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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

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PTEN (*MMAC1/TEP1*), a tumor suppressor gene on chromosome subband 10q23.3, is variably mutated and/or deleted in a variety of human cancers. Germline mutations in *PTEN*, which encode a dual-specificity phosphatase, have been implicated in at least two hamartoma tumor syndromes that exhibit some clinical overlap, Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome. Among several series of ovarian cancers, the frequency of loss of heterozygosity (LOH) of markers flanking and within *PTEN*, is ~30 to 50%, and the somatic intragenic *PTEN* mutation frequency is <10%. In this study, we screened primary adenocarcinomas of the ovary for LOH of polymorphic markers within and flanking the *PTEN* gene and for intragenic mutations of the *PTEN* gene and compared them to PTEN expression using immunohistochemistry. Furthermore, we sought to detect the expression of the presumed downstream targets of PTEN, such as P-Akt, p27, and cyclin D1 by immunohistochemistry. LOH at 10q23 was observed in 29 of 64 (45%) cases. Of the 117 samples, 6 somatic intragenic *PTEN* mutations, 1 germline mutation, and 1 novel polymorphism were found in 7 (6%) patients. Immunostaining of 49 ovarian cancer samples revealed that 13 (27%) were PTEN immunostain-negative, 25 (51%) had reduced staining, and the rest (22%) were PTEN expression-positive. Among the 44 informative tumors assessed for 10q23 LOH and PTEN immunostaining, there was an association between 10q23 LOH and decreased or absent staining ($P = 0.0317$). Of note, there were five (11%) tumors with neither mutation nor deletion that exhibited no PTEN expression and 10 (25%) others without mutation or deletion but had decreased PTEN expression. Among the 49 tumors available for immunohistochemistry, 28 (57%) showed P-Akt-positive staining, 24 (49%) had decreased p27 staining, and cyclin D1 was overexpressed in 35 (79%) cases. In general, P-Akt expression was inversely correlated with PTEN expression ($P = 0.0083$). These data suggest that disruption of *PTEN* by several mechanisms, allelic loss, intragenic mutation, or epigenetic silencing, all contribute to epithelial ovarian carcinogenesis, and that epigenetic silencing is a significant mechanism. The Akt pathway is prominently involved, but clearly not in all cases. Surprisingly, despite *in vitro* demonstration that p27 and cyclin D1 lies downstream of PTEN and Akt, there was no correlation between p27 and cyclin D1 expression and PTEN or P-Akt status. Thus, *in vivo*, although PTEN and Akt play a prominent role in ovarian carcinogenesis, p27 and cyclin D1 might not be the primary downstream targets. Alternatively, these observations could also suggest that pathways involving other than Akt, p27 and cyclin D1 that lie downstream of PTEN play roles in ovarian carcinogenesis. *(Am J Pathol 2001, 158:2097–2106)*

Ovarian cancer is the most common cancer in women to be diagnosed at an advanced stage and is the fifth leading cause of cancer deaths among women in the United States.[1] Various genetic alterations have been associated with ovarian cancer, such as somatic ampli-

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fication of the oncogenes HER-2/*neu*, *KRAS*, and *C-MYC*, somatic mutation of the *TP53* tumor suppressor gene and germline mutations of *BRCA1* and *BRCA2*.[2–4] Because carcinogenesis is a multistep process,[5] it is likely that several as yet unknown genes play a role in the development and/or progression of ovarian cancer. Previous studies focusing on genetic changes occurring during ovarian cancer development have revealed clonal abnormalities in many chromosomal regions. Earlier allelotyping studies of ovarian cancer found loss of heterozygosity (LOH) at 10q in 11 to 21% of ovarian cancers, although only three markers, not in the 10q23 region, were used.[6,7] Recent reports using microsatellite markers that were specifically chosen for 10q23 identified a LOH frequency of 31 to 52%.[8–10]

The tumor suppressor gene *PTEN/MMAC1/TEP1*, encoding a dual-specificity phosphatase, has been cloned and mapped to chromosome subband 10q23.3.[11–13] Germline *PTEN* mutations have been found in the inherited autosomal-dominant Cowden and Bannayan-Riley-Ruvalcaba syndromes, which are characterized by multiple hamartomas and by an increased risk of malignant and benign breast and thyroid tumors.[14–19] Recently, a *Proteus*-like syndrome was found to result from germline and germline mosaic *PTEN* mutations.[20] Ovarian cancer, in general, is not considered part of these syndromes. Somatic mutation and/or deletion of *PTEN* occurs to a greater or lesser extent in a wide variety of human cancers that show LOH in this region, including glioblastoma, endometrial cancer, prostate cancer, and breast cancer.[12,13,21–24]

