Short Communication

Considerations When Analyzing the Methylation Status of *PTEN* Tumor Suppressor Gene

Michele A. Zysman,* William B. Chapman,^{†‡} and Bharati Bapat*

From the Samuel Lunenfeld Research Institute,* Mount Sinai Hospital; Toronto; the University Health Network,[†] Toronto; and the Department of Laboratory Medicine and Pathobiology,[‡] Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada

Epigenetic mechanisms of gene silencing, including promoter hypermethylation of tumor suppressor genes, have been shown to contribute to tumorigenesis. *PTEN* is an important tumor suppressor implicated in the pathogenesis of a number of familial and sporadic cancers. Germline mutations of *PTEN* predispose to dominantly inherited hamartomatous disorders Cowden syndrome and Bannayan-Zonana syndrome. Somatic *PTEN* mutations commonly occur in endometrial, breast, prostate, and thyroid tumors. Several investigators have speculated on *PTEN* promoter hypermethylation as a possible mechanism of *PTEN* inactivation but data supporting such observations is not forthcoming. The genomic sequence of *PTEN* is 98% identical to a highly conserved processed *PTEN* pseudogene (*psiPTEN*) and this sequence identity extends 841 base pairs into the promoter region. This high degree of homology has made analysis of the methylation status of the *PTEN* promoter quite challenging. We have investigated the methylation profiles of the promoter region of *PTEN* in endometrial, breast, and colon cancer cell lines, as well as in a panel of primary endometrial tumors using a combination of methylation-specific polymerase chain reaction, methylation-sensitive restriction analysis, and bisulfite sequencing. Our results show that the pseudogene, and not *PTEN*, is predominantly methylated in cell lines and tumors. Without careful consideration of the critical nucleotide differences between the two sequences, results obtained from *PTEN* analysis may not necessarily represent the methylation status of *PTEN*. (Am J Pathol 2002, 160:795–800)

Tumor suppressor gene *PTEN*, also known as *MMAC1/TEP1*, has been implicated in a number of familial and sporadic cancers. Germline mutations of *PTEN* are responsible for Cowden syndrome¹ and Bannayan-Zonana² syndrome, dominantly inherited conditions, causing predisposition to a variety of cancers. Inactivation of *PTEN* is observed in a variety of sporadic cancers including glioblastomas,³ melanomas,⁴ and carcinomas of the endometrium,⁵ ovary,⁶ thyroid,⁷ breast,⁸ and prostate.⁹ Among these cancers, *PTEN* is considered to be a “gatekeeper” in the endometrium.¹⁰ *PTEN* mutations occur in 50 to 80% of endometrial adenocarcinomas.^{5,11} Furthermore, *PTEN* alterations are observed in up to 30% of complex atypical hyperplasias, which are considered to be the direct precursor lesions of endometrial adenocarcinomas and even in early lesions that are intermediate between normal endometrium and benign hyperplasias.¹² This progressive accumulation of *PTEN* mutations may contribute to the transition from premalignant to malignant disease. In a study by Mutter et al,¹¹ computerized morphometric analysis and selective UV irradiation were used to identify and obtain endometrial adenocarcinomas and associated hyperplasias. Mutations of *PTEN* were shown to occur in 83% of endometrial adenocarcinomas and 55% of hyperplasias. Most of these mutations occurred in only one allele. However, complete loss of *PTEN* expression, as seen from immunohistochemical analysis, was observed in 61% of tumors and 97% showed at least some degree of diminished expression.¹¹ These observations suggest that other mechanisms, including the epigenetic regulation of *PTEN* by differential methylation may contribute to the loss of *PTEN* expression.⁴

Differential methylation is an important epigenetic control mechanism, which has been implicated in the development of a variety of cancers. Research has shown that methylation of promoter regions of normally unmethylated

Supported in part by funds provided by Concern Foundation (B.B.) and American Institute for Cancer Research Grant 99BO55 (B.B.). M.Z. is a recipient of a University of Toronto Open Fellowship.

