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The expression of PTEN is associated with improved prognosis in patients with ampullary adenocarcinoma after pancreaticoduodenectomy

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Abstract

Context—Phosphatase and tensin homolog (PTEN) is one of the most frequently inactivated tumor suppressor genes in many sporadic cancers. Somatic mutations of *PTEN* occur frequently in many tumors including those of the gastrointestinal and hepatobiliary tracts. Loss of PTEN expression has been shown to be associated with poor prognosis in patients with metastatic colonic adenocarcinoma, gastroesophageal junction adenocarcinoma, gastric adenocarcinoma and pancreatic ductal adenocarcinoma.

Objective—To study the expression of PTEN and its significance in ampullary adenocarcinoma (AA).

Design—We constructed tissue microarrays using archival tissue from 92 patients (55 males, 37 females) with previously untreated AA who underwent pancreaticoduodenectomy at our institution. The age range was 37 to 87 years (median: 63 years). Each tumor was sampled in duplicate with a 1.0 mm punch from representative areas. PTEN expression was evaluated by immunohistochemistry and scored semiquantitatively based on staining intensity and percentage positive tumor cells. Staining results were correlated with clinicopathologic features and survival.

Results—Twenty-three of 92 (25.0%) cases were PTEN-negative. Loss of PTEN expression in AA correlated with lymph node metastasis ($P = .004$), advanced American Joint Committee on Cancer (AJCC) stage ($P = .02$) and higher frequency of recurrence ($P = .03$). Patients whose tumors had loss of PTEN expression had shorter disease-free survival (DFS, mean: 89.0 ± 20.8 months) and overall survival (OS, mean: 93.1 ± 19.1 months) compared to those whose tumors were PTEN-positive (mean DFS: 161.4 ± 11.7 months, $P = .02$; mean OS: 175.4 ± 11.0 months, $P = .002$).

respectively). In multivariate analyses, PTEN expression was a prognostic factor for both DFS and OS independent of AJCC stage, lymph node status, pathologic tumor (pT) stage, and differentiation.

Conclusion—Loss of PTEN expression is associated with poor disease-free and overall survival in patients with AA after curative surgery. PTEN expression may be used as a prognostic marker for patients with resected AA.

INTRODUCTION

Ampullary adenocarcinomas (AA) are rare malignant epithelial neoplasms arising from the ampulla of Vater and constitute approximately 2% of all gastrointestinal malignancies and 20% of all tumors of the extrahepatic biliary tree. The overall incidence is less than one per 100,000 with a higher incidence in the male population (0.7/100,000) than female population (0.45/100,000) ¹. The frequency of ampullary adenocarcinoma has been increasing in the last four decades ². AAs are biologically less aggressive than pancreatic ductal carcinomas. Patients with AA tend to present early due to biliary outflow obstruction and less aggressive compared to those with pancreatic ductal adenocarcinoma who often present with advanced disease at the time of diagnosis³. Therefore patients with AA have better overall survival compared to pancreatic ductal adenocarcinomas ^{1, 4, 5}. Compared to pancreatic ductal adenocarcinomas, loss of tumor suppressor *DPC4/SMAD4* expression and *K-ras* mutation are less frequent in AAs ^{6, 7}. In a study on 140 AAs conducted by McCarthy *et al.*, *DPC4/SMAD4* expression was lost in about a third of their cases ⁶. In addition, mutations in *p53* tumor suppressor gene have been observed during the progression of ampullary adenomas and low-grade AA to high-grade AA ⁷.

The *PTEN* gene is located on chromosome 10q23.31. In addition to its role as a tumor suppressor, it has important roles in embryogenesis and maintenance of physiological functions in many organ systems, and is constitutively expressed in normal tissues ⁸. It is one of the most frequently inactivated genes in sporadic cancer. Somatic mutations of *PTEN* occur frequently in many tumors such as glioblastoma, breast carcinoma, endometrial carcinoma, thyroid neoplasms, skin neoplasms and advanced stage prostate cancer ^{9, 10}. The role of *PTEN* as a tumor suppressor is due to its lipid phosphatase activity, including dephosphorylating phosphatidylinositol-3,4,5 triphosphate (PIP3), the product of phosphatidylinositol-3 kinase (PI3K) function, to form phosphatidylinositol-3,4 biphosphate (PIP2). The dephosphorylation of PIP3 antagonizes the PI3K function, thereby abolishing the PIP3-mediated activation of survival kinases, such as phosphoinositide-dependant kinase 1 (PDK1), and the AKT/mammalian target of Rapamycin (mTOR) pathway ^{9, 10}. PTEN is the only known lipid phosphatase abrogating PI3K pathway, and therefore, loss of PTEN has a significant impact on multiple aspects of tumorigenesis ¹⁰.