Genetic, functional, and animal modeling studies have substantiated the tumor suppressor function of PTEN. PTEN is a lipid phosphatase whose major substrate is phosphatidylinositol-3,4,5-triphosphate [PtdIns(3,4,5)P_3], downstream of which lies the Akt (PKB) pathway.[25–29] The serine-threonine kinase Akt, when phosphorylated, protects cells from apoptosis.[30,31] PTEN may also be involved in cell migration, spreading, and focal adhesion formation through dephosphorylating focal adhesion kinase, presumably through its protein phosphatase activity.[32,33] Ectopic expression of PTEN results in cell-cycle arrest at G_1 and/or apoptosis at least in the glioma and breast cancer cell line models.[27,28,34] It has been demonstrated that this function of PTEN is Akt-dependent, and can be rescued by expression of phosphorylated Akt.[27,35] One of the targets of PTEN in its ability to block cell-cycle progression at the G_1 phase has been suggested to be the cyclin-dependent kinase inhibitor p27.[28,36–38] It has been shown that up-regulation of p27 by PTEN has occurred in different cell lines.[36,37] However, it remains to be established whether the increase of p27 expression is truly a direct effect of PTEN′s action *in vivo* and which intermediate steps are involved in synthesis of this cell-cycle inhibition signal. Another potential mechanism of cell-cycle control by *PTEN* may be through inhibition of cyclin D1 accumulation. AKT phosphorylates GSK3 (glycogen synthase kinase 3), leading to its inactivation.[39] Active GSK3 phosphorylates cyclin D1, targeting it for degradation.[40] Therefore, Akt seems to promote cyclin D1 accumulation.[41,42]

Although only rare mutations of the *PTEN* gene were reported in ovarian cancer,[8–10,43–46] because of PTEN′s role in the cell cycle and cell death as well as the gene's localization to 10q23, *PTEN* is an excellent candidate to play an important role in ovarian carcinogenesis. Therefore, we sought to determine whether structural alterations in *PTEN* occurred with any frequency in ovarian cancer, if loss of PTEN expression, detected by immunohistochemistry, is a major mechanism of loss of function, and if there is a correlation between structural alterations of *PTEN* gene and PTEN protein expression. Further, we looked for any alteration in the expression of the presumed downstream targets of PTEN, such as P-Akt, p27, and cyclin D1 by immunohistochemistry, and investigated the correlation among all these variables.

Materials and Methods

Tumor Samples and DNA Extraction

One hundred seventeen epithelial ovarian tumors were obtained from patients undergoing surgery for primary epithelial ovarian cancer. Thirty-eight tumors were obtained from The Ohio State University, Columbus, OH (OSU), 25 were from the Beth Israel-Deaconess Medical Center, Boston, MA (BOS), 31 were from the University of Birmingham, UK (UK), and 23 were from Nippon Medical School, Tokyo, Japan (NMS). Apart from the 31 ovarian carcinomas from the UK that are of unknown histological sub-type, 32 were serous tumors, 28 were endometrioid cancers, 7 were clear cell cancers, 6 were mucinous tumors, 6 were mixed epithelial tumors, 6 were undifferentiated tumors, and 1 was a squamous cell carcinoma. Corresponding noncancerous tissues were available only from OSU, BOS, and NMS samples. Corresponding paraffin blocks were available only from OSU and BOS samples. Genomic DNA was extracted from tumor and matched normal tissue with a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions, or by microdissection of normal and tumor areas of paraffin-embedded archival tissue using standard protocols.[47]

LOH Analysis

Sixty-eight ovarian cancers (35 from OSU, 11 from BOS, and 22 from NMS) in which both tumor and corresponding normal DNA were available were analyzed for LOH at five polymorphic markers flanking and within the *PTEN* gene. The markers are ordered from centromere to telomere: D10S579-D10S1765-IVS4 + 109ins/delTCTTA-IVS8 + 32T/G-D10S541. *PTEN* lies between D10S1765 and D10S541, a genetic distance of 1 cM but a physical distance of only several hundred kilobase pairs. D10S1765 is within 500 kb upstream of the transcriptional start site of *PTEN* and D10S541 is within 300 kb of the translational stop site. IVS4 + 109ins/delTCTTA and IVS8 + 32T/G lie within *PTEN*.[19,48,49] The IVS4 + 109ins/delTCTTA and IVS8 + 32T/G polymorphisms were screened for by differential digestion with *Afl*II and *Hinc*II, respectively, according to

the manufacturer's guidelines (New England Biolabs, Beverly, MA) as described previously.[16,48] D10S579, D10S1765, and D10S541 were screened as documented previously.[50] Polymerase chain reaction (PCR) conditions for these markers are described elsewhere.[51,52]

PTEN Mutation Analysis

All samples were scanned for mutations by denaturing gradient gel electrophoresis (DGGE) as previously described.[24] The entire *PTEN*-coding region, exon-intron boundaries, and flanking intronic sequences were PCR amplified and fractionated through denaturing gradient gels according to the conditions described previously.[24,51] Samples showing DGGE variation were re-amplified with another set of primers, specifically for sequence analysis, gel- and column-purified and subjected to semi-automated sequence analysis as previously published.[48]

RNA Extraction and Reverse Transcriptase (RT)-PCR

From ~100 mg of ovarian cancer tissue, total RNA was extracted by the guanidine thiocyanate method,[53] using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's guidelines. Total RNA was treated with DNase I (Boehringer Mannheim, Mannheim, Germany) to remove any contaminating genomic DNA before reverse transcription. This RNA was reverse-transcribed with a first strand cDNA synthesis kit for RT-PCR (AMV; Boehringer Mannheim) according to the manufacturer's recommendations. RT-PCR using the *PTEN* exonic primers [1F, 5′-TCAAGAGGATGGATTC-GACTT-3′ (*PTEN* exon 1), and 5.1R, 5′-TCATTACAC-CAGTTCGTCC-3′ (*PTEN* exon 5)] was performed to determine whether splicing abnormalities would result from intronic mutation detected in the sequencing analysis.

