Combined Loss of PTEN and p27 Expression Is Associated with Tumor Cell Proliferation by Ki-67 and Increased Risk of Recurrent Disease in Localized Prostate Cancer[1]

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ABSTRACT

Purpose: **Recent experimental work indicates a major role for *PTEN* and *p27* in prostate cancer. The combined loss of *PTEN* and *p27* was found to strongly increase the development of prostatic carcinomas in an animal model, and a prognostic value in human tumors was postulated. The purpose of our study was to examine the impact of PTEN and p27 on prognosis in a series of prostate cancer patients, using high-density tissue microarray technology for expression profile analysis of PTEN, p27, and tumor cell proliferation.**

Experimental Design: **The expression of PTEN and p27 was examined in primary prostatic carcinomas from 104 patients treated with radical prostatectomy and with complete follow-up available. Using high-throughput tissue microarrays, the expression of PTEN and p27 was examined by immunohistochemistry, and the results were related to clinicopathological variables, tumor cell proliferation (Ki-67), and time to disease progression.**

Results: **PTEN was negative in 28 of 103 tumors (27.2%), and median p27 expression was 64%. Combined loss of PTEN and p27 expression defined a group of 18 tumors (17.5%) associated with increased tumor diameter, seminal vesicle invasion, increased pathological stage, and elevated tumor cell proliferation by Ki-67. Cox regression analysis revealed that loss of PTEN/p27 expression and histological grade were both independent predictors of time to biochemical failure and clinical recurrence.**

Conclusions: **Our findings strongly support the importance of PTEN and p27 for the progression of human prostate cancer because loss of PTEN/p27 expression was associated with adverse pathological parameters, tumor cell proliferation, and increased risk of recurrence.**

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INTRODUCTION

The *PTEN* (*MMAC1*) and *p27* (*p27^{KIP1}*) genes are both considered to be important for the development of prostate cancer (1, 2). *PTEN*, which is a tumor suppressor gene located at 10q23, encodes a dual activity phosphatase and has been involved in cell cycle regulation and cell adhesion properties including migration (1). Loss of *PTEN* function leads to increased Akt activity and, subsequently, cell survival (3). The cyclin-dependent kinase inhibitor p27^{KIP1}, a target of Akt, has been proposed as a downstream mediator through which *PTEN* may negatively regulate cell cycle progression (4). A high frequency of *PTEN* mutations has been reported in several tumor types, such as endometrial carcinoma and brain and breast cancer (5). Inactivation of *PTEN* is frequent in prostate cancer, with deletions in 30–60% of prostate cancers (6, 7), whereas point mutations seem to be fewer, around 10–16% of prostate cancers (7, 8). *PTEN* inactivation might also occur at the transcriptional level in 20–50% of prostate cancers (9, 10), and the need for protein expression studies has been focused (9). Furthermore, reduced levels of p27 are found in several tumors and generally indicate a poor prognosis (2). In prostate cancer, 16–68% of the cases reveal loss of p27 protein or low-grade expression along with significant associations with other adverse prognostic factors and impaired prognosis (11–14). The function of p27 protein is mainly regulated by posttranslational ubiquitin-mediated proteasomal proteolysis (15).

In a recent experimental study, loss of a single *PTEN* allele promoted prostate cancer progression in the transgenic adenocarcinoma of mouse prostate (TRAMP) model (16). In another study by Di Cristofano *et al.* (17), the combined loss of *PTEN* and *p27* was found to strongly increase the development of prostate cancer in double mutant mice, with elevated tumor cell proliferation. The authors postulated that inactivation of *PTEN* and *p27* might have prognostic value in human prostate cancer. The aim of our present study was to test this hypothesis in a series of prostate cancer patients with complete follow-up information, using high-density TMA[3] technology for expression profile analysis of PTEN and p27. Concomitant loss of PTEN and p27 expression was recorded in 18 (17.5%) of our cases and was associated with a strong and independent prognostic influence, thus validating the recent experimental findings at the protein level.