Accepted for publication December 18, 2001.

Address correspondence to Bharati Bapat, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada. E-mail: bapat@mshri.on.ca.

Table 1. PCR Primers Used for MSP and MSRA

Primer set	Forward primer 5' to 3'	Reverse primer 5' to 3'	Size (bp)	Genomic position	Anneal temperature (°C)
MSP					
Set I-M	<u>TTTTTTTTCGGTTTTTCGAGGC</u>	CAATCGCGTCCCAACGCCG	134	-984	59
Set I-U	<u>TTTTGAGGTGTTTGGGTTTTGGT</u>	ACACAATCACATCCCAACACCA	124	-971	59
Set II-M	TGTTTTTAATACGGCGGCGGC	AAACGAATAATCCTCCGAACG	143	-392	62
Set II-U	<u>TTTTTATTGTTTTAATATGGTGGT</u>	ACCAACAACCTCCAAAACCCA	120	-399	57
Set III-M	<u>GGTTTCGGAGGTCGTCGGC</u>	CAACCGAATAATACTACTACGACG	155	-298	58
Set III-U	<u>TGGGTTTTGGAGGTTGTGGT</u>	ACTTAACTCTAAACCAACAACCA	173	-300	56
MSRA					
Set IV-A	TCCGGAGGCCGCCGGCGGAA	CCGGGTAATGGCTGCTGCGGCG	151	-295	65
Set IV-B	TCCGGAGGCCGCTGGAGGAG	GGGTAATGGCTGCGGCAGCA	149	-295	72
Set IV-C	CAGCCGTTCCGAGGATTATT	GTTTCTGCTAACGATCTCTTTG	310	-273	56

Sequence differences between the bisulfite modified and untreated genomic DNA are shown in bold. Differences between methylated and unmethylated sequences are underlined. Genomic position is defined by the location of the 5' nucleotide of the sense primer from the translational start site of *PTEN* (Genbank accession number AF040103). PCR primers and annealing temperatures are indicated.

Set IV-A and B were used to amplify *PTEN* and the pseudogene, respectively, following *AclI* digestion. Nucleotide differences between *PTEN* gene and pseudogene are underlined. Set IV-C primers are the same as reported by Salvesen et al.²³ Genomic position is defined by the location of the 5' nucleotide of the sense primer from the translational start site of *PTEN* (Genbank accession number AF040103).

genes may lead to transcriptional inactivation and ultimately to tumorigenesis. Hypermethylation of several tumor suppressor genes has been associated with the development of various cancers. For example, methylation of *p16* has been implicated in the development of gliomas,¹³ while methylation of *APC* has been observed in the development of colon¹⁴ and breast¹⁵ tumors. Methylation of *MLH1*, a mismatch repair gene, has been shown to occur in sporadic colon and endometrial tumors and is associated with the development of the microsatellite instability (MSI) phenotype.¹⁶ Approximately 90% of endometrial carcinomas with MSI have been shown to display *MLH1* methylation. Whether hypermethylation of other tumor suppressor genes including *PTEN* contribute to endometrial cancer development is not yet known.

PTEN (GenBank accession number AF143312) is localized to chromosome 10q23.¹⁷⁻¹⁹ A highly conserved processed *PTEN* pseudogene (GenBank accession number AF040103, *PTEN* pseudogene; AF029308, Homo sapiens chromosome 9 duplication of the T cell receptor β locus and trypsinogen gene families) is located on chromosome 9p21.²⁰ Examination of the two sequences indicates that the sequence identity extends -841 nucleotides upstream of the *PTEN* translational start site. Because of the high degree of homology between *PTEN* and its pseudogene, analysis of *PTEN* has proven to be difficult. We have examined the methylation status of *PTEN* in a panel of carcinoma cell lines and endometrial tumors. In this study, we report the technical challenges and possible pitfalls faced when undertaking the analysis of methylation profiles of *PTEN*.