Immunohistochemistry

The monoclonal antibody 6H2.1 raised against the last 100 C-terminal amino acids of PTEN[54] was used. Specificity of this antibody for *PTEN* has been documented elsewhere.[50,54] Polyclonal antibody against P-Akt was obtained from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibodies against p27 and cyclin D1 were obtained from Transduction Laboratories (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

The tissue samples were fixed by immersion in buffered formalin and embedded in paraffin according to standard procedures.[47] Sections (4 μm) were cut and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Subsequently, the sections were deparaffinized and hydrated by passing through xylene and a graded series of ethanol. Antigen retrieval was performed for 20 minutes at 98°C in 0.01 mol/L sodium citrate buffer, pH 6.4, in a microwave oven. To block endogenous peroxidase activity, the sections were incubated with 0.3% hydrogen peroxide for 30 minutes. After blocking for 30 minutes in 0.75% normal serum, the sections were incubated with each antibody overnight at 4°C. The sections were washed in phosphate-buffered saline and then incubated with biotinylated second antibody followed by avidin peroxidase using the Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA). The chromogenic reaction was performed with 3–3′ diaminobenzidine using nickel cobalt amplification.[55] After counterstaining with Nuclear Fast Red (Rowley Biochemical, Danvers, MA) and mounting, the slides were evaluated under a light microscope. The immunostaining patterns and intensities were independently evaluated by two investigators (KK and X-PZ) and randomly spot evaluated by a third investigator (CE). The multiple independent reading of the slides was performed to ensure quality control and consistency of results. Further, all sections, especially those that are immunostain-negative, were checked to contain internal positive and negative staining controls. Such consistency and accuracy have been previously documented over a broad range of tissue types throughout time [24,50,54,56,57].

Immunohistochemical Analyses

Immunostaining was evaluated without knowledge of the clinical and pathological parameters. For PTEN immunostaining, according to the amount of staining, the tumors were divided into three groups: the group assigned ++ showed increased or equal staining intensity compared to the corresponding normal tissue; the group assigned + had decreased staining intensity; and the group assigned − had no trace of staining. For P-Akt, the samples were divided into two groups according to the amount of staining. The group assigned + showed increased staining intensity compared to the corresponding normal tissue and the group assigned − had no staining. For p27, positive samples were scored according to the frequency of immunopositive cells as <5%, 5 to 50%, >50% cells immunopositive. Samples from patients with <50% p27-positive tumor cells were considered low expressors, whereas those with >50% p27-positive tumor cells were considered high expressors according to the published conventions.[58,59] We also examined cyclin D1 immunostaining. We scored cyclin D1 immunostaining by the percentage of total cells that were positive in both the nucleus and cytoplasm. Slides were graded as: negative (0 to 10% cells stained), + (10 to 50% cells stained), ++ (>50% cells stained). A cutoff value of 10% staining was used to separate normal staining (<10%) from cyclin D1-overexpressing cells (>10%).

The Fisher exact test and Wilcoxon rank sum test were used for statistical analysis of the results. The nonparametric Wilcoxon test was also chosen because immunostaining intensities (especially for cyclin D1 and p27) may be considered a continuous variable. A $P < 0.05$ was considered statistically significant.

Table 1. Results of PTEN Mutation Analysis in Primary Ovarian Cancer

Tumor	10q23 LOH	Exon/intron	Mutation	Codon altered	Germline
NMS2	−	IVS 1	IVS1+41C>G	Polymorphism	Germline
NMS26	+	Exon 3	c.166T>G	F56V	Somatic
UK63	NA	Exon 1	c.70G>T	D24Y	NA
UK33	NA	Exon 5	c.463T>A	Y155N	NA
UK66	NA	Exon 5	469–470insG	Stop at codon 179	NA
UK33	NA	Exon 7	741–742insA	Stop at codon 252	NA
UK18	NA	Exon 8	c.862G>T	E288X	NA
BOS11	+	IVS 3	IVS3-1G>T	Stop at codon 76 (Ex. 4 deletion)	Germline

NA, not available.