PATIENTS AND METHODS

Patients. This series has been described previously in detail (18). Briefly, a consecutive series of 104 men (median

[3] The abbreviations used are: TMA, tissue microarray; HR, hazard ratio; s-PSA, serum prostate-specific antigen.

age, 62.0 years) treated by radical prostatectomy for clinically localized prostate cancer between 1988 and 1994 were included. The following variables were recorded: patient age; stage [tumor-node-metastasis (TNM) category]; largest tumor diameter; WHO histological grade; capsular penetration; seminal vesicle invasion; involvement of surgical margins; presence of lymph node metastasis; and s-PSA before and after surgical treatment. Time from surgery until biochemical failure, defined as s-PSA elevation $\geq$ 0.5 ng/ml in two consecutive blood samples, was recorded, as was time to clinical locoregional recurrences or metastases. The last date of follow-up was December 31, 1997, and the median observation time was 56 months. No patients were lost to follow-up. During the first 5 years of follow-up considered in survival analyses, 43 patients experienced biochemical failure, whereas clinical recurrence was recorded in a total of 23 patients (21 local recurrences, 1 lymph node recurrence, and 6 distant metastases). Two of 104 patients underwent neoadjuvant androgen blockage.

TMA. The technique of TMA has recently been introduced (19). In our study, the area of highest tumor grade was selected from the representative paraffin block on the basis of H&E-stained whole mount sections, and three tissue cores (0.6 mm in diameter) were sampled from this area in the "donor" block and mounted into a "recipient" paraffin block by the use of a custom-made instrument (Beecher Instruments, Silver Springs, MD), and 5-μm paraffin sections were then made by standard technique.

Immunohistochemistry. TMA slides were used for antibodies against PTEN and p27 proteins, but Ki-67 immunohistochemistry was performed on standard tissue sections as described previously (20). The tissue sections were subjected to heat-induced epitope retrieval in citrate buffer (pH 6.0) using a microwave oven at 350 W for 4 $\times$ 5 min for PTEN and 6 $\times$ 5 min for p27, respectively. Microwave treatment for Ki-67 was performed for 3 $\times$ 5 min in an EDTA buffer (pH 8.0). Immunostaining of tissue sections was performed on the TechMate 500 (Dako A/S, Copenhagen, Denmark) automatic staining instrument. The TMA slides were incubated with a mouse PTEN antibody (1:75; clone 6H2.1; Cascade Bioscience, Winchester, MA) overnight at room temperature and with mouse $p27^{KIP1}$ antibody (1:500; clone 57; Transduction Laboratories, Lexington, KY) for 60 min at room temperature. Incubation with a polyclonal rabbit Ki-67 antibody (1:50; Dako A/S) was performed for 25 min at room temperature. Antigen localization was achieved by an indirect streptavidin-biotin-peroxidase method using the 3-amino-9-ethylcarbazole peroxidase reaction for PTEN and the diaminobenzidine peroxidase reaction for p27 and Ki-67, counterstained with hematoxylin. For all antibodies, a case of colon carcinoma was included as a positive external control. Slides in which the primary antibody was omitted served as negative controls for Ki-67. Slides incubated with isotypic IgG1 (Dako A/S) served as negative controls for PTEN and p27.

Evaluation of Staining Results. PTEN expression was evaluated subjectively by one observer (O. J. H.), who estimated the staining intensity and percentage positivity. A staining index (values 0–9), obtained as a product of cytoplasmic staining intensity (0–3) and the proportion of immunopositive tumor cells ($\leq$10% = 1, 10–50% = 2, and $>$50% = 3), was calculated, based on an evaluation of all three cores from each case. Genetic alterations correlate with reduced cytoplasmic PTEN staining in various tumors and are most pronounced in tumors with absent or weak PTEN cytoplasmic staining (21–23), and cases with a PTEN staining index of $\leq$1 were defined *a priori* as negative.