Materials and Methods

Tissues and Cell Lines

All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). RL95-2 cells were maintained in a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium (DMEM) supplemented

with 10% fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA). HEC-1B, AN3CA and MCF-7 cell lines were maintained in modified Eagle's medium (MEM) supplemented with Earle's salts (Sigma Genosys, Oakville, Canada) and 10% FBS (Invitrogen). MDA-MB231, MDA-MB468, SW-48, and SW-480 cells were maintained in Leibovitz's L-15 media with 10% FBS (Invitrogen). HT-29 cells were maintained in McCoy's 5A media with 10% FBS (Invitrogen). DNA was extracted from cells using the Dneasy Tissue Kit (Qiagen Inc., Mississauga, Canada).

Formalin-fixed, paraffin-embedded tissue sections of endometrial adenocarcinomas and matched normal specimens ($n = 20$) were obtained from patients according to a protocol approved by the Ethics Committee, University of Toronto. Tissue was microdissected from 10- μ m cut sections, then transferred into lysis buffer (10 mmol/L Tris-Cl, pH 8; 100 mmol/L KCl; 2.5 mmol/L MgCl₂; 0.45% Tween 20) with 6 μ l of Proteinase K (20 mg/ml) and incubated at 65°C for 16 hours to obtain genomic DNA.

Methylation-Specific Polymerase Chain Reaction (MSP)

Genomic DNA (18 μ l) obtained from tissues was treated with 5 mol/L sodium metabisulfite (Fisher Scientific Ltd., Ottawa, Canada) for 16 hours (protocol kindly provided by Peter Laird, University of Southern California), while cell line DNA (1 μ g) was modified using the CpGenome DNA Modification Kit (Intergen Company, Purchase, NY). Three primer sets were designed as outlined in Table 1. For sets I and II, the polymerase chain reaction (PCR) mixture contained 1X PCR buffer,²¹ dNTPs (each at 1.28 mmol/L; Invitrogen), primers (4 ng/ μ l; Sigma Genosys), Platinum *Taq* polymerase (1.5 units; Invitrogen), and bisulfite-modified DNA (~100 ng) or unmodified DNA (50 to 100 ng) in a final volume of 25 μ l. The reactions were carried out in a DNA Engine Dyad Peltier Thermal Cycler (MJ Research Inc., Waltham, MA) at 95°C for 5 minutes,

cycled at 30 seconds at 95°C, 30 seconds at the annealing temperature listed in Table 1, and 1 minute at 72°C (35 cycles), followed by a 4 minute extension at 72°C. For set III, the PCR mixture contained 1X PCR buffer,²¹ dNTPs (each at 1.28 mmol/L; Invitrogen), primers (4 ng/ μ l; Sigma Genosys), HotStarTaq DNA polymerase (1.5U; Qiagen), and bisulfite-modified DNA (~100 ng) or unmodified DNA (50 to 100 ng) in a final volume of 25 μ l. The reactions were carried out in a Dyad thermal cycler at 95°C for 15 minutes, cycled at 30 seconds at 94°C, 30 seconds at the annealing temperature listed in Table 1, and 1 minute at 72°C (35 cycles), followed by a 10 minute extension at 72°C. Normal lymphocyte DNA supermethylated with SssI methyltransferase (New England Biolabs, Beverly, MA), and subsequently treated with sodium metabisulfite served as the methylated control. Normal lymphocyte DNA treated with sodium metabisulfite alone was included as an unmethylated control. DNA not treated with sodium metabisulfite was also included as a negative control. Ten microliters of PCR product was visualized on a 2% agarose gel.