Results

LOH Analysis

We analyzed 68 ovarian cancers (35 from OSU, 11 from BOS, and 22 from NMS), for which tumor and corresponding normal DNA were available, for LOH at five polymorphic markers flanking *PTEN* (D10S579, D10S1765, and D10S541) and within *PTEN* (IVS4 + 109ins/delTCTTA and IVS8 + 32G/T). Among the 68 samples examined, 4 were excluded from final analysis because they were not informative at all markers. LOH at 10q23 was scored when one or more of the panel of five polymorphic loci showed LOH, as is standard. Of the 64 informative samples that were informative for at least one marker, 29 (45%) had LOH at one or more loci.

PTEN Mutation Analysis

To determine whether *PTEN* is genetically altered during ovarian carcinogenesis, we screened primary ovarian cancers for mutations in the *PTEN* gene by DGGE analysis. Samples that showed variant bands on DGGE analysis were used as template for direct sequence analysis. Of the 117 total samples, DGGE analysis revealed 8 variants in 7 samples (6%), and sequence analysis of these samples confirmed the presence of sequence variation. The identified variants included three missense, one nonsense, two frameshift mutations, and two intronic variants (Table 1). The UK samples showed the most frequent mutations (4 of 31, 13%), and Japanese and United States samples harbored mutations in 2 of 23 (9%) and 1 of 63 (2%), respectively. There was a statistical significance in mutation frequency between UK and US samples ($P = 0.022$, chi-square test). The one missense mutation in tumor NMS26 was somatic, and the corresponding germline was wild type. Tumors NMS2 and BOS11 had intronic variants in both tumor and corresponding germline DNA. Germline and somatic DNA from tumor NMS2 showed a C-to-G change in intron 1, 41 bases from the exon-intron boundary (IVS1 + 41C>G). Germline and somatic DNA from BOS11 revealed a G-to-T change in intron 3, one base from exon-intron boundary (IVS3–1G>T). Corresponding germline DNA of the remaining samples that showed variants were not available.

RT-PCR was performed to determine whether these intronic variants affected splicing. In tumor NMS2, no abnormal fragments were detected. In tumor BOS11, a fragment that was 44 bp shorter than the predicted size was noted. Sequence analysis of this aberrant fragment revealed skipping of exon 4, resulting in a frameshift and a truncated product of 76 amino acids (data not shown). We concluded that the variant in tumor NMS2 is a novel, naturally occurring, polymorphism and the variant in tumor BOS11 is a germline mutation. Interestingly, LOH at 10q23 was also found in these two tumors that showed intragenic *PTEN* mutations. In addition, UK33 showed two different pathogenic mutations, located in exons 5 and 7 (Table 1).

PTEN Immunohistochemistry in Ovarian Cancer

Samples from 49 sporadic ovarian epithelial carcinomas (38 from OSU and 11 from BOS), which had paraffin-embedded sections available, were examined for PTEN expression using the monoclonal antibody 6H2.1, raised against the terminal 100 amino acids of human PTEN. Vascular endothelial cells showed strong PTEN expression with a nuclear predominance and served as an internal positive control for this study (Figure 1, A and C).[50,54] In contrast, nuclear- and cytoplasmic-staining intensity of fibrocytes was very heterogeneous and varied from strong to weak. Of 49 ovarian cancer samples, 13 (27%) lost all PTEN immunoreactivity and was graded − (Figure 1, A and B). Twenty-five of the 49 (51%) ovarian cancer specimens stained weakly, graded +, in comparison to the normal tissue (Figure 1C). The remaining 11 (22%) tumors stained positively, graded ++ (increased or equal staining intensity compared to the corresponding normal tissue) (Figure 1D). In general, the quality and intensity of PTEN immunostaining in the nucleus and cytoplasm was relatively uniform throughout each specimen.

Comparison of Immunohistochemical and Structural Alteration Data of PTEN Gene

There were a total of 46 samples in which both immunohistochemical data and LOH data are available. Among these 46 samples, LOH data from 44 tumors were infor-

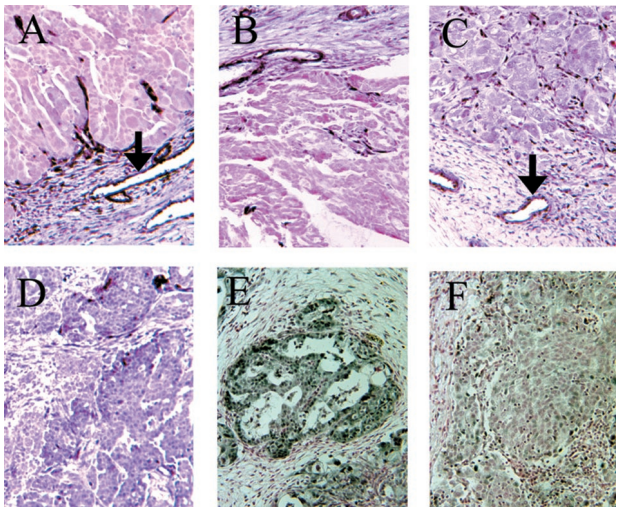
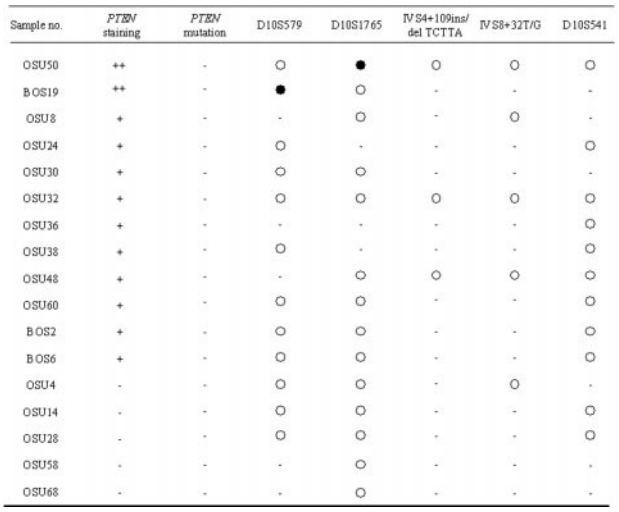