With regard to p27 expression, only nuclear staining was considered. All three cores were examined subjectively for p27 staining, and the core with the most intense p27 staining was selected from each case and subjected to quantitation of p27 expression by visually counting up to 500 nuclei. Visual scoring of p27 is easily applicable on routine histological material and has been validated by image analysis (13). Selection of the most positive core ("hot core") was done to avoid overestimation of reduced p27 expression, in accordance with others, selecting the area of highest p27 intensity to be scored (24). The nuclei were evaluated systematically by one observer (O. J. H.), using high power ($\times$63) and an eyepiece grid. The findings were recorded as the percentage of immunopositive nuclei.

Ki-67 staining was performed on regular sized tissue sections as described in an earlier study from the same patient series, and the results from this study were included in the present study for comparison (20). Briefly, Ki-67 expression was estimated in "hot spot" areas on regular slides essentially in a similar way as for p27, as described previously (20), corresponding to the area from which the TMA was generated. One case, in which tumor tissue was insufficient in the TMA, was excluded from the final analyses.

When classifying 20 randomly selected cases into groups with high (positive) and low (negative) expression, the intraobserver agreement was 90% for PTEN (K = 0.80) and 95% for p27 (K = 0.88).

Statistics. Analyses were performed using the statistical package SPSS (SPSS, Inc., Chicago, IL). Associations between different variables were assessed by Pearson's χ^2 test or the Mann-Whitney U or Kruskal-Wallis H tests, when appropriate. Continuous variables were analyzed after categorization by median, tertiles, or quartiles (25, 26). Univariate survival analysis of time to biochemical failure or clinical recurrence was performed by the product-limit method (Kaplan-Meier), with the log-rank test for differences between categories of each variable. Cox proportional hazards method was applied for multivariate survival analyses, including only significant variables ($P \leq$ 0.05) from univariate analyses, and tested by the likelihood ratio test. Model assumptions were examined by log minus log plots. Variables with a significant outcome in the first multivariate model ($P \leq 0.10$) were analyzed in a second (final) multivariate model. Validation of staining estimates was evaluated by using κ (K) statistics for intraobserver agreement.

RESULTS

Associations. PTEN was negative in 28 of 103 tumors (27.2%; Fig. 1) and was significantly associated with increasing tumor diameter (P = 0.002, Pearson's χ^2 test) and seminal vesicle invasion (P = 0.007). Median tumor diameter in PTEN-negative cases was 30 mm, compared with 27 mm in PTEN-positive tumors.