Loss of PTEN expression has been shown to be associated with poor prognosis in patients with malignancies from gastrointestinal and hepatobiliary tracts ^{11–16}. In patients with metastatic colonic adenocarcinoma and wild-type *Kras* who received cetuximab-based treatment, loss of PTEN expression by immunohistochemistry has been identified in 20% of the patients and is an independent prognostic factor for poor overall survival by multivariate

analysis¹³. Similarly, loss of PTEN expression has also been shown to be associated with poor disease-free and overall survivals in patients with advanced gastroesophageal junction cancer who received Cetuximab with irinotecan, and 5-fluorouracil as first-line treatment. Loss of heterozygosity of *PTEN* was reported in 17% of patients with gastric cancer and correlated with patient survival¹⁶. Other studies have shown that loss of nuclear staining for PTEN is associated with poor survival in colon cancer^{11, 12}. On other hand, Lee *et al.* showed that overexpression of PTEN is an independent prognostic factor associated with better patient survival in patients with intrahepatic cholangiocarcinoma¹⁴. In AAs, allelic imbalance at *PTEN* tumor suppressor gene has been reported to be present in 13% of cases¹⁷. However, the expression and the role of *PTEN* in AA have not been studied in detail. In this study, we examined 92 previously untreated cases of AA for loss of PTEN expression by immunohistochemistry. Using univariate and multivariate analyses, we determined whether the loss of expression of PTEN was associated with overall and disease-free survival and other clinicopathologic features in patients with AA. Our data showed that loss of PTEN expression was associated with poor prognosis in patients with AAs. Targeting the PI3K/AKT pathway may potentially be an effective treatment option for patients with ampullary adenocarcinoma.

MATERIALS AND METHODS

Case selection

We retrospectively reviewed medical records and tissue specimens of 92 patients with AA who underwent pancreaticoduodenectomy at the University of Texas MD Anderson Cancer Center between 1995 and 2009. Patients who received pre-operative neoadjuvant chemotherapy and/or radiation were not included. There were 55 males and 37 females with mean patient age at time of surgery of 63 years (range 37–87 years). This study was approved by the Institutional Review Board of the University of Texas MD Anderson Cancer Center.

Tissue Microarray construction

To construct the tissue microarray used in this study, formalin-fixed, paraffin-embedded archival tissue blocks and their matching hematoxylin and eosin-stained (H & E) slides were retrieved, reviewed and screened for representative tumor regions by two pathologists (S.S. and H.W.). For each patient, two cores of tumor were sampled from representative areas using a 1.0-mm punch. The tissue microarray was constructed with a tissue microarrayer (Beecher Instruments, Sun Prairie, WI) as described previously¹⁸.

Immunohistochemical analysis for PTEN

Immunohistochemical stain for PTEN was performed on 4- μ m unstained sections from the tissue microarray blocks using a mouse monoclonal antibody against PTEN (6H2.1, Dako, Carpinteria, CA). Following deparaffinization, antigen retrieval was performed on the tissue sections at 100 °C in a steamer containing Tris-EDTA buffer (pH 9.0) for 20 min. The sections were then immersed in anti-PTEN antibody (1:100 dilution) at 35 °C for 15 min. Subsequently, they were immersed in 3.0% hydrogen peroxidase at 35 °C for 5 min to block the endogenous peroxidase activity. A primary enhancer solution was then applied to the

slides and incubated at 35 °C for 8 minutes. The sections were then incubated with secondary anti-mouse immunoglobulin at 35°C for 8 min. Diaminobenzidine (DAB) was used as a chromogen and DAB enhancer was applied, and hematoxylin was used for counterstaining.