Figure 1. PTEN and P-Akt immunohistochemistry in ovarian cancers. Positive staining (++) of vascular endothelial cells serves as an internal positive control (**arrows**). Original magnification, ×10. **A** and **B:** Ovarian cancer with PTEN-negative staining (−). **C:** Ovarian cancer showing weak staining (+). **D:** Ovarian cancer exhibiting positive staining (++) for PTEN. **E:** Ovarian cancer with positive (+) P-Akt staining. **F:** Ovarian cancer exhibiting negative (−) P-Akt staining.



Sample no.	*PTEN* staining	*PTEN* mutation	D10S579	D10S1765	IVS4+109ins/del TCTTA	IVS8+32T/G	D10S541
OSU50	++	-	○	●	○	○	○
BOS19	++	-	●	○	-	-	-
OSU8	+	-	-	○	-	○	-
OSU24	+	-	○	-	-	-	○
OSU30	+	-	○	○	-	-	-
OSU32	+	-	○	○	○	○	○
OSU36	+	-	-	-	-	-	○
OSU38	+	-	○	-	-	-	○
OSU48	+	-	-	○	○	○	○
OSU60	+	-	○	○	-	-	○
BOS2	+	-	○	○	-	-	○
BOS6	+	-	○	○	-	-	○
OSU4	-	-	○	○	-	○	-
OSU14	-	-	○	○	-	-	○
OSU28	-	-	○	○	-	-	○
OSU58	-	-	-	○	-	-	-
OSU68	-	-	-	○	-	-	-

Figure 2. Correlation between PTEN immunostaining and LOH at 10q23 with apparently discordant tumors in ovarian cancer. **Open circles**, retention of heterozygosity; **filled circles**, LOH; **−**, noninformative or failed PCR.

mative. Ten (23%) of the 44 samples were graded ++ for PTEN expression by immunohistochemistry, and 8 of these 10 did not show evidence of 10q23 LOH (Table 2 and Figure 2). The remaining two samples (OSU50 and BOS19) might be viewed as apparently discordant when LOH data are compared to those obtained from immunohistochemistry (Figure 2). Sample OSU50 showed LOH at D10S1765 (5′ of *PTEN*) and retention of both alleles at the remaining four loci, notably those within *PTEN*. Sample BOS19 showed LOH at D10S579 (5′ of *PTEN*), retention of both alleles at D10S1765, and noninformative at the remaining three loci (Figure 2). Because these samples showed LOH only 5′ of *PTEN*, it is doubtful whether it is deleted within *PTEN*, thus explaining the ++ immunohistochemistry.

Thirteen samples (30%) were immunostain-negative. Among these, eight showed evidence of 10q23/*PTEN* deletion and five had no evidence of *PTEN* allele loss (Table 2 and Figure 2). Four of these five samples (OSU14, OSU28, OSU58, and OSU68) showed retention of both alleles at the flanking markers, and noninformative at the intragenic *PTEN* polymorphic markers. Therefore, there is a possibility that a part or entire *PTEN* might be deleted (Figure 2).

Twenty-one samples (48%) were immunostain-positive. Of these 21, 11 might be classified as having LOH representing hemizygous *PTEN* allelic loss that could correspond to the diminished immunostaining. The remaining 10 with weak immunoreactivity retained heterozygosity at their respective informative loci (Table 2 and Figure 2).

Table 2. Summary of PTEN Immunostaining and 10q23 LOH

	PTEN staining ++	PTEN staining +	PTEN staining −
Loss of heterozygosity	2 (20%)	11 (52%)	8 (62%)
Retention of heterozygosity	8 (80%)	10 (48%)	5 (38%)
Total	10	21	13

Statistical correlation of PTEN intensity *versus* LOH at 10q23 performed using a Wilcoxon rank sum test yielded results of P = 0.0317, indicating a significant increase in LOH at 10q23 with decreasing PTEN stain intensity.

