C for 30 sec/61°C for 30 sec/72°C for 30 sec, followed by a final extension of 72°C for 10 min. Prior to pyrosequencing 3-5 μl of amplicon were checked on a 1.5% agarose gel. For pyrosequencing, biotinylated amplicon were first immobilized using streptavidin-coated sepharose beads (Amersham Biosciences) and binding buffer according to the manufacturer’s instructions. The strands were separated using a Vacuum Prep Station (Biotage), and diluted in 40 μl annealing buffer containing 0.4 μM sequencing primer. The sequencing primers (Table 1) were annealed to the template at 90°C for 2 min. For region 2, the nucleotide dispensation order used was GATGTCGCTATGTAGTCGCTATTAGTCGATTAGTCGCTATG. For region 5, the nucleotide dispensation order used was TGTACGTAGTCAAGTCGCTATGTAGTCGCTATG. Pyrosequencing was performed using the PyroGoldReagents (Biotage) and PyroMark ID system (Biotage). The software PyroMark CpG Software (version 1.0) was used for evaluation of peaks for methylation by SQA analysis.

**Allelic bisulphite sequencing**

Individual alleles were cloned and bisulphite sequenced for regions 1, 3, 5 and 6. Amplicons were cloned into the pCR®2.1-TOPO® vector (Invitrogen) and transformed into TOP10 One Shot Cells or DH5a One Shot Cells (Invitrogen) according to the manufacturers’ instructions. Following transformation and selection of bacteria on agar plates we performed colony PCR using M13 primers. The PCR mixture contained 1x PCR buffer, 0.25 mM each dNTP, 2.5 mM MgCl₂, 0.4 μM M13 forward and M13 reverse primers and 1.25 U Platinum Taq polymerase, and inoculated with part of a discrete bacterial colony. This PCR mixture was subject to thermocycling at 95°C for 5 min, 35 cycles of 95°C for 30 sec/52°C for 30 sec/72°C for 30 sec, followed by a final extension of 72°C for 10 min. PCR products were purified using MultiScreen PCR plates (Millipore) and then subject to sequencing using the ABI BigDye Cycle Sequencing kit (Perkin Elmer) using the M13 reverse primer. Following ethanol precipitation of the sequenced products, samples were analysed using an ABI 3730 DNA sequencer (Perkin Elmer).

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**Authors’ contributions**

DP and LBH were involved in assay design, as well as acquisition and analysis of data. LBH and RLW were involved in the study concept, and interpretation of the data. EP, PF, and CE provided expertise with methodology. RLW and LBH obtained funding. All authors were involved in drafting and critical revision of the manuscript and gave final approval of the version published. All authors read and approve the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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