Measurement of PTEN expression levels

The immunohistochemically stained slides of AA tissue microarrays were examined using standard light microscopy (Olympus BX41). The staining results were independently scored semiquantitatively based on staining intensity (0-negative, 1-weak, and 2-moderate to strong) and percentage of positive tumor cells by two pathologists (S.S. and H.W.), who were blinded to the clinicopathologic data. The background stromal or non-neoplastic epithelial cells were used as an internal positive control. The PTEN expression score was calculated as the product of the staining intensity and the percentage of positive tumor cells. PTEN expression was categorized as loss of PTEN (no positive staining for PTEN, PTEN expression score=0) and PTEN-positive. To further examine the effect of PTEN expression levels on survival in patients with PTEN positive AA, we further classified the PTEN-positive cases into PTEN-low (PTEN expression score 1–100) and PTEN-high (PTEN expression score>100) using the median score for PTEN expression as the cut off.

Patient follow up and statistical analysis

The patients' follow-up information through May, 2011 was extracted from the medical records and, if necessary, updated by review of the U.S. Social Security Index. The recurrence information was updated when a patient came to the clinic for a follow-up visit. Chi-square analysis or Fisher's exact tests were used to compare categorical data. The survival curves were constructed using the Kaplan-Meier method, and the log-rank test was used to evaluate the statistical significance of differences. Disease-free survival (DFS) and overall survival (OS) were calculated as previously described¹⁹. The prognostic significance of clinicopathologic characteristics were determined using univariate and multivariate Cox regression analysis. Two multivariate models for survival analyses were used to determine the prognostic significance of loss of PTEN expression for DFS and OS. In the first model, tumor stage (pT) and lymph node status (pN) were included as two independent variables. In the second model, pT and pN were combined as one variable (AJCC stage). All statistical analysis was performed using Statistical Package for Social Sciences software (for Windows 12.0, SPSS Inc., Chicago, IL). We used a two-sided significance level of 0.05 for all statistical analyses.

RESULTS

PTEN expression in ampullary adenocarcinoma

Immunohistochemical stain for PTEN showed predominantly cytoplasmic staining with scattered nuclear staining in benign stromal cells, benign ampullary/duodenal mucosa (Figures 1A–1F) and AAs that were positive for PTEN. Among the 92 cases, 69 (75%) were PTEN-positive and 23 (25%) had complete loss of PTEN expression (PTEN-negative). The lowest PTEN expression score in the PTEN-positive AAs was 30. Fifty-seven (62%) AA

samples showed low levels of PTEN expression (PTEN-low) and 12 (13%) were PTEN-high.

Correlation of loss of PTEN expression with clinicopathologic features and other molecular markers

The clinicopathologic characteristics of the study population are correlated with the loss of PTEN expression in Table 1. The patients whose tumor had loss of PTEN expression had more frequent lymph node metastasis, higher stage, developed subsequent recurrence or metastasis compared to PTEN-positive tumors. Lymph node metastasis was present in 19 of 23 (83%) AAs with loss of PTEN expression compared to 32 of 69 (46%) of the AAs that were PTEN-positive ($P < .001$). At the time of surgical resection, the tumor was stage I, II and III in 2 of 23 (9%), 16 of 23 (70%), and 5 of 23 (22%) of patients whose tumor had loss of PTEN expression compared to 28 of 69 (47%), 32 of 69 (46%) and 9 of 69 (13%), respectively in patients whose tumors were PTEN positive ($P = .02$). No patient had stage IV disease. During follow up, 52% of the patients whose tumor had loss of PTEN expression developed recurrence/metastasis compared to 27% in patients whose tumor were PTEN-positive ($P = .03$). There were no correlation between loss of PTEN expression and the histologic subtype, tumor differentiation, presence of adenomatous component, the resection margin status, pT stages, adjuvant therapy status or expression of cytokeratin 7 (CK7), CK20 and CDX2.