We investigated potential relationships between the presence of LOH at 10q23 and the intensity of PTEN immunostaining. Among the 44 informative tumors assessed for LOH and PTEN immunostaining, there seemed to be an association between decreased or absent staining and 10q23 LOH. The frequency of tumors that showed LOH at 10q23 steadily increased from tumors with ++ immunostaining (20% LOH), + staining (52% LOH), to negative (−) staining (62% LOH) (P = 0.0317 Wilcoxon rank-sum test) (Table 2). However, it is also clear that one-third (15 of 44) of tumors did not show structural deletion or mutation but showed diminished or no PTEN expression.

P-Akt, p27, Cyclin D1 Immunohistochemistry in Ovarian Cancer

Of 49 ovarian cancer samples, 28 (57%) stained positive, graded +, in comparison to the normal tissue (Figure 1E). Twenty-one of the 49 (43%) ovarian cancer specimens lost all P-Akt immunoreactivity and showed negative immunostaining, graded − (Figure 1F). P-Akt immunostaining showed a nuclear predominance, however, weak cytoplasmic staining was also observed. Among these same 49 ovarian cancers, 4 (8%) showed negative immunostaining with p27 antibody, graded − (Figure 3A). Twenty of the 49 (41%) ovarian cancer specimens stained weakly, graded +, in comparison to the normal tissue. Thus, 24 samples (49%) were classified as low expressors. The remaining 25 (51%) tumors stained positively, graded ++ (Figure 3B) and were classified as high expressors. p27 immunostaining was mostly nuclear; however, weak cytoplasmic staining was also observed. Overexpression of cyclin D1 was detected in

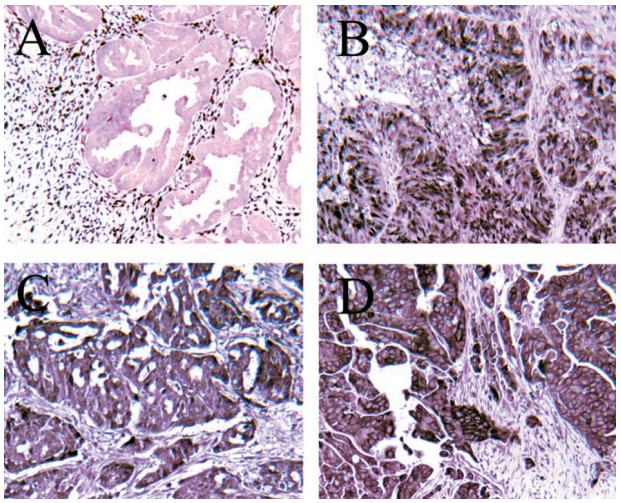


Figure 3. p27 and cyclin D1 immunohistochemistry in ovarian cancers. Original magnification, ×10. Ovarian cancer with p27-negative (−, **A**), and p27-positive (++, **B**) staining. **C:** Ovarian cancer showing cyclin D1 overexpression (++) in simultaneous nucleus and cytoplasm. **D:** Ovarian cancer showing cyclin D1 overexpression (++) in exclusively cytoplasm.

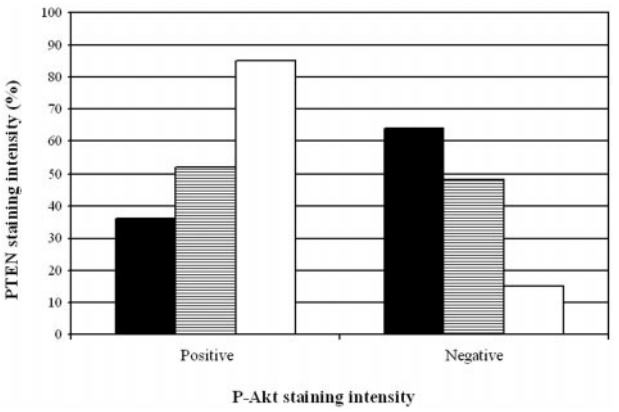


Figure 4. Correlation between PTEN and P-Akt immunostaining intensity. Strong (**filled square**), decreased (**lined box**), and absent (**open square**) PTEN staining. The *y*-axis represents percentage of samples with various intensities of PTEN staining. Statistical correlation was found between PTEN and P-Akt staining ($P = 0.0083$).

35/49 (71%) of the ovarian cancers examined. The level of overexpression was variable between the slides and was graded accordingly. Cyclin D1 was overexpressed predominantly in the nuclei of tumor cells in 6 of 49 (12%) of the samples, and simultaneously in the nucleus and cytoplasm of tumor cells in 10 of 49 (20%) (Figure 3C). Therefore, 16 of 49 (33%) of the tumors examined showed nuclear overexpression of cyclin D1 (Table 3). In 19 of 49 (39%) of the tumors examined, overexpression was detected only in the cytoplasm of the tumor cells (Figure 3D). Therefore, 29 of 49 (59%) of the tumors examined showed cytoplasmic overexpression of cyclin D1 (Table 3). Representative examples of simultaneous nuclear and cytoplasmic, and exclusively cytoplasmic staining are shown (Figure 3, C and D).