Methylation-Sensitive Restriction Analysis (MSRA)

Three hundred nanograms of genomic cell line DNA was incubated with 10 units of *Acil* (New England Biolabs) at 37°C for 16 hours. *Acil* digests only unmethylated DNA at its recognition sequence (5'-C[↓]CGC-3'), leaving methylated sites intact. Two microliters of digested DNA was then used for PCR. Primers were designed as outlined in Table 1. Set IV-A and -B primers encompass 6 *Acil* sites, while set IV-C primers flank 7 sites. For set IV-A primers, the PCR mixture contained 1X PCR buffer (Qiagen), dNTPs (each at 0.2 mmol/L; Invitrogen), primers (4 ng/ μ l; Sigma Genosys), HotStarTaq DNA polymerase (1.5 units; Qiagen), and digested DNA (~30 ng) or undigested DNA (~100 ng) in a final volume of 25 μ l. The reactions were carried out as described above for MSP set III primers. For sets IV-B and -C, the PCR mixture contained 1X PCR buffer (Qiagen), dNTPs (each at 0.2 mmol/L; Invitrogen), primers (4 ng/ μ l; Sigma Genosys), Platinum Taq polymerase (1.5U; Invitrogen), and digested DNA (~30 ng) or undigested DNA (~100 ng) in a final volume of 25 μ l. The reactions were carried out as described above for MSP set I primers. Normal lymphocyte DNA alone or treated with SssI methyltransferase (New England Biolabs) followed by *Acil* digestion served as unmethylated and methylated controls, respectively. Ten microliters of PCR product was visualized on a 2% agarose gel.

Sequencing of Bisulfite-Modified DNA and MSRA Products

Manual sequencing of unmethylated and methylated bisulfite-modified DNA products (sets I and II) was conducted using the ThermoSequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) to distinguish between *PTEN*

Figure 1. Alignment of the *PTEN* gene (lower) and pseudogene (upper). The region upstream of nucleotide (-841) represents a divergent sequence. MSP primer sets I, II, and III are shown. Set IV-A, -B primers were used for MSRA to amplify the *PTEN* gene and pseudogene, respectively. *Acil* restriction sites are indicated with triangles. Differences between the two sequences are shown with the pseudogene sequence on top. Set IV-C are primers reported by Salvesen et al²³ for MSRA are shown. Genomic position is defined by the location relative to the translational start site of *PTEN* (GenBank accession number AF143312).

and the pseudogene. Manual sequencing of PCR products using set-IV-A and -B primers was conducted to verify primer specificity.

Results and Discussion

The role of methylation in *PTEN* silencing has been suggested by a number of researchers.^{4,22} However, little information has been published on the methylation status of *PTEN*. Salvesen et al²³ recently reported that 26 of 138 endometrial carcinomas displayed *PTEN* promoter methylation. To our knowledge, this is the only published study of its kind. However, these researchers did not address the issue of the *PTEN* pseudogene and its relevance to determining *PTEN* methylation status. We analyzed *PTEN* methylation status in a panel of cancer cell lines (endometrial: RL95-2, AN3CA, HEC-1B; breast: MCF-7, MDA-MB231, MDA-MB468; colon: HT-29, SW-48, SW-480) and in primary endometrial adenocarcinomas using a combination strategy of methylation-specific PCR (MSP), methylation-sensitive restriction analysis (MSRA) and bisulfite genomic sequencing. We designed three primer sets for MSP analysis of the *PTEN* promoter region (Figure 1, Table 1). Set I primers (nucleotides -984 to -848) amplified a region of the promoter specific to *PTEN* that is not homologous to the pseudogene. Set II and III primers (nucleotides -399 to -250; nucleotides -300 to -128, respectively) were downstream of set I primers and were localized to the *PTEN* sequence which shares over 98% identity with the pseudogene. Set II primers did not discriminate between *PTEN* and the pseudogene while set III primers exclusively amplified *PTEN* and were designed by taking advantage of critical nucleotide differences between the two sequences. MSP analysis using *PTEN* gene specific set I and III primers (Table 2; Figure 2a, 3a) showed that none of the cell lines were methylated. However, using set II primers we found that these same cell lines exhibited methylation (Figure 3b) with the exception of the MCF-7 cell line. Furthermore, analysis of endometrial tumors was consistent with these results. For set I and