The median value of p27 expression was 64% positive

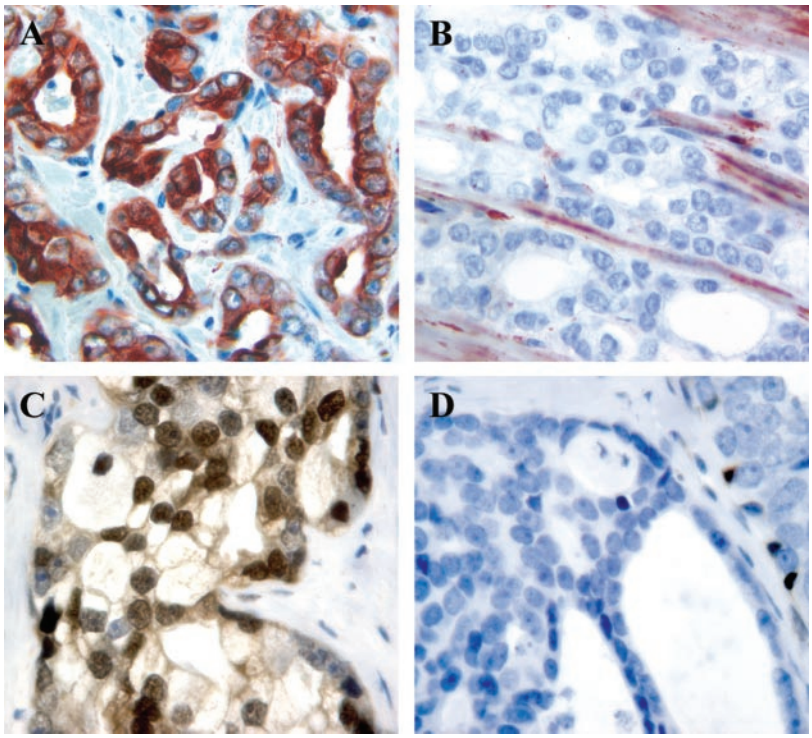

Fig. 1 Immunohistochemical expression of PTEN (*A* and *B*) and p27 (*C* and *D*) on TMAs of prostatic carcinomas. *A*, strong cytoplasmic PTEN staining (3+). *B*, PTEN-negative tumor with positive reaction in prostatic stroma. *C*, p27-positive tumor. *D*, p27-negative tumor. Note the positive staining of basal cell nuclei in a gland with severe dysplasia or high-grade prostatic intraepithelial neoplasia (*right*).

tumor cell nuclei (range, 0–97.4%), and cases below the median were defined as low expressors (Fig. 1), in line with other studies (26). Expression of p27 below the median was associated with high clinical stage ($P = 0.033$), capsular penetration ($P = 0.011$), seminal vesicle invasion ($P = 0.002$), high pathological stage ($P = 0.009$), and elevated preoperative s-PSA ($P = 0.002$), whereas the association with tumor diameter ($P = 0.054$) was of borderline significance (by Mann-Whitney or Kruskal-Wallis tests). Reduced expression of p27 was weakly associated with loss of PTEN expression ($P = 0.049$); the median value of p27 expression was 68.0% and 58.7% in PTEN-positive and -negative tumors, respectively. In PTEN-negative tumors, low expression of p27 was significantly associated with elevated proliferation by Ki-67 ($P = 0.010$, Mann-Whitney *U* test; Fig. 2).

Combined loss of PTEN/p27 expression was found in 18 of 103 tumors (17.5%). Lack of PTEN/p27 expression was associated with increased tumor diameter ($P = 0.003$), capsular penetration ($P = 0.013$), seminal vesicle invasion ($P < 0.001$), and pathological stage ($P = 0.019$), whereas preoperative s-PSA ($P = 0.059$) was of borderline significance (by Pearson's χ^2 test).

Univariate Survival Analysis. Expression of PTEN ($P = 0.010$), expression of p27 ($P = 0.030$), expression of combined PTEN/p27 ($P = 0.002$; Fig. 3), tumor diameter, histological grade (WHO), pathological stage, positive surgical margins, and preoperative s-PSA were all significant predictors of time to biochemical failure. PTEN expression ($P = 0.003$) and combined PTEN/p27 expression ($P = 0.001$) were also significant predictors of clinical recurrence (Fig. 3), as was WHO histological grade ($P = 0.006$), whereas p27 expression

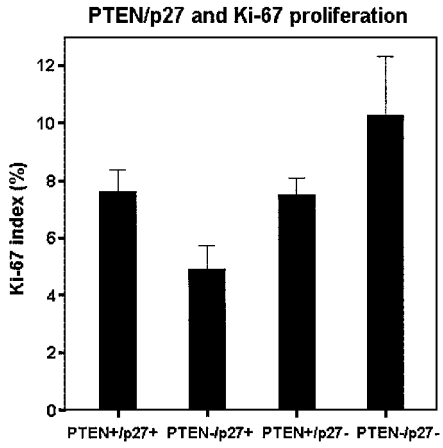

Fig. 2 Tumor cell proliferation by mean (±SE) Ki-67 expression in 103 human prostatic carcinomas according to PTEN and p27 status. PTEN+, PTEN positive; PTEN−, PTEN negative. p27+, p27 expression above the median; p27−, p27 expression below the median. Ki-67 expression was significantly associated with loss of p27 expression within PTEN-negative tumors ($P = 0.010$, Mann-Whitney *U* test).

was of borderline significance ($P = 0.062$). With regard to the site of clinical recurrence, loss of PTEN expression was significantly associated with time to local recurrence ($P = 0.005$) but not distant spread (skeletal metastases), although this subgroup is small. Reduced p27 expression was not associated with time to clinical recurrence by specific site.