We investigated potential relationships of expression of PTEN, P-Akt, p27, and cyclin D1. Among the 49 informative tumors assessed for immunostaining, there seemed to be an association between positive P-Akt staining and decreased or absent staining of PTEN (Figure 4). The proportion of tumors that had positive P-Akt immunostaining steadily increased from tumors with ++ PTEN expression (36%), to those with no PTEN expression (85%) ($P = 0.0083$ Wilcoxon rank sum test; Figure 4). Of the 11 tumors that showed ++ PTEN expression, 7 (64%) of these tumors stained p27 ++, 4 (36%) of these showed p27 + staining, and no tumors showed negative p27 staining. (Figure 4). However, this correlation between PTEN expression and p27 expression did not achieve statistical significance ($P > 0.05$, chi-square test; $P = 0.4$, Fisher two-tailed exact test). No significant association was found between PTEN or P-Akt expression and cyclin D1.

Discussion

Our study of PTEN in primary epithelial ovarian carcinomas has revealed that genetic and epigenetic inactivation of PTEN together play a significant role in tumorigenesis. In this study, we have found that the incidence of LOH at 10q23 was 45% in primary epithelial ovarian cancers, a figure consistent with previous allelotyping.[8–10] Examining 117 sporadic ovarian cancers, we found that 6 cases (5%) harbored intragenic *PTEN* mutations, again consistent with previous reports (0 to 10%).[8–10,43–46] Obata and colleagues[9] identified that intragenic *PTEN* mutations are much more common in endometrioid histology (7 of 34; 21%). However, Saito and colleagues[10] reported intragenic *PTEN* mutations were found not only in endometrioid type (1 of 5; 20%) but also in serous (1 of 10; 10%) and mucinous (1 of 4; 25%) type. Unfortunately, histological information of UK samples was not available. NMS26 and BOS11, two *PTEN* mutation-positive samples in which histological information was available, were classified as mucinous (1 of 6; 17%) and endometrioid (1 of 28; 4%) type, respectively. Of 13 samples that showed negative PTEN immunoreactivity, 6 were endometrioid cancers (6 of 13; 46%), 4 were serous tumors (4 of 21; 19%), 2 were mixed epithelial tumors (2 of 6; 33%), and 1 was an undifferentiated tumor (1 of 4; 25%). Thus, PTEN seems to play a role across several subtypes of ovarian cancer and is not predominant among the endometrioid subtype, as previously believed.

Table 3. Cyclin D1 Immunostaining and Localization in Ovarian Cancer

	Cyclin D1 (cytoplasm only)	Cyclin D1 (nuclear only)	Cyclin D1 (both)	Negative
Overexpression*	19 (39%)	6 (12%)	10 (20%)	14 (29%)

*, >10% cells stained.

Among the samples that had intragenic *PTEN* mutations, only two samples had both tumor and normal DNA available for investigation. Interestingly, both these samples (NMS26 and BOS11) showed biallelic structural inactivation by intragenic *PTEN* mutation plus loss of the remaining wild-type allele. Although normal DNA was not available, the exon 5 and exon 7 double-intragenic mutations in UK33 almost certainly represents mutations involving both alleles (Table 1). Previous studies have shown that biallelic structural inactivation occurs in ovarian cancer, either by somatic intragenic *PTEN* mutation plus loss of the remaining wild-type allele, by double-somatic intragenic *PTEN* mutations, or by homozygous deletion at 10q23.[9,10] Our study and these data suggest that a subset of ovarian cancer, albeit small, might be in the same category as glioblastoma multiforme, a subset of endometrial cancers and cervical cancers, where biallelic structural *PTEN* inactivation is also an important mechanism.[21,23,60,61]

In case BOS11, mutation within intron 3, one base from the exon-intron boundary (IVS3–1G>T), has been shown to cause aberrant splicing and almost certainly also results in a transcript with decreased stability. This mutation was also identified in the corresponding germline DNA; thus, among 107 apparently sporadic ovarian cancer cases, we detected at least one (1%) carrying an occult germline *PTEN* mutation. It is, therefore, vital that this patient be followed closely for development of Cowden-related cancers, especially those of the breast, thyroid, and endometrium, because the presence of a germline *PTEN* mutation is a sensitive molecular diagnostic marker for Cowden syndrome.[18,62] If our observations can be replicated, clinicians might wish to inquire about other features of Cowden syndrome and to take a good family history when faced with apparently sporadic ovarian cancer cases. Hereditary ovarian cancer occurs as a part of three clinically distinct syndromes, site-specific ovarian cancer and breast-ovarian cancer, both of which are because of germline mutations in *BRCA1* and *BRCA2* in >90% of such cases,[63] and hereditary nonpolyposis colorectal cancer. It is possible that Cowden syndrome might be included as a minor differential diagnosis of genetic ovarian cancer cases.