Table 2. MSP and MSRA Results for Cell Lines

Tissue type	Cell line	MSP						MSRA		
		Set I		Set II		Set III		Set IV-A	Set IV-B	Set IV-C
		M	U	M	U	M	U	M	M	M
Endometrial	RL95-2	—	+	+	+	—	+	—	+	+
Endometrial	AN3CA	—	+	+	+	—	+	—	+	+
Endometrial	HEC-1B	—	+	+	+	—	+	—	+	+
Breast	MCF-7	—	+	—	+	—	+	—	—	—
Breast	MDA-MB231	—	+	+	+	—	+	—	+	+
Breast	MDA-MB468	—	+	+	+	—	+	—	+	+
Colon	HT-29	—	+	+	+	—	+	—	+	+
Colon	SW-48	—	+	+	+	—	+	—	+	+
Colon	SW-480	—	+	+	+	—	+	—	+	+

MSP (primer sets I, II, III) and MSRA (primer sets IV-A, B, and C) analysis of cancer cell lines. U, unmethylated; M, methylated.

III primers, no tumors were found to exhibit methylation, while all tumor and matched normal tissues displayed methylation for set II primers (Figure 3, a and b).

We verified these results by performing MSRA on the same cancer cell line DNA. MSRA primers were designed (Table 1) in the same region as the MSP set III primers to amplify either *PTEN* (set IV-A; nucleotides –295 to –147) or the *PTEN* pseudogene (set IV-B; nucleotides –295 to –149). This was important so that results from MSP could be correlated with those of MSRA

based on the same CpG sites. In the study published by Salvesen et al,²³ *PTEN* promoter methylation status was analyzed by performing MSRA using primers that were essentially located in the same region as our set IV primers, but extended approximately 150 base pairs (bp) further downstream. We found that none of the cell lines showed *PTEN* methylation using set IV-A (Figure 2b) following *AccI* digestion, while 8 of 9 cell lines showed methylation of the pseudogene using set IV-B (Figure 2c). When we performed MSRA using the same primers as reported by Salvesen et al,²³ (primers IV-C) we again found that all cell lines, with the exception of MCF-7, exhibited methylation (Figure 2d).

To further confirm the specific contributions of *PTEN* and the pseudogene to these observations, we sequenced MSP and MSRA products. Sequencing of set I unmethylated tumor products and control supermethylated NL DNA, confirmed the *PTEN*-specific sequence (Figure 3c). As expected, sequencing of set II unmethylated cell line and tumor PCR products demonstrated nucleotide sequences corresponding to both *PTEN* and the pseudogene. This was evident by the presence of both a TT from the *PTEN* gene (CC modified to TT following bisulfite treatment) and GG from the pseudogene (Figure 3d). While for methylated PCR products, only the pseudogene was amplified as determined from the presence of only GG which is encoded by the pseudogene sequence (Figure 3e; note the absence of a TT at this location as compared to the unmethylated sequencing products).

From our results, we conclude that *PTEN* methylation is likely to be a rare event since set I and III primers, which were specific to the *PTEN* gene, showed absence of methylation in all cell lines and endometrial tumors examined. However, methylation was observed in 8 of 9 cell lines and in all endometrial tumors tested using set II primers which could not discriminate between *PTEN* and the pseudogene. Taken together, our findings confirm an absence of *PTEN* methylation. Our results demonstrate the extreme difficulties in determining the methylation status of the promoter/5' untranslated region of *PTEN* due to the high homology in this region with that of the *PTEN* pseudogene. It is important that researchers pay particular attention to the few critical residues that differ be-

Figure 2. Methylation-specific PCR using set III *PTEN* specific primers (a) and methylation-sensitive restriction analysis (b, c, d) using primer set IV-A (*PTEN*-specific), IV-B (pseudogene-specific), and IV-C (reported by Salvesen et al²³) respectively for cell lines. MSP analysis, as well as MSRA using primer set IV-A (*PTEN*), show lack of methylation in cell lines (a, b). Results for MSRA using set IV-B and -C primers (c, d) show methylation positive products in the same cell lines. NL treated with *SssI* methyltransferase was used as a methylated positive control. Following *AccI* digestion, equal amounts of cell line DNA were used for PCR analysis. U, unmethylated; M, methylated; N, undigested DNA; D, digested DNA; NL, normal lymphocyte DNA.