Multivariate Survival Analysis. In Cox regression analysis, the three standard prognostic variables [histological grade

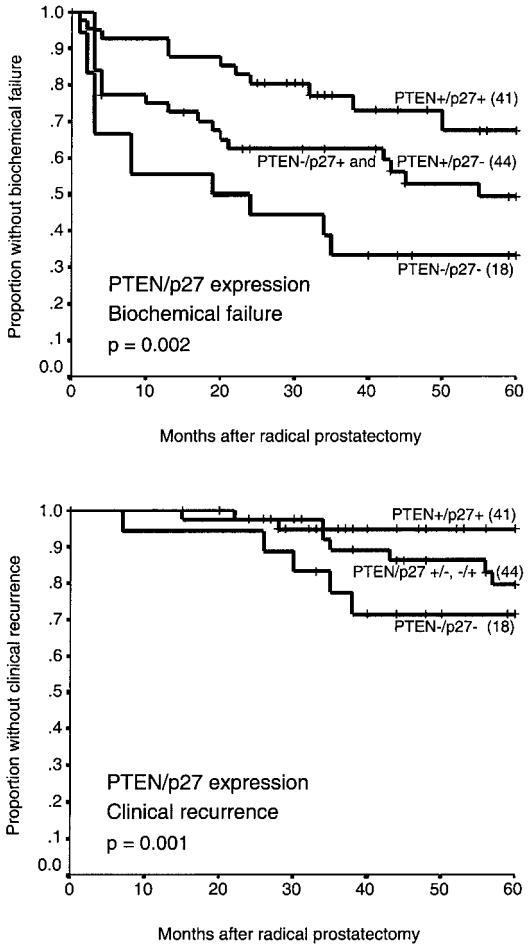

Fig. 3 PTEN/p27 status and prostate cancer progression: univariate analysis (Kaplan-Meier method) of time to biochemical failure (*top panel*) and clinical recurrence (*bottom panel*) after radical prostatectomy in 103 patients. p27+, p27 expression above the median; p27−, p27 expression below the median.

(poorly *versus* well/moderately differentiated), pathological stage (pT3 *versus* pT2), and preoperative s-PSA (>20 *versus* <20 ng/ml)] were included as covariates if statistically significant prognostic impact was recorded in univariate analyses. Because preoperative s-PSA was available in a subgroup only, and pathological stage is not a relevant variable in the setting of PTEN/p27 as a potential prognostic marker on preoperative prostate biopsies, separate regression analyses including histological grade and PTEN/p27 only were performed.

In multivariate analysis including the three standard covariates, pathological stage and s-PSA remained in the final model as independent predictors of time to biochemical failure, whereas PTEN expression (HR = 1.9; $P = 0.062$) and WHO histological grade were of borderline significance. In a separate analysis including histological grade as the sole covariate, PTEN expression (HR = 2.4; $P = 0.007$) and WHO histological grade (HR = 2.1; $P = 0.024$) remained as independent predictors of biochemical failure. In multivariate analysis of time to clinical recurrence, PTEN expression (HR = 2.9; $P = 0.011$) and WHO histological grade (HR = 3.0; $P = 0.014$) both remained as independent predictors. PTEN expression (HR = 3.0; $P = 0.016$) was the only independent significant predictor of time to recurrence broken down by site, *i.e.*, locoregional recurrence, as histological grade (HR = 2.4; $P = 0.055$) was of only borderline significance.

Table 1 Multivariate Cox regression analysis (proportional hazards method) of time to biochemical failure and clinical recurrence after radical prostatectomy in 103 prostate cancer patients

	Biochemical failure			Clinical recurrence		
Variables	HR	95% CI[a]	P^b	HR	95% CI	P^b
PTEN/p27[c]	2.2	1.2–4.2	0.035	2.7	1.2–6.5	0.029
Histological grade (WHO)[d]	2.3	1.2–4.3	0.001	2.7	1.2–6.2	0.024

[a] CI, confidence interval.
[b] Likelihood ratio test.
[c] PTEN/p27 −/− versus +/+, −/+, +/−.
[d] Poorly versus well/moderately differentiated.