Among the samples that were available for both PTEN immunohistochemistry data and LOH analysis, we found that 77% (34 of 44) of these tumors had either partial or complete expressional loss of PTEN at the protein level. Of these 34 samples, only 3% (1 of 34) had structural biallelic inactivation, 53% (18 of 34) had structural monoallelic deletion of *PTEN*, and the remaining 15 samples (44%) had no evidence of *PTEN* structural anomalies and thus, lost of PTEN expression might be because of epigenetic silencing (Table 2). Especially worthy of note are the 13 tumors with no PTEN expression (−): only 1 had structural biallelic inactivation by intragenic *PTEN* mutation and loss of the remaining wild-type allele; 7 had evidence of loss of one allele only and so, silencing of the remaining wild-type allele might be by epigenetic means; and 5 had complete silencing of both *PTEN* alleles via mechanisms beyond structural alteration. Similarly, among the 21 tumors with weak (+) PTEN immunostaining, 11 had monoallelic deletion that would account for the decreased PTEN expression. However, the remaining 10 had neither *PTEN* mutation nor deletion. These 10 tumors might have monoallelic silencing via epigenetic mechanisms (Table 2 and Figure 2). In contrast, all tumors with strong PTEN expression had both intact *PTEN* alleles. The two seemingly discordant samples with strong expression but LOH only had LOH 5′ of PTEN. Recent accumulating knowledge has suggested that PTEN may be inactivated by several mechanisms other than mutations and/or deletions in a tissue-specific manner [24,29,50,54,56,57,64]. In general, the data to date would suggest that more than one mechanism of PTEN inactivation can occur in a given tumor type. It would seem, however, that certain mechanisms of *PTEN* inactivation predominate in certain tissues. For example, in primary cervical carcinomas and glioblastoma multiforme, the predominant mechanism of PTEN silencing is biallelic structural alteration (mutation; deletion).[61] In metastatic malignant melanoma, biallelic epigenetic mechanisms of PTEN silencing predominate.[57] In nonmedullary thyroid cancers and endocrine pancreatic tumors, PTEN inactivation seems to be mediated by differential subcellular compartmentalization.[50,56] In the case of primary epithelial ovarian cancers (this study), biallelic structural alterations are rare whereas a mixed genetic/epigenetic and biallelic epigenetic silencing are evident in the great majority of tumors with decreased or absent PTEN expression. In contrast to thyroid tumors and endocrine pancreatic tumors, there is no evidence of subcellular compartmentalization as a mechanism of PTEN inactivation in ovarian carcinomas. Nonetheless, the precise epigenetic or other mechanisms, such as methylation or degradation, involved in modulating *PTEN* expression are yet to be elucidated. Further, why subcellular compartmentalization involving the nuclear compartment for a molecule such as PTEN lacking a nuclear localization signal is actively being investigated.[32,50,56,65,66]

It has been well established that PTEN signals down the PI3K-Akt pathway and that PTEN inversely correlates with P-Akt.[25–27,29] Of the 49 tumors examined, 28 (57%) showed positive (+) P-Akt staining and 21 (43%) showed negative (−) staining, a general trend showing this inverse correlation. However, it should be noted that the inverse correlation is not observed among all tumors: six (12%) did not show this inverse correlation. These observations might suggest that in the subset of PTEN null-P-Akt null ovarian cancers, at least one other non-PI3K-Akt pathway downstream of PTEN is the major pathway involved. In contrast, in the subset of PTEN and P-Akt co-expressors, PTEN is likely not involved and P-Akt is anti-apoptotic via other upstream mechanisms. These *in vivo* observations in noncultured tumors corroborate our *in vitro* functional analyses that demonstrate that PTEN can induce cell-cycle arrest and apoptosis via PI3K-Akt-dependent and -independent pathways.[67]

Because of several reports of finding a relationship between PTEN and p27 and between PTEN and cyclin D1, albeit *in vitro*, we sought to examine differential expression levels among these proteins. Several reports have suggested that PTEN induced p27 expression

downstream of Akt[36–38] and that this was a mechanism of cell-cycle arrest mediated by PTEN. If this were true, we would have observed a direct correlation between PTEN expression and p27 expression. Although we saw an associative trend, this was not statistically significant nor was it convincing. Similarly, the literature has suggested that PTEN down-regulates cyclin D1.[68] If this were true, then we should have observed a trend of increased cyclin D1 expression with decreased PTEN expression. In our series, cyclin D1 expression levels and subcellular location were random irrespective of PTEN or P-Akt status. Our observations might suggest that *in vivo*, at least in primary epithelial ovarian carcinomas, p27 and cyclin D1 are not prominent or direct targets of PTEN action.

In summary, despite the low frequency of intragenic somatic *PTEN* mutations in primary ovarian cancer, we have found a high frequency of decreased or absent PTEN protein expression, associated with increased P-Akt expression. Our data argues for the prominent role of PTEN inactivation in ovarian carcinomas via multiple mechanisms, ranging from biallelic genetic alteration to biallelic epigenetic silencing. Further, although the Akt pathway is involved in PTEN's role in a proportion of ovarian tumorigenesis, p27 and cyclin D1, previously described to be downstream of PTEN, do not seem to have this relationship with PTEN, at least not in epithelial ovarian carcinogenesis. Therefore, it would seem that not only are mechanisms of PTEN inactivation tissue-specific but downstream pathways of PTEN as well.

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