Survival analysis

After pancreaticoduodenectomy, the median follow-up was 48 months ranging from 8 to 222 months. No patients were lost to follow-up. Loss of PTEN expression in patients with AA correlated with poor DFS and OS. The patients whose tumor had loss of PTEN expression had a shorter mean DFS of 89.0 ± 20.8 months than those whose tumors were PTEN positive (mean DFS: 161.4 ± 11.7 months, $P = .02$, Figure 2A). The mean OS was 93.1 ± 19.1 months for patients whose tumor had loss of PTEN expression compared to 175.4 ± 11.0 months for patients whose tumor was PTEN positive ($P = .002$, Figure 2A). To examine the effect of PTEN expression levels on survival, we further classified our cases that were PTEN positive into PTEN-low and PTEN-high. We found that patients with PTEN-low tumors had a better OS (mean survival: 164.5 ± 12.8 months) than the patients whose tumors had loss of PTEN expression (mean survival: 93.1 ± 19.1 months, $P = .007$). Patients with PTEN-low tumors had a shorter DFS and OS than those with PTEN-high tumors (Figure 3A and 3B), however the difference in either DFS or OS between these two groups was not statistically significant.

In univariate analysis, both DFS and OS correlated with pathologic tumor (pT) stage, lymph node status, AJCC stage and loss of PTEN expression. However, no significant correlation between either DFS or OS with CDX2 expression, adjuvant therapy, or other clinicopathologic parameters was observed (Table 2). Two multivariate models for survival analyses were used to determine the prognostic significance of loss of PTEN expression for DFS and OS and the results are shown in Table 3. In the first model, lymph node status was an independent prognostic factor for DFS ($P = .002$) and pT stage was an independent prognostic factors for OS ($P = .03$). In the second model, AJCC (pathology) stage was an

independent prognostic factor for both DFS ($P = .003$) and OS ($P = .046$). The loss of PTEN expression was a prognostic factor for OS in both models independent of tumor differentiation, pT stage, lymph node status, and AJCC stage ($P < .05$, Table 3). However, the correlation between the loss of PTEN expression and DFS was not statistically significant in either model ($P > .05$, Table 3).

DISCUSSION

In this study, we examined the protein expression of PTEN tumor suppressor gene by immunohistochemistry in 92 previously untreated AAs. We found loss of PTEN expression in 25% of AAs. Loss of PTEN was associated with lymph node metastasis, AJCC stage, and recurrence in our study population. In addition, we showed that loss of PTEN expression in AAs correlated with poor DFS and OS and was an independent prognostic factor for OS in multivariate analysis. Our data suggest that loss of PTEN expression plays a role in AAs and may be used as a prognostic factor in patients with resected AA.

PTEN is an important tumor suppressor gene involved in many types of human malignancies. Loss of function of PTEN leads to constitutive activation of the PI3K-AKT pathway, which, in turn, activates various downstream target genes and results in cellular growth, proliferation and survival^{9, 10}. In this study, we found that PTEN expression was lost in a fourth of the resected AA samples. However, the mechanism(s) of the loss of PTEN expression in AAs are unclear. Multiple possible mechanisms may be involved in regulating the expression and the functions of PTEN, which include mutations of *PTEN*, heterozygous and homozygous deletions, transcriptional silencing by promoter methylation, microRNA targeting, post-translational regulation and stability of the PTEN protein^{9, 20–23}. Transcriptional silencing by *PTEN* promoter methylation has been reported in endometrial, thyroid, gastric, lung, breast and ovarian carcinomas, as well as glioblastomas^{24–30}. Downregulation of PTEN protein expression due to microRNA targeting has also been reported in gliomas, prostate and lung carcinomas^{23, 31, 32}. Given the previous report that allelic imbalance at PTEN tumor suppressor gene is present in 13% of AA cases¹⁷, it is possible that heterozygous and homozygous deletions may be involved in the loss of PTEN expression in ampullary carcinoma. Further studies are needed to elucidate the mechanism of loss of PTEN expression in these patients.

Loss of PTEN has been shown to be a prognostic factor of poor prognosis in several cancers, including carcinomas of the gastrointestinal and hepatobiliary tracts^{11–16, 33–36}. Intact PTEN expression has been shown to be associated with lower stage and negative lymph nodal status in endometrial carcinomas³⁷. Consistent with previous reports for other cancers, we found that loss of PTEN expression was associated with lymph node metastasis and higher AJCC stage at the time of surgery and was associated with higher frequency of recurrence and poor DFS and OS after curative surgery in our study population. Similar to our results, loss of PTEN expression has been reported in 25.6% of pancreatic ductal adenocarcinoma and was associated with tumor recurrence/metastasis and poor prognosis in a study on 133 cases of surgically resected stage II pancreatic ductal adenocarcinoma by Foo *et al.* (add Tim's paper here as ref) It is interesting to note that patients whose tumors were PTEN-low had a DFS and OS intermediate to the group of our patients whose tumors were