Figure 3. MSP and sodium bisulfite sequencing for set I and II primers. **a** and **b**: MSP results for *PTEN* primer sets I and II respectively. **Panels** on the **left** show results for cell lines while those on the **right** are representative of matched normal (N) and tumor (T) samples. *PTEN* set I sodium bisulfite sequencing of unmethylated cell lines and matched normal and tumors, and methylated NL is shown (**c**). Sequencing of unmethylated (**d**) and methylated (**e**) cell lines and tumors using set II primers are shown. *PTEN* and pseudogene sequences are shown with critical differences between the 2 sequences identified with an * and differences between the genomic and sodium bisulfite modified DNA are indicated. Note the presence of a T instead of a C (**arrow**) in the AN3CA cell line indicating that this C of a CpG dinucleotide is not methylated, unlike in HEC-1B (**e**). Cytosine residues of CpG dinucleotides are underlined. U, unmethylated; M, methylated; NL, normal lymphocyte DNA.

tween the *PTEN* and pseudogene sequences. Methylation detection methods which do not make the distinction between *PTEN* and the pseudogene sequences, may lead to false positives and may suggest a higher degree of *PTEN* promoter methylation than truly exists. Specific contribution of *PTEN* promoter methylation should be determined by conducting different assessment methods in parallel and should include sequencing to check the identity of the critical nucleotides that differ between the gene and its pseudogene. Studies to examine the *PTEN* expression including immunohistochemical analysis and RT-PCR should also be used. Besides promoter methylation, other genetic events such as loss of allelic expression or dysregulation of control elements may explain the high frequency of loss of *PTEN* expression observed by immunohistochemical analysis. Determining the effects of such molecular mechanisms on the regulation of *PTEN* warrants further study.

Acknowledgments

We thank Dr. P. Laird for the bisulfite-modification protocol, A. Saka and S. Esufali for technical support, A. Firestone for assistance with preparation of figures, and the members of our lab for helpful discussions in the preparation of this manuscript.