In a multivariate analysis of p27 expression, pathological stage and preoperative s-PSA remained in the final model as independent predictors of time to biochemical failure, whereas p27 expression and WHO histological grade did not. In a separate analysis including histological grade as the sole covariate, WHO grade (HR = 2.3; $P = 0.011$) remained as an independent predictor of biochemical failure, whereas p27 expression was of borderline significance (HR = 1.8; $P = 0.072$).

When multivariate analysis of combined PTEN/p27 expression was performed, pathological stage (HR 3.1; $P = 0.025$) and s-PSA (HR 2.4; $P = 0.012$) remained in the model as independent predictors of time to biochemical failure, whereas PTEN/p27 expression and grade did not reach statistical significance. In a separate analysis including histological grade as the sole covariate, PTEN/p27 expression (HR = 2.2; $P = 0.035$) and WHO histological grade (HR = 2.2; $P = 0.001$) remained as independent predictors of biochemical failure (Table 1). In a multivariate analysis of time to clinical recurrence (locoregional recurrence or metastasis), combined PTEN/p27 expression (HR = 2.7; $P = 0.029$) and WHO histological grade (HR = 2.7; $P = 0.024$) remained as significant and independent predictors of clinical recurrence (Table 1).

When we excluded two patients on neoadjuvant hormone blockade, PTEN/p27 expression was still an independent predictor of biochemical failure (HR = 2.0; $P = 0.038$) or clinical recurrence (HR = 3.0; $P = 0.014$), as was histological grade, and other conclusions, such as associations with tumor cell proliferation or adverse pathological variables, were not altered.

DISCUSSION

Previous studies have indicated that both *PTEN* and *p27* tumor suppressor genes are important in prostate cancer (1, 2, 14, 17). Haploinsufficiency of the *PTEN* gene promotes prostate cancer progression in mice, a finding that may explain the difference in loss of heterozygosity and biallelic inactivation (16). Recent experimental work has strongly indicated that *PTEN* and *p27* might cooperate in tumor suppression and stimulate tumor cell proliferation, with decreased survival being

related to allelic loss (17). The present study was performed to validate these findings in a series of 104 localized and potentially curable prostate cancer patients with detailed follow-up information. Our results indicate a significant and independent prognostic influence of the combined PTEN/p27 variable in addition to traditional factors such as histological grade and primary tumor stage. Thus, our data support the recent mouse model findings at the protein level in human prostatic carcinomas.

When studied individually, lack of PTEN protein expression was found in 27% of the cases, comparable with 20% negative cases in a study of McMenamin *et al.* (10). PTEN expression was found to be associated with increasing tumor diameter and advanced primary tumor stage, as indicated by carcinomatous infiltration of the seminal vesicles, and was also related to time to local recurrence, supporting the importance of PTEN for prostate cancer growth and local invasion, possibly reflecting changes in cell cycle regulation, migration, or loss of cell cohesion influenced by PTEN (1). Low expression of p27 was also associated with advanced local growth. These data strongly suggest an important role of both PTEN and p27 for the progressive growth and local invasion of prostate cancer in our series of patients treated with radical prostatectomy for localized and presumed organ-confined tumors.

In keeping with mouse model findings (17), increased tumor cell proliferation by Ki-67 was most pronounced in cases negative for both PTEN and p27 expression. Although PTEN may suppress tumor formation by induction of p27 (4), the association between PTEN and p27 expression was only marginally significant in our study. Lack of convincing correlation between PTEN and p27 expression has also been reported for ovarian carcinomas (23), indicating the possible existence of *p27*-independent pathways downstream of *PTEN*.

In conclusion, our findings strongly support recent experimental data (17) and indicate a synergistic influence of *PTEN* and *p27* inactivation for the progressive growth and prognosis of human prostate cancer. Thus, the combined effect of these factors was found to be significantly associated with increased tumor cell proliferation by Ki-67 expression, and the PTEN/p27 variable strongly and independently indicated an elevated risk for clinical tumor recurrence in this important and increasing group of treatable prostate cancer patients. Because the outcome of this patient group might be difficult to predict, our results increase the availability of prognostic information, and identification of tumors with specific immunophenotypes may prove valuable in the future when selecting patients for experimental treatment protocols.

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