PTEN-high and the groups whose tumor had loss of PTEN expression, although the difference did not reach statistical significance. It is therefore possible that PTEN may affect the prognosis in patients with AA in a dose-dependent manner. In contrast to the previous studies which showed that loss of nuclear staining for PTEN is associated with poor survival in colon cancer^{11, 12}, we did not observe significant correlation between nuclear PTEN expression and either survival or other clinicopathologic parameters (data not shown). This may reflect the difference in the antibodies used for immunohistochemistry among these studies or difference in molecular alterations between colonic adenocarcinoma and AAs.

In addition to the loss of PTEN expression as an independent prognostic factor for DFS and OS, our data also showed that advanced tumor (pT) stage, lymph node metastasis and AJCC stage were correlated with both DFS and OS by univariate analysis and AJCC stage was an independent prognostic factor for both DFS and OS by multivariate analysis. Our findings are corroborated by several previous studies which demonstrated prognostic significance of pathologic stage in patients with AA following curative surgery^{38–40}. Consistent with previous studies^{38–40}, patients with AAs of intestinal subtype also had better prognosis than those with pancreaticobiliary subtype in our patient population (data not shown). However, we did not observe significant correlation of either DFS or OS with the commonly used intestinal markers such as CK20 or CDX2.

In summary, our study showed that loss of PTEN expression is an independent prognostic factor for poor disease-free and overall survival in patients with ampullary adenocarcinoma after curative surgery. Immunohistochemical analysis of PTEN expression can be useful as a prognostic factor, and have therapeutic implications in this patient population. Targeting the PI3K/AKT pathway via PI3K inhibitors and mTOR inhibitors may prove to be an effective treatment for patients with ampullary adenocarcinoma.

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Figure 1.

Representative micrographs show PTEN expression in ampullary adenocarcinoma. A and B, a moderately differentiated ampullary adenocarcinoma with complete loss of PTEN expression (negative staining for PTEN); C and D, weak cytoplasmic and nuclear staining for PTEN in a moderately differentiated ampullary adenocarcinoma (PTEN-low); E and F, a moderately differentiated ampullary adenocarcinoma with strong positive staining for PTEN (PTEN-high). The intervening tumor stromal cells serve as internal positive control. Original magnification, 40 $\times$ for A, C, and E; 200 $\times$ for B, D, and F).

Figure 2.

Kaplan-Meier curves for disease-free survival (A) and overall survival (B) by loss of PTEN expression in patients with ampullary adenocarcinoma after curative surgery. Patients whose tumors had loss of PTEN expression had shorter disease-free survival and overall survival than patients whose tumors retained PTEN expression.

Figure 3. Kaplan-Meier curves for disease-free survival (A) and overall survival (B) by PTEN expression levels in patients with ampullary adenocarcinoma after curative surgery.

Table 1

Characteristics	N	Loss of PTEN	PTEN Positive	p value
Gender				
Male	55	13	42	0.81
Female	37	10	27	
Mean age (range, years)	92	63.0 (37.0–76.0)	63.1 (56.0–87.0)	0.96
Histology Pattern				
Intestinal	30	5	25	0.60
Pancreaticobiliary	32	10	22	
Mixed	30	8	22	
Presence of adenoma				
No	52	15	37	0.47
Yes	40	8	32	
Tumor Differentiation				
Well to Moderate	59	14	45	0.80
Poor	33	9	24	
CDX2 expression				
Negative	52	17	35	0.05
Positive	40	6	34	
Margin status				
Negative	86	20	66	0.16
Positive	6	3	3	
Tumor Stage				
T1 and T2	50	9	41	0.09
T3 and T4	42	14	28	
Lymph Node Positivity (pN)				
0 (0)	41	4	37	0.004
1–3 positive nodes (1)	32	10	22	
> 3 positive nodes (1)	19	9	10	
AJCC Pathologic stage				
Stage I	30	2	28	0.02
Stage II	48	16	32	
Stage III	14	5	9	
Postoperative therapy				
No	49	9	40	0.12
Yes	43	14	29	
Recurrence				
No	61	11	50	0.03
Yes	31	12	19	