References

1. Nelen MR, Padberg GW, Peeters EA, Lin AY, van den Helm B, Frants RR, Coulon V, Goldstein AM, van Reen MM, Easton DF, Eeles RA, Hodgson S, Mulvihill JJ, Murday VA, Tucker MA, Mariman EC, Starink TM, Ponder BA, Ropers HH, Kremer H, Longy M, Eng C: Localization of the gene for Cowden disease to chromosome 10q22-23. *Nat Genet* 1996, 13:114-116
2. Marsh DJ, Dahia PL, Zheng Z, Liaw D, Parsons R, Gorlin RJ, Eng C: Germ-line mutations in *PTEN* are present in Bannayan-Zonana syndrome. *Nat Genet* 1997, 16:333-334
3. Rasheed BK, Stenzel TT, McLendon RE, Parsons R, Friedman AH, Friedman HS, Bigner DD, Bigner SH: *PTEN* gene mutations are seen in high-grade but not in low-grade gliomas. *Cancer Res* 1997, 57:4187-4190
4. Zhou XP, Gimm O, Hampel H, Niemann T, Walker MJ, Eng C: Epigenetic *PTEN* silencing in malignant melanomas without *PTEN* mutation. *Am J Pathol* 2000, 157:1123-1128
5. Tashiro H, Blazes MS, Wu R, Cho KR, Bose S, Wang SI, Li J, Parsons R, Ellenson LH: Mutations in *PTEN* are frequent in endometrial carcinoma but rare in other common gynecological malignancies: *Cancer Res* 1997, 57:3935-3940
6. Kurose K, Zhou XP, Araki T, Cannistra SA, Maher ER, Eng C: Frequent loss of *PTEN* expression is linked to elevated phosphorylated Akt levels, but not associated with p27 and cyclin D1 expression, in primary epithelial ovarian carcinomas. *Am J Pathol* 2001, 158:2097-2106
7. Dahia PL, Marsh DJ, Zheng Z, Zedenius J, Komminoth P, Frisk T, Wallin G, Parsons R, Longy M, Larsson C, Eng C: Somatic deletions and mutations in the Cowden disease gene, *PTEN*, in sporadic thyroid tumors. *Cancer Res* 1997, 57:4710-4713
8. Perren A, Weng LP, Boag AH, Ziebold U, Thakore K, Dahia PL, Komminoth P, Lees JA, Mulligan LM, Mutter GL, Eng C: Immunohistochemical evidence of loss of *PTEN* expression in primary ductal adenocarcinomas of the breast. *Am J Pathol* 1999, 155:1253-1260
9. Cairns P, Okami K, Halachmi S, Halachmi N, Esteller M, Herman JG, Jen J, Isaacs WB, Bova GS, Sidransky D: Frequent inactivation of *PTEN/MMAC1* in primary prostate cancer. *Cancer Res* 1997, 57:4997-5000
10. Ali IU: Gatekeeper for endometrium: the *PTEN* tumor suppressor gene. *J Natl Cancer Inst* 2000, 92:11:861-863
11. Mutter GL, Lin MC, Fitzgerald JT, Kum JB, Baak JP, Lees JA, Weng LP, Eng C: Altered *PTEN* expression as a diagnostic marker for the earliest endometrial precancers. *J Natl Cancer Inst* 2000, 92:924-930
12. Levine RL, Cargile CB, Blazes MS, van Rees B, Kurman RJ, Ellenson LH: *PTEN* mutations and microsatellite instability in complex atypical hyperplasia, a precursor lesion to uterine endometrioid carcinoma. *Cancer Res* 1998, 58:3254-3258
13. Costello JF, Berger MS, Huang HS, Cavenee WK: Silencing of p16/*CDKN2* expression in human gliomas by methylation and chromatin condensation. *Cancer Res* 1996, 56:2405-2410
14. Esteller M, Sparks A, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Gonzalez S, Tarafa G, Sidransky D, Meltzer SJ, Baylin SB, Herman JG: Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. *Cancer Res* 2000, 60:4366-4371
15. Virmani AK, Rathi A, Sathyanarayana UG, Padar A, Huang CX, Cunningham HT, Farinas AJ, Milchgrub S, Euhus DM, Gilcrease M, Herman J, Minna JD, Gazdar AF: Aberrant methylation of the adenomatous polyposis coli (*APC*) gene promoter 1A in breast and lung carcinomas. *Clin Cancer Res* 2001, 7:1998-2004
16. Esteller M, Catusas L, Matias-Guiu X, Mutter GL, Prat J, Baylin SB, Herman JG: *hMLH1* promoter hypermethylation is an early event in human endometrial tumorigenesis. *Am J Pathol* 1999, 155:1767-1772
17. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliareis C, Rodgers L, McCombie R, Bigner SH, Giovannella BC, Iltmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R: *PTEN*, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 1997, 275:1943-1947
18. Li DM, Sun H: TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Res* 1997, 57:2124-2129
19. Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH, Tavtigian SV: Identification of a candidate tumor suppressor gene, *MMAC1*, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* 1997, 15:356-362
20. Dahia PL, Fitzgerald MG, Zhang X, Marsh DJ, Zheng Z, Pietsch T, von Deimling A, Haluska FG, Haber DA, Eng C: A highly conserved processed *PTEN* pseudogene is located on chromosome band 9p21. *Oncogene* 1998, 16:2403-2406
21. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996, 93:9821-9826
22. Mutter GL: *Pten*, a protean tumor suppressor. *Am J Pathol* 2001, 158:1895-1898
23. Salvesen HB, MacDonald N, Ryan A, Jacobs IJ, Lynch ED, Akslen LA, Das S: *PTEN* methylation is associated with advanced stage and microsatellite instability in endometrial carcinoma. *Int J Cancer* 2001, 91:22-26