PTEN, Phosphatase and tensin homolog

AJCC, American Joint Committee on Cancer

Table 2

Characteristics	Number of patients	Disease-free survival HR (95% CI)	P value	Overall HR (95% CI)	Survival P value
Gender					
Male	55	1.00		1.00	
Female	37	1.08 (0.53, 2.21)	0.83	0.90 (0.41, 1.99)	0.80
Age (years)	92	1.01 (0.98, 1.04)	0.49	1.01 (0.97, 1.04)	0.65
Adenoma					
No	52	1.00		1.00	
Yes	40	0.73 (0.36, 1.51)	0.40	0.77 (0.35, 1.68)	0.51
Tumor Stage (pT)					
T1 and T2	50	1.00		1.00	
T3 and T4	42	2.61 (1.26, 5.44)	0.01	2.90 (1.30, 6.47)	0.009
Tumor Differentiation					
Well to Moderate	59	1.00		1.00	
Poor	33	1.88 (0.93, 3.81)	0.08	1.96 (0.91, 4.22)	0.09
Lymph Node Positivity (pN)					
Negative	41	1.00	0.002	1.00	0.05
1–3 positive nodes (1)	32	2.05 (0.82, 5.10)	0.12	1.89 (0.75, 4.81)	0.18
> 3 positive nodes (1)	19	5.02 (2.03, 12.38)	<0.001	3.38 (1.26, 9.05)	0.02
Pathology stage (AJCC stage)					
Stage 1	30	1.00	0.003	1.00	0.01
Stage 2	48	7.22 (2.17, 24.03)	0.00	6.09 (1.80, 20.59)	0.004
Stage 3	14	2.84 (0.57, 14.13)	0.20	3.41 (0.68, 17.11)	0.14
Postoperative therapy					
No	49	1.00		1.00	
Yes	43	1.19 (0.59, 2.41)	0.63	1.15 (0.53, 2.49)	0.72
CDX2 expression					
Negative	52	1.00		1.00	
Positive	40	0.68 (0.32, 1.41)	0.30	0.67 (0.30, 1.50)	0.33
Loss of PTEN					
No	69	1.00		1.00	

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Characteristics	Number of patients	Disease-free survival HR (95% CI)	P value	Overall HR (95% CI)	Survival P value
Yes	23	2.42 (1.17, 4.99)	0.02	3.41 (1.57, 7.42)	0.002

PTEN, Phosphatase and tensin homolog
HR, Hazard Ratio
CI, Confidence interval
AJCC, American Joint Committee on Cancer

Table 3

Characteristics	No. of patients	Disease-free survival HR (95% CI)	p value	Overall Survival HR (95% CI)	p value
Tumor Differentiation					
Well to Moderate	59	1		1	
Poor	33	1.42 (0.68, 2.97)	0.36	1.61 (0.73, 3.55)	0.23
Tumor Stage (pT)					
T1 and T2	50	1		1	
T3 and T4	42	1.68 (0.74, 3.79)	0.21	2.55 (1.13, 5.75)	0.03
Lymph Node Positivity (pN)					
Negative	41	1	0.002	1	0.99
1–3 positive nodes (I)	32	2.05 (0.82, 5.10)	0.12	1.05 (0.38, 2.91)	0.92
> 3 positive nodes (I)	19	5.02 (2.03, 12.38)	<0.001	1.07 (0.31, 3.68)	0.92
Loss of PTEN					
No	69	1		1	
Yes	23	1.56 (0.71, 3.41)	0.27	3.00 (1.37, 6.59)	0.006
Tumor Differentiation					
Well to Moderate	59	1		1	
Poor	33	1.52 (0.74, 3.09)	0.25	1.46 (0.66, 3.20)	0.35
Pathology stage (AJCC)					
Stage 1	30	1	0.003	1	0.046
Stage 2	48	7.22 (2.17, 24.03)	0.001	4.63 (1.31, 16.30)	0.02
Stage 3	14	2.84 (0.57, 14.13)	0.2	2.56 (0.49, 13.31)	0.26
Loss of PTEN					
No	69	1		1	
Yes	23	1.64 (0.78, 3.43)	0.19	2.33 (1.05, 5.18)	0.04

PTEN, Phosphatase and tensin homolog

HR, Hazard Ratio

CI, Confidence interval

AJCC, American Joint Committee on Cancer